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CHLOROPLAST DNA REGION BASED PHYLOGENETIC RELATIONSHIPS WITHIN GENUS *ABUTILON* MILL.

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Abstract

The genus *Abutilon* belongs to the family Malvaceae. It is a taxonomically complex genus due to the high degree of morphological variations observed among its members, both within and between species. Describing *Abutilon* species is particularly challenging because of these extensive morphological variations. Consiquently, the interspecific relationships within the genus remain unclear. In the current study, phylogenetic relationships within the genus *Abutilon,* was studied using sequence data from the noncoding (*psb*A-*trn*H) region of the chloroplast genome. Fifteen *Abutilon* species that are found in Pakistan were used to reconstruct the phylogenetic tree. Bayesian Inference analysis was applied to generate the phylogenetic tree, resulting in a well resolved phylogenetic tree with strong statistical support. Phylogenetic relationships were further discussed by comparing the morphological characteristics. Based on the p*sb*A-*trn*H sequence data, two major groups with further clades were identified which generally confirmed the traditional grouping with some exceptions. The genetic profile of *A. alii* was found to be similar to *A. muticum and A. pannasum*, suggesting that these species are closely related and should be placed in the same group. Similarly, the study inferred the close association of *A. pakistanicum* to *A. grandifolium*; *A. fruticosum* to *A. ramosum; A. karachianum* to *A. hirtum* and *A. sepalum; A. bidentatum* to *A. indicum, A. figarianum* and *A. gafoorianum.* One of the most significant findings of this analysis was the identification of *A. theophrasti* as a basal species, with unique sequence characteristics suggesting that it should be considered both basal and primitive. In this study, the associations among *Abutilon* species were explored using DNA sequence data from noncoding region of the chloroplast genome and the inferred relationships were compared with previous morphology-based classification of *Abutilon*. Prior to this study, the discrimination of *Abutilon* species and their associations had not been explored using molecular data. The findings provide molecular evidence that largely supports the previously established morphological based classification with some exceptions. The study also suggests that *psbA-trn*H could serve as a reliable marker for *Abutilon* species delimitation. In the future, with an increased sample size, this molecular marker (*psbA-trn*H) could be utilized to discriminate *Abutilon* species and explore interspecific and infrageneric association among them. The species delimitation and inferred association presented in this study will contribute to updating the classification of *Abutilon* at molecular level.

Key words: *Abutilon*, Chloroplast genome, Interspecific, Intraspecific. *psbA-trn*H, Phylogenetics relationships.

Introduction

The genus *Abutilon* belongs to the family Malvaceae and subfamily Malvoideae. *Abutilon* species are annual or perennial herbs, shrubs, undershrub, rarely small trees and found in tropics and subtropics regions of the world. *Abutilon* is most divers in Neotropics (Areces Berazain & Fryxell, 2007). The number of its recognized species varies considerably. Nowadays, the number of accepted species is about 150-160 worldwide. (Abedin, 1979; Bayer & Kubitzki, 2003; Mabberley, 2008; Verdcourt & Mwachala, 2009). In Pakistan the genus is represented by 18 specific and intraspecific taxa (Abedin, 1979). The genus *Abutilon* achieved considerable economic importance due to its medicinal properties (de Melo *et al*., 2011; Gaoue *et al*., 2021: Hassan *et al*., 2021; Sikorska and Matlawska, 2008; Sasikala and Meena, 2018) and the fiber that could be exploited as a substitute for jute (Zhang *et al*., 2014; Reddy & Yang, 2008).

Abutilon has been considered as the most difficult genus of Malvaceae (Kearney, 1958). It is also considered as a heterogenous genus with many taxonomic problems (Fryxell, 2002). The reason behind it is the high degree of variations found in morphological characters of its species and the adaptations to climatic conditions. These high variations lead to misidentification, improper naming and inappropriate placement of species (Fryxell, 2002). The species delimitation and phylogenetic relationship of *Abutilon* species at interspecific and infrageneric level is poorly understood.

Previous morphology-based studies classified the *Abutilon* species into sections and subsections. But these studies were restricted for geographical regions such as Neotropical region (Presl, 1835), West India (Grisebach, 1864), Brazil (Schumann, 1891), East Africa (Mattei, 1915). Mattei (1915) classified the East African species into three strips namely Capsulati, Cephalocarpi, Monospermi and seven substrips namely Fruticosi, Cuspidati, Indici, Graveolenti, Microcarpi, Blepharocarpi and Mutici. This classification was based on leaves, carpels and seeds morphology. Hutchinson in 1967 treated *Abutilon* in tribe Abutileae and subtribe Abutilneae. The interspecific relationships and infrageneric classification of *Abutilon* species is still unclear (Fryxell, 2002) as the species of *Abutilon* showed morphological variations. Therefore, there is a need to explore phylogenetic relationships using molecular markers.

The DNA sequence data obtained from different noncoding regions of chloroplast genome has found tremendous applications in the field of plant molecular phylogenetics (Shinwari *et al*.,1994a; Dong *et al*., 2012; Jamil *et al*., 2014; Zahra *etal.,*2016; Shinwari *et al*., 2018; Sun *et al*., 2020; Idress *et al*., 2021). Among different noncoding regions, *psbA-trn*H intergenic spacer region considered potential molecular marker in discriminating

the species (Channa *et al*., 2018; Feng *et al*., 2018; Yang *et al*., 2020) and inferring their interspecific and infrageneric relationships (Tseng *et al*., 2019).

The infrageneric and interspecific relationships of *Abutilon* species is still unclear as it is very difficult to delineate the species and exhibit their association with other species based on morphological features (Fryxell, 2002, Donnell *et al*., 2012). The *psbA-trn*H is one of the most variable region of CpDNA in terms of having the highest percentage of variable sites (Shaw *et al*., 2007) and it can provide high levels of species discrimination (Kress *et al*., 2005; Shaw *et al*., 2007). In *Abutilon*, no work has been done for determination of interspecific association using molecular data. Therefore, the current study aimed to explore the phylogenetic association among *Abutilon* species and to explore the potential of *psbA-trn*H region as a molecular marker for *Abutilon* species identification and phylogenetic studies.

Material and Method

Species collection: Fifteen species of the genus *Abutilon* and one species of the genus *Sida* were included in the study. Since *Sida ovata* is a cloosely related species to the genus *Abutilon* therefore it was used as an outgroup. Fresh leaf samples of eight *Abutilon* species namely *A. indicum, A. bidentatum, A. fruticosum, A. pakistanicum, A. karachianum, A. sepalum, A. pannosum, A. theopharasti* and one *Sida* species *(Sida ovata)* were collected from different areas of Sindh and Karachi during field survey. While the dried leaf samples of remaining *Abutilon* species*; A. ramosum, A. grandifolium, A. hirtum, A. muticum, A. alii, A. figarianum* and *A. gafoorianum* were obtained from Center for Plant Conservation, Herbarium, University of Karachi. The collected species were identified using flora of Pakistan and their herbarium sheets were deposited at the Center for Plant Conservation, University of Karachi. The collected species along with their names, localities and voucher specimen numbers are presented in (Table 1).

Total genomic DNA extraction: Total genomic DNA was isolated from fresh leaf samples using a modified Cetyle Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1987). While genomic DNA from dried leaf material was extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The extracted DNA was quantified using nano photometer (Implan, Germany) and its quality was checked by agarose gel electrophoresis (Cleaver Scientific HU10, UK). The isolated DNA was diluted and stored at -20ºC for further use.

PCR and DNA sequencing: The *psbA-trn*H intergenic spacer region was amplified using forward (5`GTTATGCATGAACGTAATGCTC 3`) and reverse (5` CGCGCATGGTGGATTCACAATCC 3`) primers of about 22-23 bp in length. For the amplification of *psbA-trn*H region, a PCR master mix was prepared in a 30 µl volume containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.1 µM each of forward and reverse primers, 1 unit of DNA Taq polymerase, 50 ng of DNA template, and appropriate amount of milli Q water. The thermal cycler conditions were optimized as follows: initial denaturation at 94ºC for 1 minute, followed by 30 cycles of denaturation at 94ºC for 30 seconds, primer annealing at 50 ºC for 30 seconds, extension at 72ºC for 1 minute. For the amplification of DNA isolated from dried leaf samples, a ready to use Master mix (MOLEQULE-ON, New Zealand) was used with the same thermal cycler condition. The PCR products were purified using a PCR product purification kit (Bioneer, Korea) and then sequenced from commercial laboratory (MOLEQULE-ON, New Zealand). The sequences were analyzed using bioinformatics tools to identify any discrepancy present in sequences. Sequences similarity was assessed using Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990). The analyzed sequences were submitted to the National Center for Biotechnology Information (NCBI) nucleotide sequence database (GenBank). The accession numbers issued by GenBank for the submitted sequences were recorded and are listed in (Table 1).

Table 1. Species with locality, General Herbarium Numbers (G.H.NO.) and accession numbers of *psbA-trn*H intergenic spacer.

S. No.	Name of species	Locality	G.H. No.	Accession No. *psbA-trn*H
1.	*Abutilon indicum*	Karachi	95761	OR188577
2.	*Abutilon bidentatum*	Rawalpindi	95762	OR188578
3.	*Abutilon fruticosum*	Karachi	94792	OR188579
4.	*Abutilon pakistanicum*	Karachi	95766	OR188580
5.	*Abutilon karachianum*	Karachi	94793	OR188581
6.	*Abutilon sapalum*	Karachi	95759	OR188582
7.	*Abutilon pannosum*	Peshawar	95757	OR188583
8.	*Abutilon grandifolium*	Peshawar	35246	OR188584
9.	*Abutilon ramosum*	Karachi	35651	OR188585
10.	*Abutilon gafoorianum*	Changa manga	35237	OR188586
11.	*Abutilon figarianum*	Sonmiani	35197	OR188587
12.	*Abutilon hirtum*	Gwadar	KUH538	OR188588
13.	*Abutilon muticum*	Gwadar	35594	OR188589
14.	*Abutilon alii*	Karachi	35115	OR188590
15.	*Abutilon theopharasti*	Chitral	35663	OR188591
16.	*Sida ovata*	Karachi	95753	OR188592

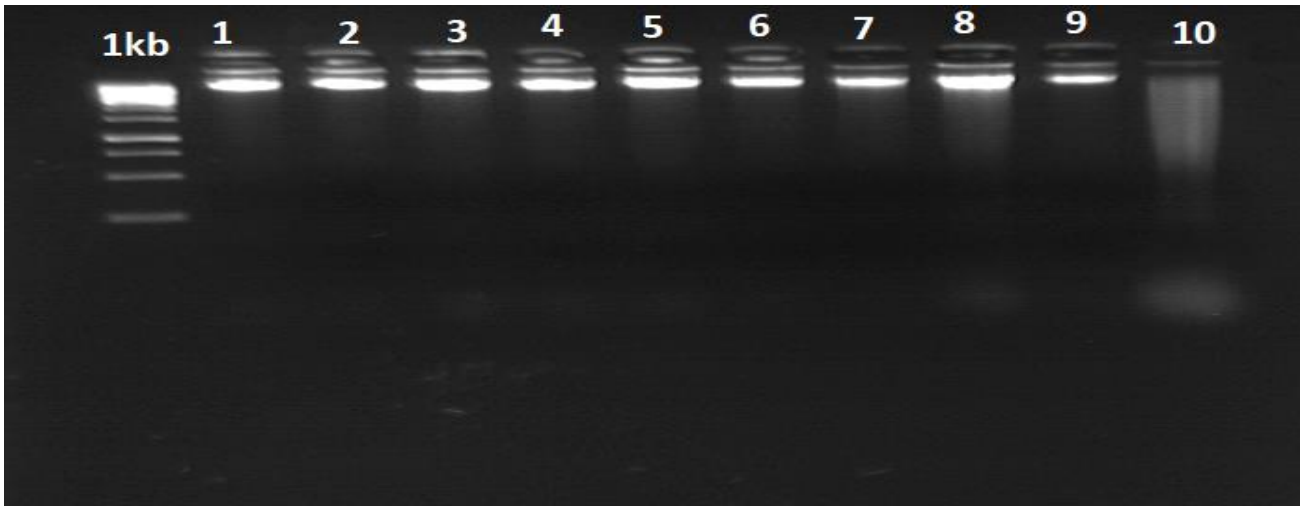

Fig. 1. Image showing the extracted DNA from some *Abutilon* species.

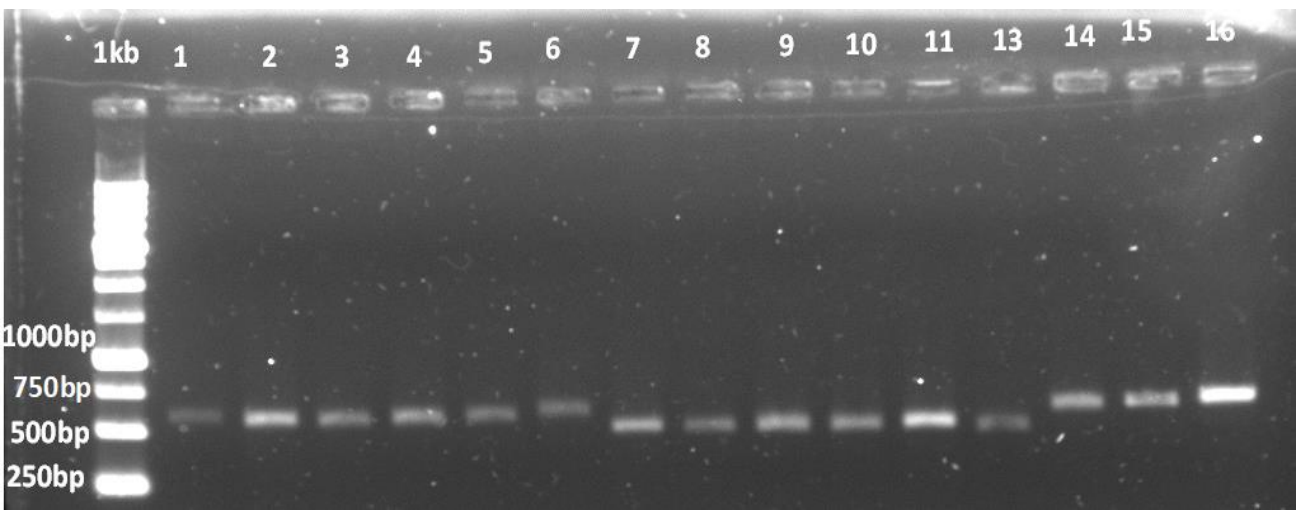

Fig. 2. The image of amplified PCR product of *psbA-trn*H intergenic spacer region of *Cp*DNA.

Sequence data analysis: To generate the phylogenetic tree, all analyzed sequences of the *psbA-trn*H were aligned using the online sequence alignment tool ClustalW (Larkin *et al*., 2007). The tree was reconstructed using Bayesian Inference analysis through the Bayesian Evolutionary Analysis Sampling Trees (BEAST) software version 1.7.5 (Drummond *et al*., 2012). The General Time Reversible model with Invariant sites and Gamma distribution (GTR+I+G) was applied as the nucleotide substitution model for *psbA-trn*H sequence data as it was identified the best fit model for nucleotide substitution (Abadi *et al*., 2019). In the analysis, the sequence data of all *Abutilon* species was considered as the ingroup while the sequence data of the species of *Sida ovata* (s species form genus *Sida*) was used as the outgroup. Forthe Bayesian analysis following parameters were applied: 800000 generations were set in which trees were screened at every 200 generations and the first 4% of sampled trees were calculated as burn-in using Markov Chain Monte Carlo (MCMC) and posterior probability of the remaining tree was also calculated using MCMC. The generated phylogenetic tree was viewed in Fig tree software version 1.4 (Rambaut, 2012).

Results

The genomic DNA was successfully extracted using CTAB and Kit method (Fig. 1). In addition, the *psbA-trn*H intergenic spacer region was also successfully amplified (Fig. 2).

The phylogenetic tree (Fig. 3) generated from Bayesian analysis, grouped all investigated species of genus *Abutilon* into two lineages: lineage I and Lineage II. The lineage I, consists of only one species *A. theopharasti*. While lineage II was divided into two groups: group I and group II.

Within group I, four species: *A. pakistanicum, A. grandifolium, A. fruticosum*, and *A. ramosum* were found in cluster. The group I was divided into two clades. In one clade, *A. pakistanicum and A. grandifolium* revealed close relationships with strong support (1PP). Similarly, in second clade, *A. fruticosum* and *A. ramosum* depicted close association by receiving strong support (1PP). However, the connection between both clade was not strongly supported (0.62 PP).

Within group II, the remaining *Abutilon* species were clustered into two clades: clade I and clade II. In clade I, four species: *A. bidentatum, A. indicum, A. figarianum* and *A. gafoorianum* were found in a cluster and showed close

affinity. In this cluster, *A. bidentatum* was obtained as sister to *A. indicum, A. figarianum* and *A. gafoorianum*.

In clade II, six *Abutilon* species: *A. karachianum, A. hirtum, A. sepalum, A. muticum, A. pannosum* and *A. alii* were grouped into two subclade. In the first subclade, *A. karachianum* and *A. hirtum* exhibited close relationships by receiving powerful support (0.92 PP) while *A. sepalum* was observed as sister to these two species with weak support (0.41 PP). In subclade II, three species: *A. muticum, A. pannosum* and *A. alii* showed close relationships. Within this subclade, the close relationships between *A. pannosum* and *A. alii* was moderately supported (0.78 PP). Similarly, the sister association of *A. muticum* to these species was also moderately supported (0.84). The overall topology of the tree was fully resolved.

Discussion

In the current study, fifteen species of the genus *Abutilon* that are found in Pakistan were included to infer their interspecific and infrageneric association using the intergenic spacer region (*psb*A-*trn*H) of the chloroplast genome. Bayesian analysis was performed to generate the phylogenetic tree, which was well-resolved. Based on the *psb*A-*trn*H sequence data, the *Abutilon* species were clustered into two groups. One group was composed of four *Abutilon* species namely A. *pakistanicum, A. grandifolium, A. fruticosum* and *A. ramosum.* The second group with further division includes ten *Abutilon* species *namely A. bidentatum, A. indicum, A. figarianum, A. gafoorianum, A. karachianum, A. hirtum, A. sepalum, A. muticum, A. pannosum* and *A. alii*. One species, *A. theopharasti* was found to be sister to these two groups.

The *psbA-trn*H sequence data showed that four *Abutilon* species, *A. pakistanicum, A. grandifolium, A. fruticosum* and *A. ramosum* were grouped together. This grouping may be based on the number of carpels, as in all four species, the number of carpels does not exceed 12. Within this group, *A. pakistanicum* and *A. grandifolium* revealed a close association. This close relationships may be attributed to some shared external morphological features, such as both species having 10 carpels, ovoid fruit shape, carpel lengths and fruit diameters exceeding 1cm (Abedin, 1979; Personal communication). However, these species differ in other characteristics. For example, in *A. grandifolium*, the flowers are more than 2 cm across, orange yellow in color, calyx is larger than the fruit, and the plant is covered with markedly long hairs. In contrast, in *A. pakistanicum,* the flowers are less than 2 cm across, yellow in color, the calyx is smaller than the fruit and markedly long hairs are absent on the plant (Husain & Baquar, 1974; Abedin, 1979). The *psbA-trn*H is a rapidly evolving region of chloroplast genome and accumulates genetic variations (phylogenetically important variations) that are useful to discriminate the taxa at species level (Feng *et al*., 2018; Hao *et al*., 2010; Štorchová & Olson, 2008; Hamilton *et al*., 2003). In the current study, *psbA-trn*H region successfully defined the association and discriminated the *Abutilon* species.

Similarly, within this group, *A. fruticosum* and *A. ramosum* were found to be closely related. This close affinity may be attributed to similarities in having 8-12 carpels, a cylindric fruit shape, a carpel length and fruit diameter of less than 1cm, and filiform stipules. However, these species can be differentiated by certain characteristics. For instance, *A. fruticosum* has truncated carpels (carpels without beaks or awns) and hoary (grayish white) leaves with 7 nerves. While *A. ramosum* has beaked or awned carpels, and glabrescent leaves with 9 nerves. The number of carpels, fruit shapes, carpels having beak or not are considered reliable characteristics for the discrimination, and classification of *Abutilon* species (Chaudhary, 2001; Alfarhan *et al*., 2005; Anatriello, 2007; Kunnur & Kkotresha, 2011; Brown, 2012; Gill & Kaur, 2015; Bano and Deora, 2017; Migahid, 1988; Abedin, 1979; Husain & Baquar, 1974). The *psbA-trn*H sequence data strongly supported the close association between these species as no variation was observed in the sequence of both species. Mattei in 1915, revised the infrageneric classification of East African Species of *Abutilon* and placed the *A. fruticosum* and *A. ramosum* in strip Capsulati but in different substrip Fruticosi and Cuspidati respectively. This placement in Capsulati was based on species having similar number of carpels that are not exceeding from the central axis, forming true capsule that dehiscent at the time of maturity. The *psbA-trn*H analysis confirmed the close association and placement of these species in strip Capsulati. and substrip Fruticosi and Cuspidati respectively.

The *psb*A-*trn*H sequence data supported the grouping of three *Abutilon* species namely *A. muticum, A. pannosum* and *A. alii.* This grouping may be attributed to some shared morphological characteristics such as all three species have globose fruit with obtuse carpels, stellate pubescent covering the entire plant, and mericarp that separate after dehiscence. within this clade, *A. pannosum* and *A. alii* showed a close association while *A. muticum* appeared as sister to these two. The association among these species can be further discussed in light of the flora of Pakistan (Abedin, 1979; Husain & Baqur, 1974; personal communication). The close affinity between *A. pannosum* and *A. alii* may be due to some shared morphological traits, such as lanceolate stipules, velvety upper leaf surfaces, fruit not more than 28 carpels, 3 seeded mericarps, and flower diameter exceeding 2.6 cm. In cintrast, *A. pannosum,* differss slightly from these species as it has linear stipules, scabrous upper leaf surfaces, fruits with more than 28 carpels, 2 seeded mericarps, and flower diameters of up to 2.6 cm. Early morphology based study suggested that *A. pannosum* and A. *muticum* are distinct species, they are not conspecific (Abedin, 1980). This study also suggested that *A. pannosum* and *A. muticum* are two distinct, but closely related species. A previous study based on ITS sequence also reported a close association between these two species (Grewal & Kaur, 2020). Mattei in 1915, placed *A. muticum* and *A. pannosum* in strips Cephalocarpi and substrip Mutici due to their obtuse carpels and dens hairs covering the plant. The *psbA-trn*H sequence data supports the placement of both species in strip Cephalocarpi and substrip Mutici and suggests the placement of *A. alii* in substrip Mutici along with *A. muticum* and *A. pannosum.*

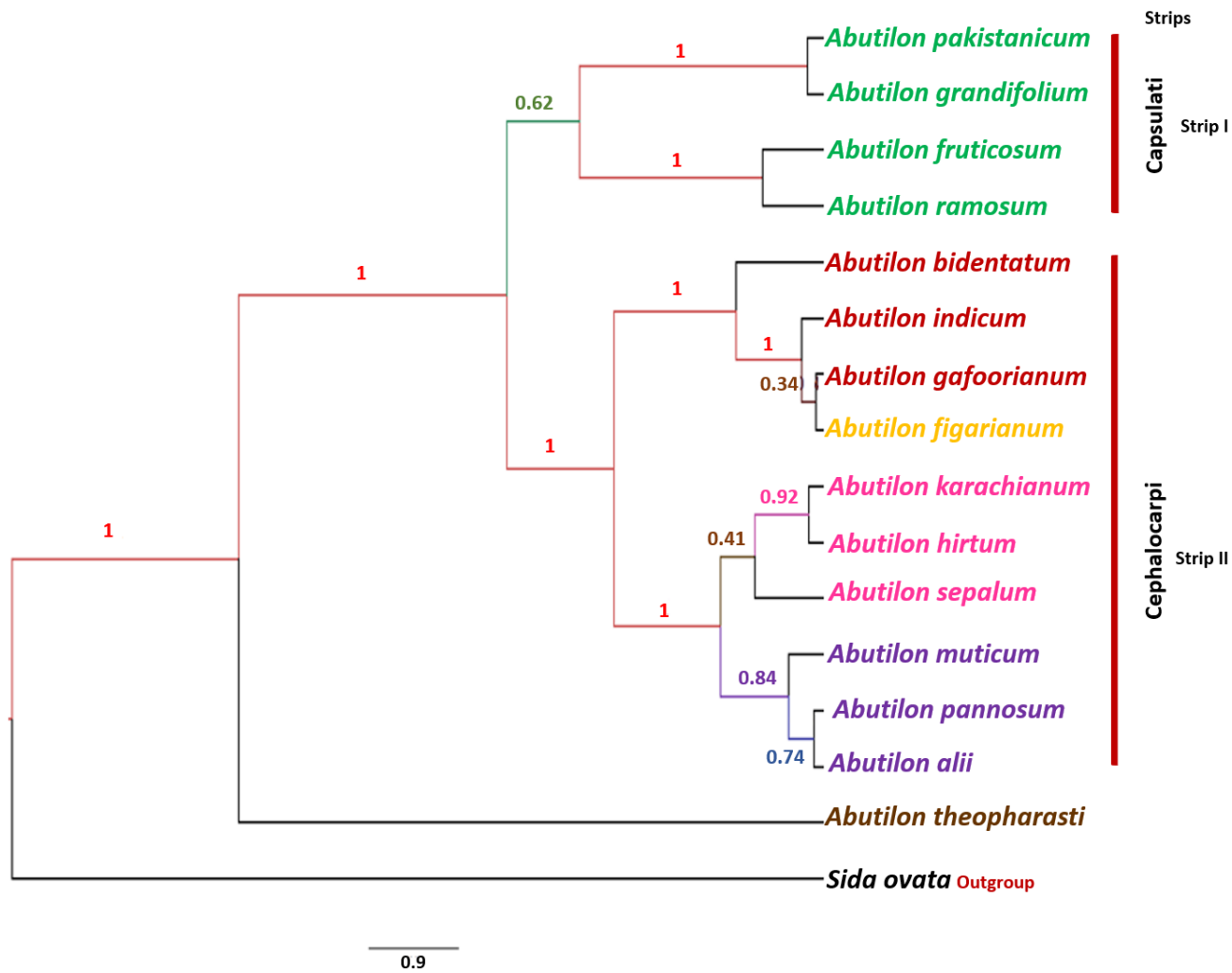

Fig. 3. Bayesian Inference (BI) trees are based on CpDNA (*psbA-trn*H) sequence data of *Abutilon* and outgroup species. Numbers above the branches are Posterior Probability (PP) obtained from Bayesian analysis.

In clade II of group II, three *Abutilon* species, namely *A. sepalum, A. karachianum* and *A. hirtum* were grouped together. The grouping of these three species into a single clade may be supported by some shared external fruit characteristics, such as the fruit of all three species being flat or truncate at the apex with mucronate (slightly pointed) carpels that remain adpressed to each other in the fruit (Abedin, 1979; Husain & Baqur, 1974; Personal Communication). In this group, *A. hirtum* and *A. karachianum* formed a close association with strong support while *A. sepalum* served as sister to these two species with weak support. The relationships between *A. hirtum* and *A. karachianum* may be based on their macronate carpels which are longer than calyx in the fruit, a carpel range of 20-30, and the presence of a non- leathery calyx in the fruit. Alternatively, it may be due to some molecular evidence that, to the best of our knowledge, has not been reported previosly. The sister species (*A. sepalum)* is different from the other two species in having carpels that are shorter than the calyx in the fruit, a carpels range of 27-33, and a leathery calyx in the fruit. The number of carpels, carpels having beak or not and the length of calyx in relation to fruit size are considered reliable characteristics for species discrimination and infrageneric classification of *Abutilon* species (Husain & Baqur, 1974). The *psb*A-*trn*H region supported the placement of *A. sepalum* in group II along with *A. hirtum* and *A. karachianum* but was unable to resolve its sister relationships. It is suggested that increasing the sample sizes may help clarify the association of *A. sepalum* with other species. Mattei (1915) Placed the *A. hirtum* in strip Cephalocarpi and substrip Microcarpi due to the presence of mucronate and minute shining carpels/ mericarp. The *psbA-trn*H analysis provided molecular evidence supporting these morphological characteristics for the placement of *A. hirtum* in substrip Microcarpi. The study also suggests the placement of *A. karachianum* and *A. sepalum* in substrip Microcarpi along with *A. hirtum*.

In the first clade of group II, four *Abutilon* species *A. bidentatum, A. indicum, A. figarianum* and *A. gafoorianum* were clustered. The clustering of these species may be attributed to the number of mericarp which do not exceed 20 (ranging from 13-20), presence of a staminal tube with stellate hairs, prominently toothed leaf margins and acute to acuminate leaf apices. Within this clade, *psbA-trn*H sequence data weakly supported the close association between *A. figarianum* and *A. gafoorianum*. These two species differ in morphological traits, such as shape of stipule and fruit. In *A. figarianum,* fruit shape is globose, and the stipule is lanceolate while in *A. gafoorianum,* the

fruit shape is cylindric and the stipule is linear. However, these species exhibit similarities in their DNA sequences. It is suggested that the placement of *A. figarianum* in this clade and its association should be further evaluated by increasing the number of replicates from both species.

Within this clade, *A. indicum* showed close relationships with *A. gafoorianum* and *A. figarianum*. Morphologically, *A. indicum* resembles *A. gafoorianum* in having a similar general plant habit (under shrub or shrub), linear stipules, cylindrical fruits, orange yellow or yellow flower, flower size (2.5-3 cm across), 15-20 mericarps and fruit with deep ridges and furrows throughout. However, these species differ in other characteristics such as in *A. indicum* mericarps remain erect at maturity while in *A. gafoorianum* mericarps spread stellately at maturity. In *A. indicum,* the mericarps dehisce after breaking away from the central axis, and short spreading hairs are present at least on the young parts of the plant. In cntrast in *A. gafoorianum*, mericarp dehisce before breaking away from the central axis and spreading hairs are lacking only present on the top of the petiole. Mattei in 1915, placed the *A. indicum* in strip Cephalocarpi and substrip Indici.

A. theopharsti is formed a strongly supported basal lineage, serving as a sister species to the rest of the *Abutilon* species. Morphology this species differs from the other species in having a glabrous staminal tube, minutely toothed leaf margins, 12- 20 scabrous mericarp, mericarps with 3-5 mm long awns that spread horizontally, and sepals that are neither leathery nor exceed the fruit. The placement of *A. theopharasti* in a basal position with strong support in the phylogenetic tree suggests that *A. theopharasti* evolved earlier than other species during evolution. Therefore, it may be inferred that *A. theopharasti* represents a primitive species.

In our analysis, the use of intergenic spacer region (*psbA-trn*H) to assess interspecific relationships proved to be highly effective. Particularly, for the genus *Abutilon,* a good resolution was achieved, as the associations of all investigated species were established with strong support, except for three species whose relatinships remained unclear. This is because the *psbA-trn*H is a rapidly evolving region of the chloroplast genome therefore, accumulating the high levels of nucleotide sequence variations. These variations are sufficient to infer evolutionary relationships, specifically at the interspecific level (Shaw *et al*., 2005, 2007). All groups were well defined and further supported by external morphological characters.

Conclusions

The relationships established in the current study provide a useful framework for future systematics reviews of genus *Abutilon* and for the selection of species for pre-breeding programs. By exploring phylogenetic relationships, underutilized species can be identified and utilized for beneficial purposes. The study concluds that our data supports the morphological classification and provides molecular evidence to morphological findings. In the future, further resolved relationships at interspecific and infrageneric level can be achieved by including an increased number of *Abutilon* species.

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Conflict of interest: The authors have no conflict of interest to declare.

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