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AUTHENTICATION OF *RUTA GRAVEOLENS* AND ITS ADULTERANT USING INTERNAL TRANSCRIBED SPACER (ITS) SEQUENCES OF NUCLEAR RIBOSOMAL DNA

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Abstract

Ruta graveolens L. (Rutaceae) is commonly known as '*Sudab'* which is well known for hippocratic medicine and is commonly used in indigenous health-care system in India. *Euphorbia dracunculoides* Lam. (Euphorbiaceae) in raw drug trading has almost similar morphology to *R. graveolens* in dried state, is being sold locally or used clinically as an adulterant of *R. graveolens* (genuine) at a relatively low price under the same name '*Sudab*' which has ultimately reduced the efficacy and quality of this herb. The internal transcribed spacer (ITS) sequence of nuclear ribosomal DNA gene of genuine and adulterant were sequenced and analyzed to assess species admixture in raw drug trading of genuine herbal drug. The BLAST search results of ITS sequence of genuine sample of '*Sudab'* i.e., *R. graveolens* showed 99% similarity to the sequence of *R. graveolens*, however, *E. dracunculoides* showed 100% similarity to the species of *Euphorbia* and did not show any similarity with *R. graveolens*. The sequence alignment of both species was entirely different to each other. Phylogenetic analysis based on ITS sequence of adulterant sample i.e., *E. dracunculoides* together with sequences of *Euphorbia* species available in the GenBank has also clearly showed its nesting within the *Euphorbia* tree. The generated ITS sequences of both samples in the present study may be referred hereafter as species-specific DNA barcode signature, which can be used in authenticating and validating the exact species identities to discriminate the genuine sample of '*Sudab*' from its adulterants if any available to guarantee the quality and purity of this drug in the herbal drug market.

Introduction

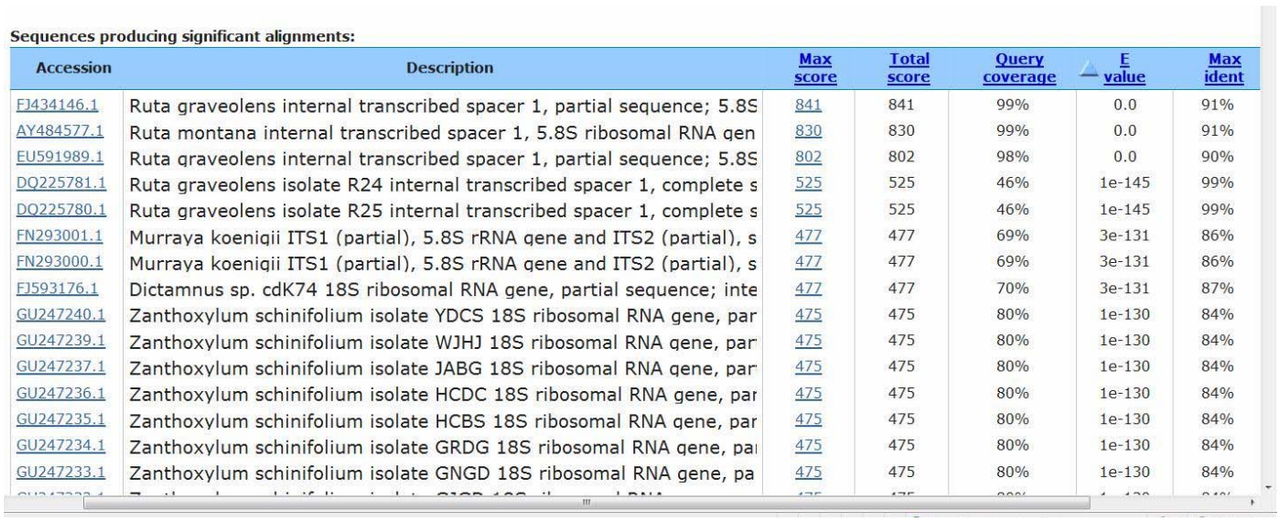
Exact identification of medicinal plants is necessary to ensure the quality of herbal drugs, because in most cases medicinal herbs are knowingly or unknowingly substituted or adulterated with similar species or varieties (Kiran *et al*., 2010). Many herbal drugs commercially available still cannot be authenticated or identified using their morphological or histological characteristics. Use of a wrong herb may be ineffective or in some cases even fatal.

A variety of plants of the family Rutaceae are used as traditional medicine world-wide. The most common medicinal plant of this family is *Ruta graveolens* L., which is commonly known as '*Rue*' or '*Sudab'* in Hindi (Indian language). Although it is native to Europe, it is distributed throughout the world. It is an ornamental evergreen shrub of up to one m tall and has considerable medicinal importance. More than 120 natural compounds mainly including acridone alkaloids, coumarines, essential oils, flavonoids, and furoquinolines have been found in the roots and aerial parts of this plant (Kuzovkinaa *et al*., 2004). Due to the presence of these potential compounds, it is being widely used for medicinal purpose from very ancient time but prudence of its use is still contentious. The plant is widely used as anti-inflammatory, antiviral, and anti-plasmodial (Raghav *et al*., 2006), antimicrobial and cytotoxic (Ivanova *et al*., 2005), as well as contraceptive (Maurya *et al*., 2004)**.** For example, the polyphenolic and alkanoid fractions of *R. graveolens* showed protective effects on acute and chronic inflammation in rat (Ratheesh *et al*., 2010). Total extract (70% ethanol) of this plant showed *in vitro* cytotoxicity against tumor cell lines of different origin (Varamini *et al*., 2009). Another species belonging to a different genus, i.e., *Euphorbia dracunculoides* Lam., (family Euphorbiaceae) has similar morphology in dried state, and is being sold locally or used clinically as a replacement of *R. graveolens* at a relatively low price under the same name "*Sudab*" (Khan *et al*., 2011). This has reduced the efficacy and quality of *R. graveolens*. The adverse effects of *E. dracunculoides* were studied as epistaxis, nausea/vomiting and haematuria (Rahman *et al*., 2003) which were found different from those of *R. graveolens*.

Limitations of biochemical and morphological markers for authentication of herbal drugs have triggered the need to develop more reproducible molecular markers for quality control of medicinal plants. Recently, efforts have been made to ensure accurate identification of medicinal plants used in raw drug trade to guarantee the purity and quality of the drugs (Jayasinghe *et al*., 2009). Despite a number of classical methods including examination of wood anatomy and morpho-taxonomical keys, a variety of DNA-based methods have been recently developed for the identification of medicinal plants (Sucher *et al*., 2008). Authentication of *R. graveolens* was earlier carried out (Khan *et al*., 2011) using RAPD markers, but due to its less reproducibility it has generated the need to develop more reproducible markers for identification of this herbal drug under raw condition for the preservation of the quality of this potential medicinal herb.

The nuclear ribosomal transcriptional unit (NRTU) comprises 18S, 5.8S and 28S genes, two ITS (ITS-1 and ITS-2), and hundreds to thousands tandem copies of an intergenic spacer (IGS) within plant genomes. The conserved regions (18S and 28S genes) of NRTU are used to draw phylogenetic relationships at higher taxonomic levels, whereas the more rapidly evolving segments, ITS and IGS, are employed for studies at the genic or population levels (Alvarez & Wendel, 2003). The nrDNA region has frequent insertions/deletions, which can be phylogenetically informative (Baldwin, 1995). Because of their different rates of evolution, the ITS regions have become preferable markers in evolutionary studies in different taxonomic levels (Gulbitti-Onarici *et al*., 2009). Internal transcribed spacer sequences (ITS) of nrDNA have been widely used for resolving phylogenetic relationships in many plant species (Gulbitti-Onarici *et al*., 2009; Pandey & Ali, 2006; Choo *et al*., 2009), molecular authentication of herbal materials (Zhang *et al*., 2007), genetic diversity assessment (Mondini *et al*., 2009), intra-specific variation study (Haque *et al*., 2009), and DNA barcoding (Zuo *et al*., 2010). Recently, ITS sequence based SCAR marker has been developed for the discrimination of *Paphiopedium armeniacum*, *P. micranthum*, *P. delinetii* and their hybrids (Sun *et al*., 2010). Genuine sample specific markers are required to maintain the quality of this medicinal herb for

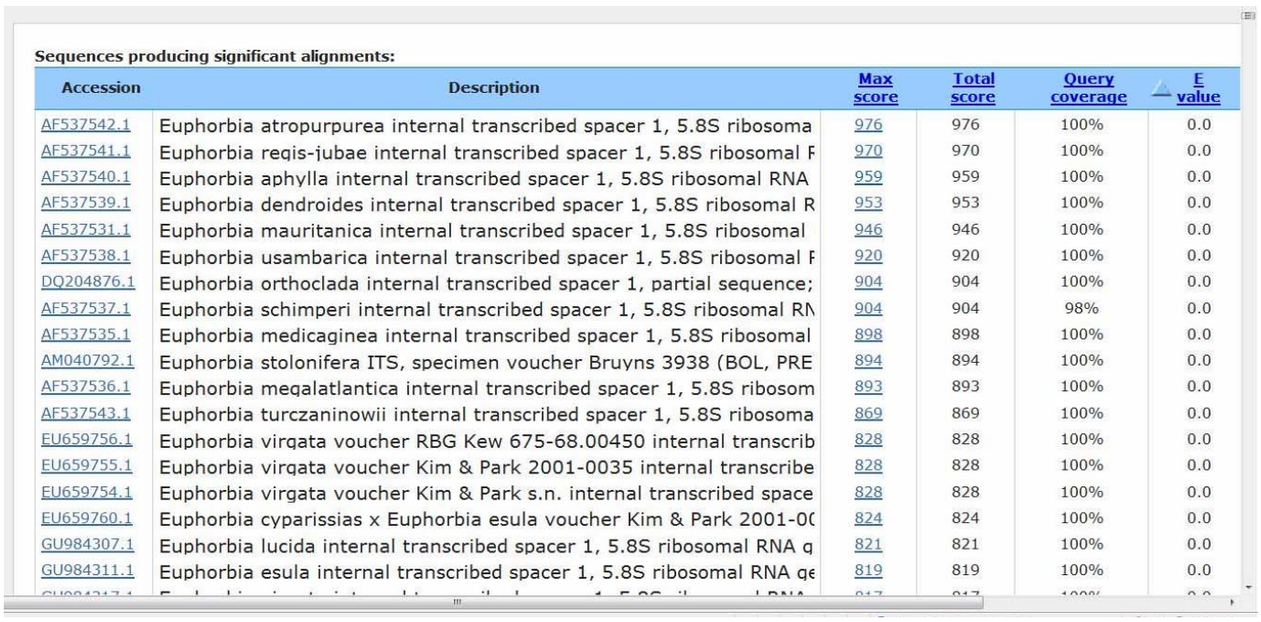
herbal formulations. Since, nrDNA ITS sequences are highly reproducible under wide laboratory conditions as compared to other DNA markers like RAPD, so our major objective was to assess utility of nrDNA-ITS sequence for accurate identification of *R. graveolens,* and differentiate and authenticate *R. graveolens* from *E. dracunculoides.*



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ434146.1	Ruta graveolens internal transcribed spacer 1, partial sequence; 5.8S	841	841	99%	0.0	91%
AY484577.1	Ruta montana internal transcribed spacer 1, 5.8S ribosomal RNA gen	830	830	99%	0.0	91%
EU591989.1	Ruta graveolens internal transcribed spacer 1, partial sequence; 5.8S	802	802	98%	0.0	90%
DQ225781.1	Ruta graveolens isolate R24 internal transcribed spacer 1, complete s	525	525	46%	1e-145	99%
DQ225780.1	Ruta graveolens isolate R25 internal transcribed spacer 1, complete s	525	525	46%	1e-145	99%
FN293001.1	Murraya koenigii ITS1 (partial), 5.8S rRNA gene and ITS2 (partial), s	477	477	69%	3e-131	86%
FN293000.1	Murraya koenigii ITS1 (partial), 5.8S rRNA gene and ITS2 (partial), s	477	477	69%	3e-131	86%
FJ593176.1	Dictamnus sp. cdK74 18S ribosomal RNA gene, partial sequence; inte	477	477	70%	3e-131	87%
GU247240.1	Zanthoxylum schinifolium isolate YDCS 18S ribosomal RNA gene, par	475	475	80%	1e-130	84%
GU247239.1	Zanthoxylum schinifolium isolate WJHJ 18S ribosomal RNA gene, par	475	475	80%	1e-130	84%
GU247237.1	Zanthoxylum schinifolium isolate JABG 18S ribosomal RNA gene, par	475	475	80%	1e-130	84%
GU247236.1	Zanthoxylum schinifolium isolate HCDC 18S ribosomal RNA gene, par	475	475	80%	1e-130	84%
GU247235.1	Zanthoxylum schinifolium isolate HCBS 18S ribosomal RNA gene, par	475	475	80%	1e-130	84%
GU247234.1	Zanthoxylum schinifolium isolate GRDG 18S ribosomal RNA gene, par	475	475	80%	1e-130	84%
GU247233.1	Zanthoxylum schinifolium isolate GNGD 18S ribosomal RNA gene, pa	475	475	80%	1e-130	84%

Fig. 1. Screenshot showing ITS sequences similarity of *Ruta graveolens* under BLAST search.



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value
AF537542.1	Euphorbia atropurpurea internal transcribed spacer 1, 5.8S ribosoma	976	976	100%	0.0
AF537541.1	Euphorbia regis-jubae internal transcribed spacer 1, 5.8S ribosomal R	970	970	100%	0.0
AF537540.1	Euphorbia aphylla internal transcribed spacer 1, 5.8S ribosomal RNA	959	959	100%	0.0
AF537539.1	Euphorbia dendroides internal transcribed spacer 1, 5.8S ribosomal R	953	953	100%	0.0
AF537531.1	Euphorbia mauritanica internal transcribed spacer 1, 5.8S ribosomal	946	946	100%	0.0
AF537538.1	Euphorbia usambarica internal transcribed spacer 1, 5.8S ribosomal R	920	920	100%	0.0
DQ204876.1	Euphorbia orthoclada internal transcribed spacer 1, partial sequence;	904	904	100%	0.0
AF537537.1	Euphorbia schimperi internal transcribed spacer 1, 5.8S ribosomal RN	904	904	98%	0.0
AF537535.1	Euphorbia medicaginea internal transcribed spacer 1, 5.8S ribosomal	898	898	100%	0.0
AM040792.1	Euphorbia stolonifera ITS, specimen voucher Bruyns 3938 (BOL, PRE	894	894	100%	0.0
AF537536.1	Euphorbia megalatlantica internal transcribed spacer 1, 5.8S ribosom	893	893	100%	0.0
AF537543.1	Euphorbia turczaninowii internal transcribed spacer 1, 5.8S ribosoma	869	869	100%	0.0
EU659756.1	Euphorbia virgata voucher RBG Kew 675-68.00450 internal transcrib	828	828	100%	0.0
EU659755.1	Euphorbia virgata voucher Kim & Park 2001-0035 internal transcribe	828	828	100%	0.0
EU659754.1	Euphorbia virgata voucher Kim & Park s.n. internal transcribed space	828	828	100%	0.0
EU659760.1	Euphorbia cyparissias x Euphorbia esula voucher Kim & Park 2001-0(	824	824	100%	0.0
GU984307.1	Euphorbia lucida internal transcribed spacer 1, 5.8S ribosomal RNA g	821	821	100%	0.0
GU984311.1	Euphorbia esula internal transcribed spacer 1, 5.8S ribosomal RNA ge	819	819	100%	0.0

Fig. 2. Screenshot showing ITS sequences similarity of *Euphorbia dracunculoides* under BLAST search.

Materials and Methods

Raw drug trade sampling: Samples of both *Ruta graveolens* and *Euphorbia dracunculoides* were collected under the same vernacular name '*Sudab'* from the local herbal market Khari Baoli, Delhi, India. In the local market, the sample of *E. dracunculoides* was found as adulterant when identified very carefully at species level morphologically.

Genomic DNA extraction and amplification of ITS region: Leaf powder (100 mg each) was used for total genomic DNA extraction in accordance with the modified CTAB method (Khan *et al*., 2007). Total genomic DNA was used in the polymerase chain reaction for the amplification of nrDNA ITS regions from *R. graveolens* as well as *E. dracunculoides*. ITS sequences of nrDNA were amplified using primers {ITS1 (Forward 5'-GTCCACTGAACCTTATCATTTAG-3') and ITS4 (Reverse 5'-TCCTCCGCTTATTGATATGC-3')} of White *et al*., (1990) using the AccuPower HF PCR PreMix (Bioneer, Daejeon, South Korea) in 20 µL volumes containing 2 µL of 10X buffer, 300 µM dNTPs, 1 µL of a 10 pm solution of each primer, 1 unit of HF DNA polymerase. One round of amplification consisting of denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min., annealing at 49°C for 1 min., and extension at 72°C for 1 min with a final extension step of 72°C for 5 min. The PCR products were purified using SolGent PCR purification Kit-Ultra (SolGent, Daejeon, South Korea) prior to sequencing.

DNA sequencing: The amplified products were directly sequenced at Macrogen Inc., South Korea using dye terminator chemistry. The sequencing reaction was performed in a 10 µL final volume with the BigDye terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems). Cycle sequencing was conducted using same primers used in amplification and

BigDye vers. 3 reagents and an ABI PRISM 3100 DNA Analyzer (Perkin-Elmer, Applied Biosystems). Cycling conditions included an initial denaturing set at 94°C for 5 min, followed by 30 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. The sequenced product was precipitated with 17 µL of deionized sterile water, 3 µL of 3 M sodium acetate solution, and 70 µL of 95% ethanol. Polyacrylamide gel electrophoresis was conducted with long ranger single packs (FMC BioProducts) and an ABI 3100 automated DNA sequencer (Perkin-Elmer, Applied Biosystems).

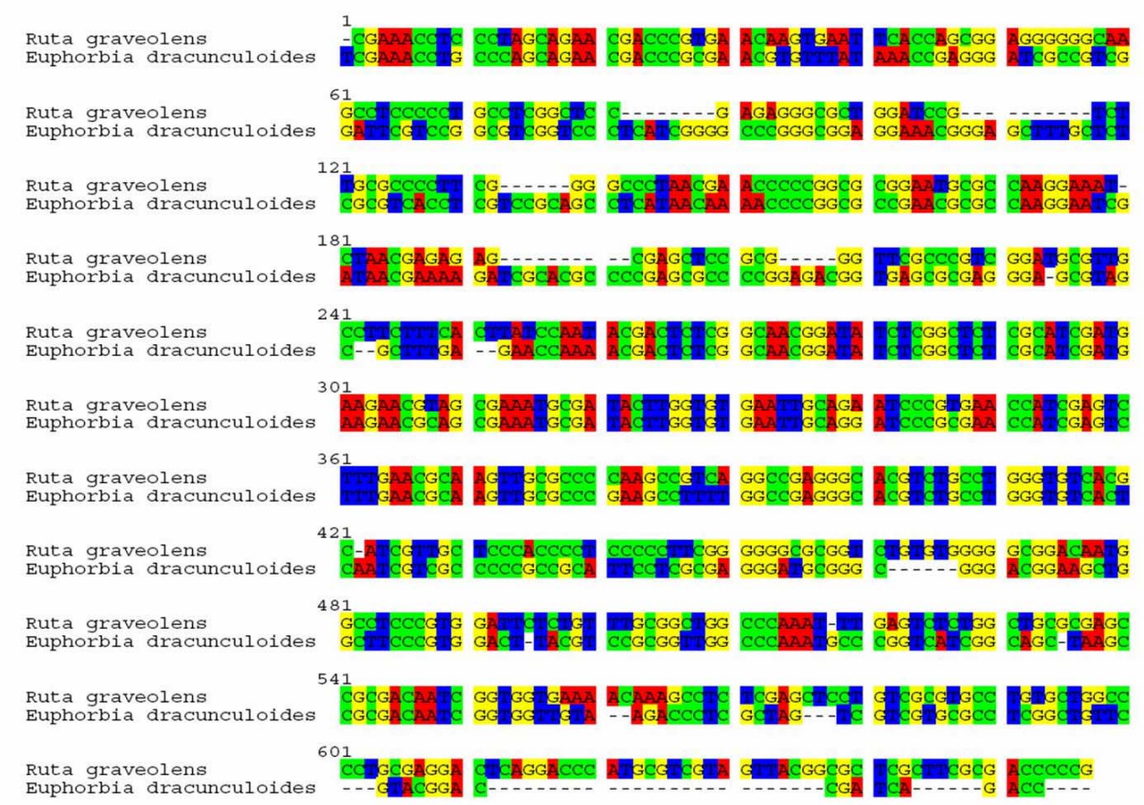
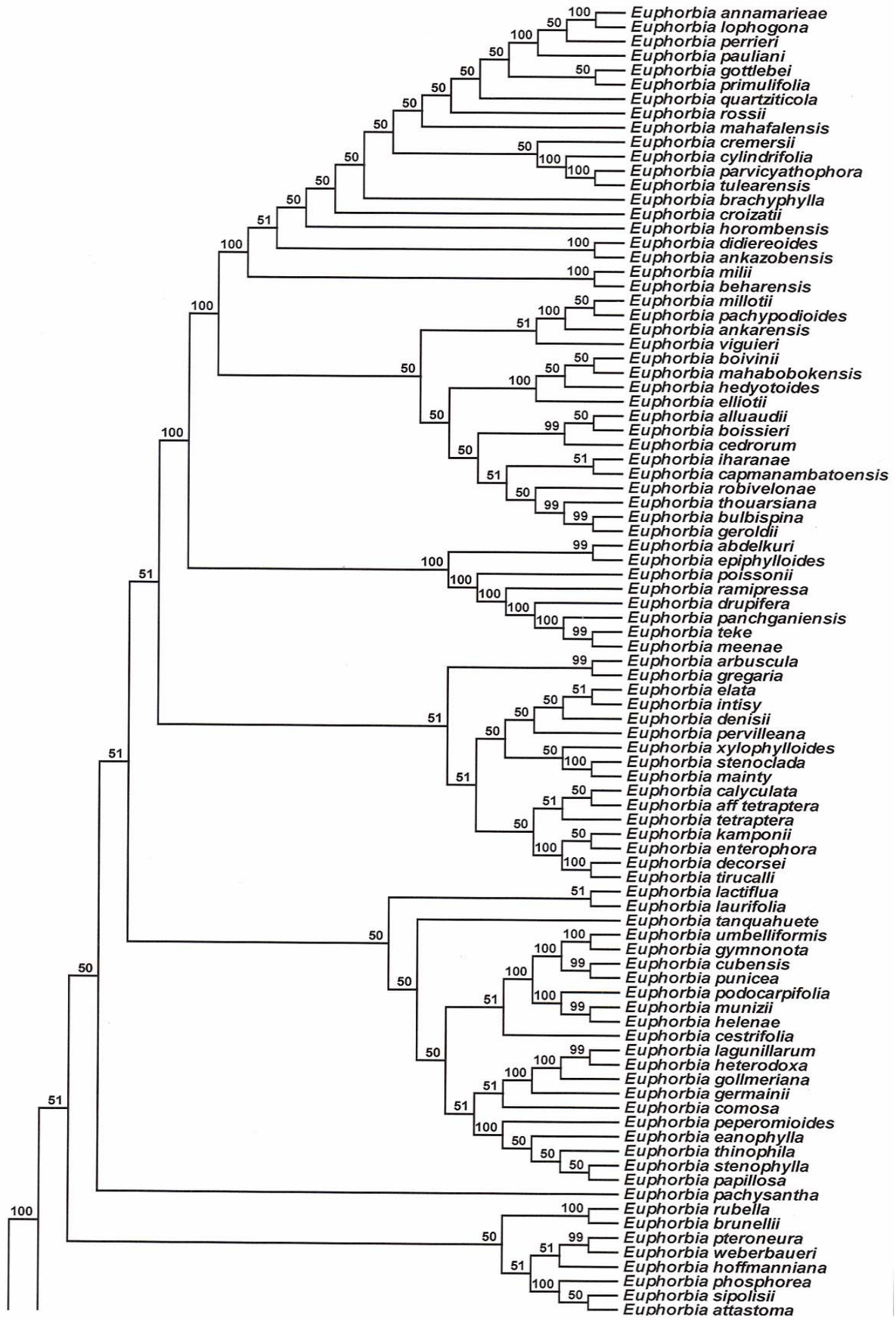


Fig. 3. Complete alignment matrix of the ITS sequences of *Ruta graveolens* and *Euphorbia dracunculoides.*

DNA analysis: Each sample was sequenced in the sense and antisense direction and analyzed with ABI Sequence Navigator software (Perkin-Elmer/Applied Biosystems). Nucleotide sequences of both DNA strands were obtained and compared to ensure accuracy. To ensure the sequence similarity, the BLAST searches of the nrDNA ITS sequences of *R. graveolens* and *E. dracunculoides* were done through http://blast.ncbi.nlm.nih.gov/Blast.cgi. The sequences generated for both samples in the present study were submitted to the GenBank (accession numbers: HQ830197 and HQ830198, respectively). The nrDNA ITS sequences of both samples were aligned using ClustalX version 1.81(Thompson *et al*., 1997) to note the differences in sequence pattern. The nrDNA-ITS sequences of *Euphorbia* sp. available in the GenBank (www.ncbi.nlm.nih.gov) were retrieved (see Appendix) and analyzed together with the sequences of *E. dracunculoides* to test its nesting in the phylogenetic tree.

Results

The internal transcribed spacer (ITS) sequence was used in the present study for the authentication of *R. graveolense* to preserve its efficacy and quality for herbal drug formulations. In the local market samples, *E. dracunculoides* was found as adulterant when later identified very carefully at species level at the National Institute of Science Communication and Information Resources (NISCAIR), New Delhi (voucher no, NISCAIR/RHMD/ consult/-2007-08/937/121) (Khan *et al*., 2011). The dried leaves, fruits and stem of *E. dracunculoides* are more or less similar in morphology with those of *R. graveolens* in dried state which make difficult to differentiate each other under naked eyes even by taxonomists. These morphological similarities have created confusion among customers, and *E. dracunculoides* is being purchased instead of *R. graveolens* by mistake from the local market. The BLAST search result of ITS sequences of genuine sample of '*Sudab'* i.e., *R. graveolens* showed 99% similarity to the sequence of *R. graveolens* (accession No. EU591989) already available in the GenBank (Fig. 1), whereas, the BLAST search result of ITS sequence of adulterant sample purchased by same name '*Sudab'* showed 100% similarity to the species of *Euphorbia* and did not show any similarity with *R. graveolens* (Fig. 2), and under sequence alignment an entirely different pattern of sequence pattern has been observed in between the sequence of genuine and adulterant samples purchased by the same name '*Sudab'* (Fig. 3). A thorough search for the available sequences of *Ruta* species available in the NCBI GenBank reveals that there is only one ITS sequences of *Ruta* i.e., *R. graveolens* (accession No. EU591989) is available, hence, it could not be possible to analyze the generated ITS sequence of genuine sample of '*Sudab'* along with the other species of the genus *Ruta* to observe its nesting within the phylogenetic tree, however, phylogenetic analysis of ITS sequence of adulterant sample, i.e., *E. dracunculoides* together with sequences of *Euphorbia* species available in the GenBank has clearly showed its deeply nesting within the *Euphorbia* tree (Fig. 4).


Euphorbia annamarieae
Euphorbia lophogona
Euphorbia perrieri
Euphorbia pauliani
Euphorbia gottlebei
Euphorbia primulifolia
Euphorbia quartziticola
Euphorbia rossii
Euphorbia mahafalensis
Euphorbia cremersii
Euphorbia cylindrifolia
Euphorbia parvicyathophora
Euphorbia tulearensis
Euphorbia brachyphylla
Euphorbia croizatii
Euphorbia horombensis
Euphorbia didiereoides
Euphorbia ankazobensis
Euphorbia milii
Euphorbia beharensis
Euphorbia millotii
Euphorbia pachypodioides
Euphorbia ankarensis
Euphorbia viguieri
Euphorbia boivinii
Euphorbia mahabobokensis
Euphorbia hedyotoides
Euphorbia elliotii
Euphorbia alluaudii
Euphorbia boissieri
Euphorbia cedrorum
Euphorbia iharanae
Euphorbia capmanambatoensis
Euphorbia robivelonae
Euphorbia thouarsiana
Euphorbia bulbispina
Euphorbia geroldii
Euphorbia abdelkuri
Euphorbia epiphylloides
Euphorbia poissonii
Euphorbia ramipressa
Euphorbia drupifera
Euphorbia panchganiensis
Euphorbia teke
Euphorbia meenae
Euphorbia arbuscula
Euphorbia gregaria
Euphorbia elata
Euphorbia intisy
Euphorbia denisii
Euphorbia pervilleana
Euphorbia xylophylloides
Euphorbia stenoclada
Euphorbia mainty
Euphorbia calyculata
Euphorbia aff tetraptera
Euphorbia tetraptera
Euphorbia kamponii
Euphorbia enterophora
Euphorbia decorsei
Euphorbia tirucalli
Euphorbia lactiflua
Euphorbia laurifolia
Euphorbia tanquahuete
Euphorbia umbelliformis
Euphorbia gymnonota
Euphorbia cubensis
Euphorbia punicea
Euphorbia podocarpifolia
Euphorbia munizii
Euphorbia helenae
Euphorbia cestrifolia
Euphorbia lagunillarum
Euphorbia heterodoxa
Euphorbia gollmeriana
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Euphorbia comosa
Euphorbia peperomioides
Euphorbia eanophylla
Euphorbia thinophila
Euphorbia stenophylla
Euphorbia papillosa
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Euphorbia rubella
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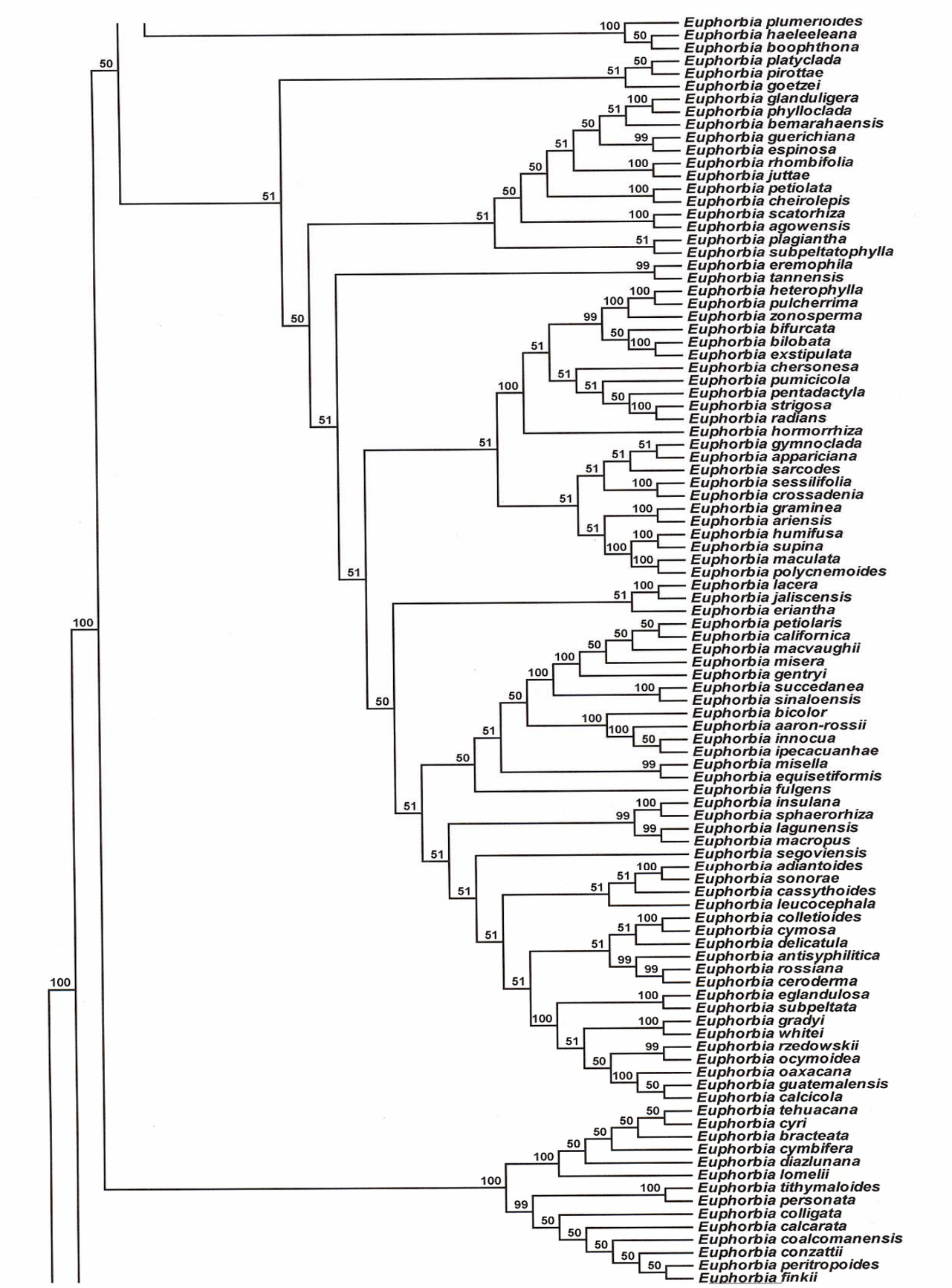
Euphorbia plumerioides
Euphorbia haeleeleana
Euphorbia boophthona
Euphorbia platyclada
Euphorbia pirottae
Euphorbia goetzei
Euphorbia glanduligera
Euphorbia phylloclada
Euphorbia bemarahaensis
Euphorbia guerichiana
Euphorbia espinosa
Euphorbia rhombifolia
Euphorbia juttae
Euphorbia petiolata
Euphorbia cheirolepis
Euphorbia scatorhiza
Euphorbia agowensis
Euphorbia plagiantha
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Euphorbia eremophila
Euphorbia tannensis
Euphorbia heterophylla
Euphorbia pulcherrima
Euphorbia zonosperma
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Euphorbia crossadenia
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Euphorbia ariensis
Euphorbia humifusa
Euphorbia supina
Euphorbia maculata
Euphorbia polycnemoides
Euphorbia lacera
Euphorbia jaliscensis
Euphorbia eriantha
Euphorbia petiolaris
Euphorbia californica
Euphorbia macvaughii
Euphorbia misera
Euphorbia gentryi
Euphorbia succedanea
Euphorbia sinaloensis
Euphorbia bicolor
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Euphorbia innocua
Euphorbia ipecacuanhae
Euphorbia misella
Euphorbia equisetiformis
Euphorbia fulgens
Euphorbia insulana
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Euphorbia lagunensis
Euphorbia macropus
Euphorbia segoviensis
Euphorbia adiantoides
Euphorbia sonorae
Euphorbia cassythoides
Euphorbia leucocephala
Euphorbia colletioides
Euphorbia cymosa
Euphorbia delicatula
Euphorbia antisyphilitica
Euphorbia rossiana
Euphorbia ceroderma
Euphorbia eglandulosa
Euphorbia subpeltata
Euphorbia gradyi
Euphorbia whitei
Euphorbia rzedowskii
Euphorbia ocymoidea
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Euphorbia tehuacana
Euphorbia cyri
Euphorbia bracteata
Euphorbia cymbifera
Euphorbia diazlunana
Euphorbia lomelii
Euphorbia tithymaloides
Euphorbia personata
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Euphorbia peritropoides
Euphorbia finkii

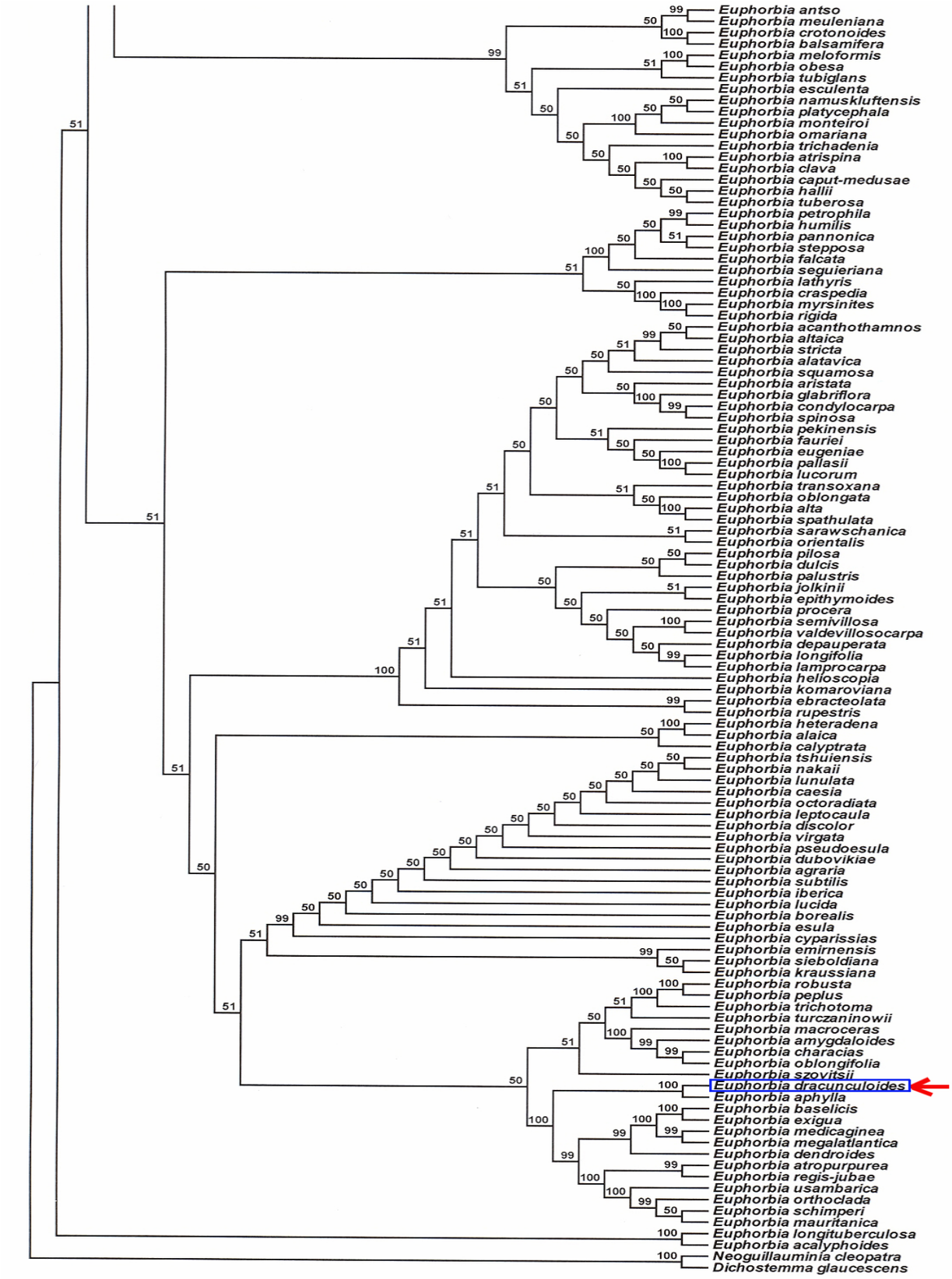

Fig. 4. The consensus tree was developed from 536 most parsimonious trees. The consistency index is (0.216553), the retention index (0.831093), and the composite index 0.202772 (0.179976) for all sites and parsimony-informative sites (in parentheses). The maximum parsimony tree was obtained using the Close-Neighbor-Interchange algorithm in which the initial trees were obtained with the random addition of sequences (10 replicates). The percentage of parsimonious trees in which the associated taxa clustered together is shown above the branches. The codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). There were a total of 271 positions in the final dataset, out of which 95 were parsimony informative. Phylogenetic analyses were conducted in MEGA4.

Discussion

Ruta graveolens is characterized by strong-smelling of ethereal oils in its leaves, slightly toxic and bitter in taste. The bruised leaves have a pleasant orange-like fragrance. Since, dried parts of this scented plant such as leaves, fruits and stem are used in herbal formulations, so morphological characteristics of *R. graveolens* and *E. dracunculoides* are more or less similar, and difficult to discriminate morphologically under naked eye. *R. graveolense* commonly known as '*Sudab*' in the local herbal markets is adulterated/substituted by *E. dracunculoides* because of morphological similarities (Khan *et al*., 2011). The leaves of *R. graveolens* are bipinnate or tripinnate and obviate-oblong, while those of *E. dracunculoides* are lanceolate or linear, oblong, subacute, base rarely rounded or sub-cordate; odour and taste not distinct. The fruit of *R. graveolens* is capsule, globosely with 4-5 lobed containing numerous seeds having tiny size, blackish and triangular in shape, while fruit of *E. dracunculoides* is capsule with subglobose shape, 3-celled with or without attached pedicel, smooth or obscurely reticulate and glabrous (Khan *et al*., 2011). Seeds of *E. dracunculoides* are ovoid-terete and gray or dark gray. These morphological characteristics sometimes misslead authentication of these medicinal plants. *R. graveolens* has a potential medicinal value which is different from *E. dracunculoides* according to their traditional usage and on animal studies. Thus, the adulteration/substitution of *R. graveolens* by *E. dracunculoides* in the herbal markets reduces the medicinal efficacy of this scented medicinal herb. The generated sequence of both species in the present study may be referred hereafter as species-specific DNA barcode signature which can be used in authenticating and validating the exact species identities to discriminate the genuine sample of '*Sudab*' from its adulterant *E. dracunculoides* or any other adulterants if any available to ensure the purity, quality and safety of this drug. There are other molecular markers available which can be used for the authentication of herbal drugs. For example, RAPD which has simplicity in practical exercise and low experimental cost as well and is being or has already been used for a number of medicinal plant species to discriminate from their morphologically allied and geographically co-occurring species or adulterants (Khan *et al*., 2009; Khan *et al*., 2010a, 2010b). *R. graveolense* was earlier authenticated from its adulterant *E. dracunculoides* based on unique bands using the RAPD marker (Khan *et al*., 2011). The present generated species-specific DNA barcode signature for *R. graveolens* and *E. dracunculoides* can make ease in authenticating and validating the exact species identities to discriminate the genuine sample and adulterant samples of '*Sudab*' to ensure the purity, quality and safety of this herbal drug as well as it may prove beneficial for pharmacologists to design safe drug.

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