



Phylogenetic analysis of *Evolvulus* species based on RBCL secondary structure

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Abstract

Context: The plant *Evolvulus alsinoides* (Linn.) Linn (Convolvulaceae), is the source of an important traditional Ayurvedic medicine. Misidentification and adulteration of the plant affects the safety and efficacy of medication. Phylogenetic relationships among the species of this family are unknown.

Objective: The present study sought to detect the phylogenetic relationships based on Ribulose-1, 5-bisphosphate carboxylase (RBCL) region of all 99 species of Convolvulaceae recorded.

Materials and methods: Accession of six species of *Evolvulus* served as reference samples. DNA of *Evolvulus alsinoides* (Linn.) Linn was isolated and the RBCL region was amplified using polymerase chain reaction (PCR). The RBCL sequence of the sample was deposited in the Genbank database and was compared with the RBCL sequences deposited in the NCBI server. Distance analysis, species discrimination and secondary structure of RBCL were used to assess the ability of RBCL sequence in authenticating. The phylogenetic relationships were detected using three methods: Bayesian inference (BI), maximum likelihood (ML), and neighbor joining (NJ).

Results: Six species of *Evolvulus alsinoides* (Linn.) Linn were well resolved in molecular phylogenetic tree. Besides, *Evolvulus nuttallianus* and *Evolvulus sericeus* was closer to *Evolvulus alsinoides* (Linn.) Linn within the tree. Further, we also found that RBCL secondary structure can be a candidate tool in distinguishing three closely related species *Evolvulus nuttallianus*, *Evolvulus sericeus* and *Evolvulus alsinoides* (Linn.) Linn. For accurate identification of different species of *Evolvulus* based on species-specific nucleotide sites, a consensus sequences database with all 7 species is established.

Discussions and conclusions: The results are able to discriminate *Evolvulus* species and illustrate the phylogenetic relationships, which are essential for the investigation of adulterants and misidentifications of *Evolvulus*.

Keywords: *Evolvulus alsinoides*, phylogenetic analysis, RBCL

Introduction

The plants of Convolvulaceae (morning glory family) are twining herbs, occasionally with milky sap, encompassing about 85 genera and 2,800 species. These plants are characterized with flowers solitary or in terminal or axillary dichasia. The leaves are simple, lobed to pinnate sect and alternate; stipules are absent (Austin, 1990) ^[1]. *Evolvulus* is a genus of flowering plants in the Convolvulaceae. They are known generally as dwarf morning glories. There are about 100 species. The genus name *Evolvulus* originates from the Latin word meaning "to unroll", inspired by its non-vining form (Van Oostroom, 1934) ^[2].

Evolvulus alsinoides (Linn.) Linn. is one among the plants of *Evolvulus* genus with immense therapeutic value mainly used for sleeplessness, anxiety and stress. Previous studies have reported that the main compounds in *Evolvulus alsinoides* (Linn.) Linn. (alkaloids, triterpenes, and uncarinic acids) have several beneficial properties, such as alexiteric, antihelmintic (Dash *et al.*, 2002) ^[15] and febrifuge (Chatterjee and Pakrashi, 1990) ^[12] The whole plant and the various parts are useful in the treatment of fever (Sivarajan and Balachandran, 1999) ^[13], loss of memory, syphilis, bronchitis, biliousness, epilepsy and leucoderma (Asolkar *et al.*, 1992) ^[14].

The demand for herbal medicine is increasing day-by-day because of their biological activity and being safer than synthetic drugs. However, due to their widespread requirement for medicinal use, plants of high value species

are now becoming endangered. Moreover, the medicinal herb *Evolvulus alsinoides* (Linn.) Linnis being misidentified and adulterated with similar but less valuable species of *Evolvulus* which is affecting the safety and efficacy of the nutraceuticals.

The identification of *Evolvulus* is based primarily on the morphological characteristics, microscopic structures and/or chemical components of the plant. Based on environmental conditions, the same genotype may express different chemical patterns or vice-versa. Therefore, it is necessary to develop the sensitive and effective technology for the characterization of medicinal plants. DNA-based molecular markers have acted as very useful tools in various fields like taxonomy, physiology, embryology, plant breeding, ecology, genetic engineering etc. Molecular markers have their applications in fingerprinting genotypes, determining the seed purity and in phylogenetic analysis by which the conservation of the plant can be made easy (Rubiolo *et al.*, 2009) ^[16].

The RBCL gene is a supportive tool to assess phylogenetic relationships. This gene is found in the chloroplasts of most photosynthetic organisms. It is an abundant protein in leaf tissue (Freeman 2008) ^[19]. Thus this gene exists as a common factor between photosynthetic organisms and can be contrasted with the RBCL genes of other plants in order to determine genetic similarities and differences. It codes for the large subunit of the protein ribulose-1, 5-bisphosphate carboxylase/oxygenase (rubisco) (Geilly, Taberlet, 1994) ^[20]. Because of its slow synonymous nucleotide substitution

rate in comparison with nuclear genes and its functional constraint that reduces the evolutionary rate of non synonymous substitutions (Wolfe *et al.*, 1987)^[17], RBCL is considered to be more useful than the isozymes and the restriction fragment length polymorphisms (Kousar *et al.*, 2016)^[18] at these taxonomic levels.

In the present study, the ability of RBCL region to differentiate among *Evolvulus* species was tested and a sequence database with species-specific positions to discriminate the 7 species of *Evolvulus* recorded in the Flora of Herbal Medicine was established. We also attempted to illustrate the phylogenetic relationships among the 7 species of *Evolvulus* and use RBCL secondary structure to distinguish potential closely related species.

Materials and methods

DNA extraction, PCR amplification and sequencing

Evolvulus alsinoides (Linn.) Linn. was collected from the Sathyamangalam Ghats of Tamilnadu and taxonomic identification was done by Botanical Survey of India (BSI/SRC/5/23/2016/Tech/1711 Dated 27.10.2016), Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Total DNA was extracted from fresh leaf tissue by CTAB method (Doyle and Doyle, 1987). Amplification of RBCL gene (BankIt2226974 BSeq#1 MK955878) was carried out using RBCL Forward primer: 5'-ATGTCACCACAAACAGAGACTAAAGC-3' and RBCL Reverse primer: 5'-GTAAAATCAAGTCCACCRCG-3'. The PCR conditions were as follows: 1 cycle (94°C for 3 min), 35 cycles (94°C for 1 min, 55°C for 1 min and 72°C for 1 min), and 1 cycle 72°C for 7 min. Amplified PCR products were checked on 1.5% agarose gel electrophoresis for the 1500 bps band which was sent to Rajiv Gandhi Centre for Biotechnology (Trivandrum, Kerala) for DNA sequencing (Shuang *et al.*, 2018)^[9].

Sequence alignment and data analyses

The RBCL sequences of the Convolvulaceae family were queried and downloaded from the National Center for Biotechnology Information (NCBI) database. Low quantity and ambiguous sequences were manually checked and deleted. A total of 99 RBCL sequences from NCBI database was compared. The query sequences were identified considering E value $< 1 * 10^{-5}$ and maximum hits (99 or 100%) with a species in the reference data base.

Phylogenetic analysis

Bayesian inference (BI), maximum likelihood (ML) and neighbor joining (NJ) methods were used to analyze the sequence datasets. Bayesian inference was performed with the computer program Mr. Bayes ver. 3.2.7. Four simultaneous chains of Markov Chain Monte Carlo (MCMC) algorithm were run twice with two million generations. Trees were sampled every hundredth generation, whereby the first 5,000 trees were discarded as burn-in. The remaining trees were used to calculate posterior probabilities (PP) of the branching pattern in the 50% majority-rule consensus tree. The ML analyses, including 1,000 nonparametric bootstrap replicates, were carried out in MEGA version 6.06 under the Tamura 3-parameter (T92) +G model (Ronquist *et al.* 2012)^[10]. The NJ tree was constructed under the K2P model, which is based on distance substitution, using MEGA version 6.06.

Bootstrap support for the NJ tree was estimated with 1000 replicates, while uninformative characters (gaps and missing data) were completely deleted.

Prediction of secondary structure

The nucleotide sequence was translated into protein sequence using ExPasy server (<https://web.expasy.org/translate/>). The output was used to predict its secondary structure using Jpred 4 (http://www.compbio.dundee.ac.uk/jpred4/cgi-bin/chklog?jp_uLNg_uE). The phylogenetic analysis was done using Jalview Desktop 2.10.5 (<http://www.jalview.org/>).

Results

DNA extraction, PCR amplification and sequencing

The amplified RBCL gene fragment corresponds to 1500 bps. The RBCL sequence of *Evolvulus alsinoides* (Linn.) Linn obtained was deposited at the Gen bank and the Accession no: MK955878 dated 22.05.2019 was obtained.

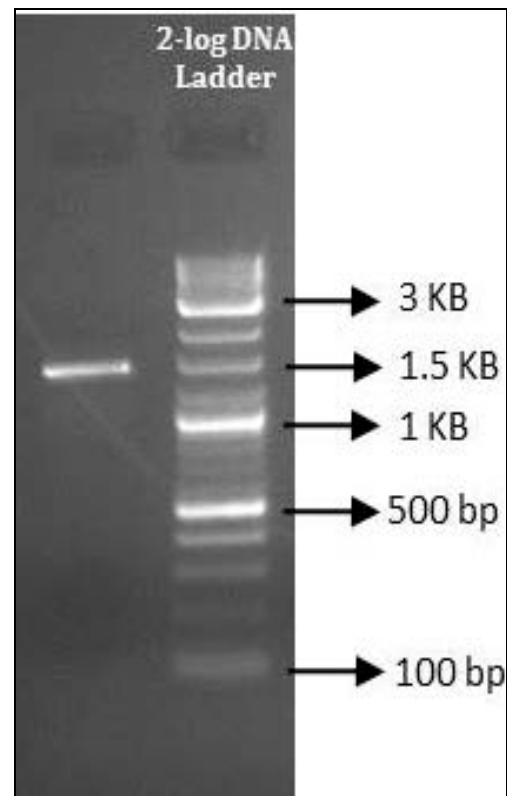


Fig 1

Sequence Alignment

The query sequence was subjected to BLAST analysis and the degree of similarity between the query sequence and the hits was determined. The hits were all eudicots and all the hits belong to the same family, Convolvulaceae. The similarity level varies between the query sequence and the hits and also within the hits. This shows that RBCL region is highly conserved for identification at the species level. Identification using RBCL gene as the marker provides significant differences between the plants of the same family.

Table 1: Degree of similarity between the query sequence and hits.

Rank	Genus	Species	Score	Similarity
1.	<i>Evolvulus</i>	<i>glomeratus</i>	662	96.9
2.	<i>Evolvulus</i>	<i>nuttallianus</i>	662	96.9
3.	<i>Cladostigma</i>	<i>hildebrandtioides</i>	662	96.9
4.	<i>Hildebrandtia</i>	<i>valo</i>	660	96.76
5.	<i>Maripa</i>	<i>paniculata</i>	658	96.61
6.	<i>Merremia</i>	<i>peltata</i>	658	96.61
7.	<i>Maripa</i>	<i>glabra</i>	658	96.61
8.	<i>Evolvulus</i>	<i>pilosus</i>	658	97.01
9.	<i>Merremia</i>	<i>dissecta</i>	656	96.47
10.	<i>Operculina</i>	<i>sp.</i>	656	96.47
11.	<i>Dicranostyles</i>	<i>mildbraediana</i>	656	96.47
12.	<i>Seddera</i>	<i>hirsuta</i>	656	96.47
13.	<i>Convolvulus</i>	<i>vollesenii</i>	654	97.4
14.	<i>Merremia</i>	<i>vitifolia</i>	654	96.33
15.	<i>Neuropeltis</i>	<i>acuminata</i>	654	96.33
16.	<i>Polymeria</i>	<i>pusilla</i>	654	96.33
17.	<i>Turbina</i>	<i>oblongata</i>	654	96.33
18.	<i>Wilsonia</i>	<i>backhousei</i>	654	97.27
19.	<i>Bonamia</i>	<i>media</i>	654	96.33
20.	<i>Calystegia</i>	<i>sepium</i>	654	96.33
21.	<i>Cressa</i>	<i>depressa</i>	654	96.33
22.	<i>Dicranostyles</i>	<i>villosus</i>	654	96.33
23.	<i>Itzaea</i>	<i>sericea</i>	654	96.33
24.	<i>Stylisma</i>	<i>patens</i>	654	96.33
25.	<i>Calystegia</i>	<i>sepium</i>	654	96.33
26.	<i>Convolvulus</i>	<i>sagittatus</i>	652	97.25
27.	<i>Ipomoea</i>	<i>purpurea</i>	652	96.06
28.	<i>Stictocardia</i>	<i>incomta</i>	652	96.19
29.	<i>Tetralocularia</i>	<i>pennellii</i>	652	96.19
30.	<i>Argyreia</i>	<i>osyrensis</i>	652	96.19
31.	<i>Argyreia</i>	<i>splendens</i>	652	96.19
32.	<i>Hewittia</i>	<i>scandens</i>	652	96.19
33.	<i>Hewittia</i>	<i>sublobata</i>	652	96.19
34.	<i>Ipomoea</i>	<i>purpurea</i>	652	96.06
35.	<i>Convolvulus</i>	<i>arvensis</i>	652	96.19
36.	<i>Ipomoea</i>	<i>wrightii</i>	652	96.19
37.	<i>Convolvulus</i>	<i>capituliferus</i> var. <i>foliaceus</i>	652	97.12
38.	<i>Convolvulus</i>	<i>maireanus</i>	652	97.12
39.	<i>Ipomoea</i>	<i>purpurea</i>	652	96.06
40.	<i>Convolvulus</i>	<i>steudneri</i>	652	97.12
41.	<i>Calystegia</i>	<i>soldanella</i>	652	96.19
42.	<i>Convolvulus</i>	<i>arvensis</i>	652	96.19
43.	<i>Convolvulus</i>	<i>eremophilus</i>	651	97.25
44.	<i>Convolvulus</i>	<i>sagittatus</i>	651	96.32
45.	<i>Convolvulus</i>	<i>farinosus</i>	651	97.25
46.	<i>Convolvulus</i>	<i>korolkowii</i>	651	97.25
47.	<i>Convolvulus</i>	<i>sagittatus</i>	651	97.25
48.	<i>Convolvulus</i>	<i>farinosus</i>	651	97.25
49.	<i>Convolvulus</i>	<i>scammonia</i>	651	97.25
50.	<i>Convolvulus</i>	<i>schulzei</i>	651	97.39
51.	<i>Convolvulus</i>	<i>hystrix</i>	650	96.98
52.	<i>Convolvulus</i>	<i>chilensis</i>	650	97.25
53.	<i>Ipomoea</i>	<i>quamoclit</i>	650	96.05
54.	<i>Iseia</i>	<i>luxurians</i>	650	96.05
55.	<i>Astripomoea</i>	<i>grantii</i>	650	96.05
56.	<i>Cressa</i>	<i>truxillensis</i>	650	96.05
57.	<i>Ipomoea</i>	<i>aquatica</i>	650	96.05
58.	<i>Erycibe</i>	<i>coccinea</i>	650	97.39
59.	<i>Aniseia</i>	<i>cernua</i>	650	96.05
60.	<i>Bonamia</i>	<i>spectabilis</i>	650	96.05
61.	<i>Erycibe</i>	<i>hellwigii</i>	650	96.98
62.	<i>Turbina</i>	<i>corymbosa</i>	650	96.05
63.	<i>Convolvulus</i>	<i>hystrix</i>	650	96.98
64.	<i>Convolvulus</i>	<i>galaticus</i>	650	96.98
65.	<i>Convolvulus</i>	<i>dorycnium</i>	650	96.98

66.	<i>Convolvulus</i>	<i>chondrilloides</i>	650	96.98
67.	<i>Convolvulus</i>	<i>reticulatus</i>	649	97.11
68.	<i>Convolvulus</i>	<i>thymoides</i>	649	97.11
69.	<i>Convolvulus</i>	<i>virgatus</i>	649	97.11
70.	<i>Convolvulus</i>	<i>asyrensis</i>	649	97.11
71.	<i>Convolvulus</i>	<i>rhyniospermus</i>	649	97.11
72.	<i>Convolvulus</i>	<i>scammonia</i>	649	97.11
73.	<i>Convolvulus</i>	<i>oppositifolius</i>	649	97.11
74.	<i>Convolvulus</i>	<i>chilensis</i>	649	97.25
75.	<i>Convolvulus</i>	<i>arvensis</i>	649	97.11
76.	<i>Convolvulus</i>	<i>oxyphyllus</i>	649	97.11
77.	<i>Convolvulus</i>	<i>virgatus</i>	649	97.11
78.	<i>Convolvulus</i>	<i>kilimandschari</i>	649	97.25
79.	<i>Convolvulus</i>	<i>persicus</i>	648	97.11
80.	<i>Ipomoea</i>	<i>obscura</i>	648	95.91
81.	<i>Lepistemon</i>	<i>owariensis</i>	648	95.91
82.	<i>Xenostegia</i>	<i>tridentata</i>	648	96.55
83.	<i>Convolvulus</i>	<i>arvensis</i>	648	95.91
84.	<i>Convolvulus</i>	<i>farinosus</i>	648	97.24
85.	<i>Dichondra</i>	<i>micrantha</i>	648	97.24
86.	<i>Convolvulus</i>	<i>arvensis</i>	648	96.3
87.	<i>Ipomoea</i>	<i>alba</i>	648	95.91
88.	<i>Convolvulus</i>	<i>reticulatus</i>	648	97.11
89.	<i>Convolvulus</i>	<i>demissus</i>	648	97.38
90.	<i>Convolvulus</i>	<i>thymoides</i>	647	97.24
91.	<i>Breweria</i>	<i>rotundifolia</i>	647	96.68
92.	<i>Convolvulus</i>	<i>compactus</i>	647	96.97
93.	<i>Convolvulus</i>	<i>compactus</i>	647	96.97
94.	<i>Convolvulus</i>	<i>leiocalycinus</i>	647	96.97
95.	<i>Convolvulus</i>	<i>acanthocladus</i>	647	96.97
96.	<i>Convolvulus</i>	<i>pseudoscammonia</i>	647	97.1
97.	<i>Convolvulus</i>	<i>pseudoscammonia</i>	647	97.1
98.	<i>Convolvulus</i>	<i>oppositifolius</i>	647	97.1
99.	<i>Convolvulus</i>	<i>virgatus</i>	647	97.1

Phylogenetic Tree

Families and clustering

The phylogenetic tree was constructed using Neighbor Joining method and the evolutionary distances were computed employing maximum composite likelihood method. The phylogenetic relationships of the medicinal species of Convolvulaceae is shown in the present study. *Evolvulus sericeus* and *Evolvulus nuttallianus* can be considered as sister species to *Evolvulus alsinoides* with minimum distance and a single node. Whereas *Evolvulus pilosus* and *Evolvulus glomeratus* has a longer distance with two nodes. The genus *Cressa* and *Evolvulus* are separated with a single clade. To summarize, the RBCL region has more variable sites to discriminate the plants of Convolvulaceae and thus has better discriminating performance.

Conclusion

A comprehensive phylogenetic analysis including all the species of Convolvulaceae recorded in the Gen bank were concluded. Firstly, degree of variation between the plants of *Evolvulus* was determined using RBCL marker from the clustered report in the molecular phylogeny tree. Secondly, we established the RBCL database with the obtained sequences and found that RBCL sequences have appropriate variable sites for discrimination most of species in Convolvulaceae. Finally, the RBCL secondary structure can be used as candidate method for distinguishing the two closely related species *Evolvulus sericeus* and *Evolvulus alsinoides*.

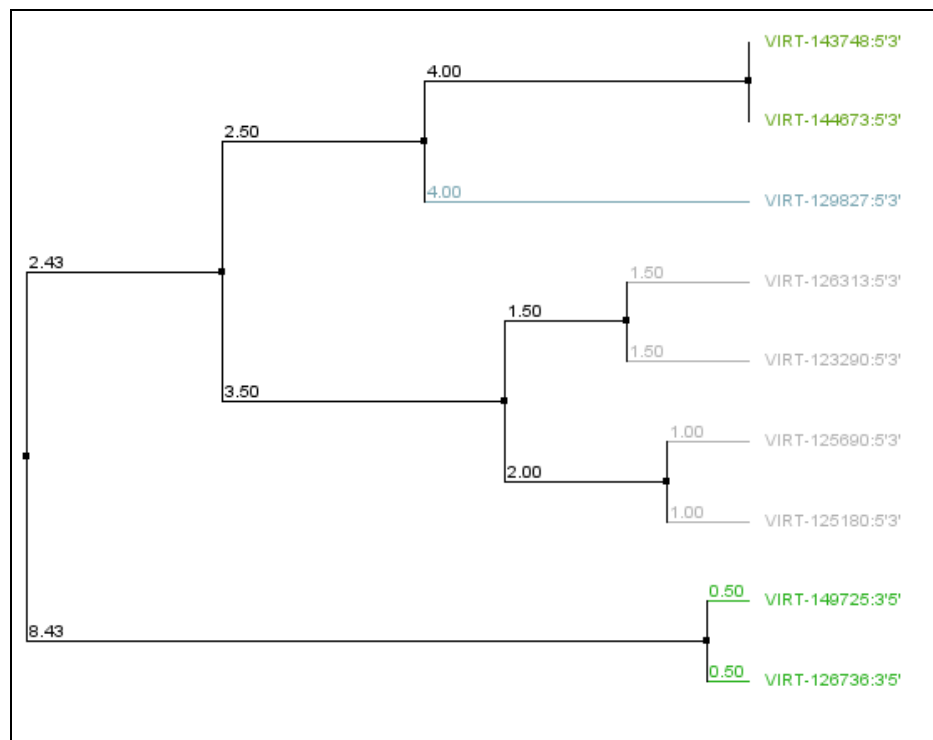


Fig 3

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