



RESEARCH PAPER

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The genetic diversity of *Convolvulus* L. species inhabited in Pakistan based on intergenic spacer *trnL-trnF*

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Article published on September 30, 2017

Key words: Intraspecific, *trnL-trnF*, Bayesian approach, Phylogenetics, *Convolvulus*

Abstract

Chloroplast non-coding regions are now frequently used in plant phylogenetic studies as they exhibit more genetic variations. The resolving power of an intergenic spacer region *trnL-trnF* was evaluated on *Convolvulus* species. Genus *Convolvulus* L. has many economic advantages. Genus specific primer set was designed and used for polymerase chain reaction and bi-directional Sanger sequencing. 15 ingroup and 2 outgroup species were used for the present study. A consensus tree based on Bayesian inference method was reconstructed to observe *Convolvulus* L. species associations. Results revealed two distinct lineages. The sister associations between *C. acanthocladus* and *C. pseudocantabricus*; *C. arvensis* and *C. kotschyanus*; *C. spinosus*, *C. prostratus* and *C. scindicus* are strongly supported by 1 posterior probability value. Another clade representing *C. lineatus*, *C. cephalopodus*, *C. fruticosus*, *C. glomeratus* and *C. rhyniospermus* also establishing close relationship between species included in the clade. The morphological data also agree the association of *C. glomeratus* and *C. rhyniospermus*. The study can help in many aspect of agronomical, medicinal and economical value of represented species in future studies.

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Introduction

The family Convolvulaceae consists of many genera of agriculture importance in which Ipomoea is the most species rich genus followed by *Convolvulus* L. The genus *Convolvulus* L. included about 200 to 250 species (Mabberley, 2008), exhibiting a remarkable diversity of morphological characters and is distributed in a wide range of ecological habitats, mainly at dry, stony and sandy ranges. In Asia, it is distributed in all countries but most of the species are diversified in Iran. Only 21 species are reported from Pakistan (Austin and Ghazanfar, 1967).

It is a cosmopolitan genus but the Mediterranean and East Asia are the major points of diversity of *Convolvulus* L. It is also diverse in Eastern Asia, Africa and Australia but rarely found in America (Mitchell *et al.*, 2016). The plants are annual, biennial or perennial herbs, shrubs or under shrubs, bearing alternate, extremely variable leaves and funnel shaped flowers mostly white or pink and rarely blue, violet, purple and yellow with smooth or hairy banded petals (Austin and Ghazanfar, 1967, Wood *et al.*, 2015).

Many species of *Convolvulus* L. are economically important. In different areas of Pakistan *C. spinosus*, *C. scindicus* and *C. glomeratus* are used as fodder and medicinally as purgative (Austin and Ghazanfar, 1967; Abbas and Qaiser, 2011). *C. leiocalycinus* is a forage species (Hussain and Durrani, 2009) while *C. prostratus* and *C. arvensis* are familiar therapeutic species, used to treat many disorders (Qureshi and Bhatti, 2008; Qureshi *et al.*, 2010; Bhowmik *et al.*, 2012; Canbolat, 2012; Agarwal *et al.*, 2014; Brian and Lauriault, 2015). The *Convolvulus* L. species have showy flowers due to which many species like *C. arvensis*, *C. tricolor* and *C. lineatus* are used as ornamental (Sa'ad, 1967).

The genus *Convolvulus* L. has great economic value; however, little attention has been paid to the taxonomical relationships within the genus. Boisser (1875) and Sa'ad (1967) presented infrageneric classification of *Convolvulus* L. Many earlier taxonomists included different taxa within the genus

Convolvulus L. Brown (1810) moved it to a new genus *Calystegia* L. Web (1841) transferred some species of *Convolvulus* L. into a new genus *rhodorhrrizaon* the basis of the short style and the irregular capsule dehiscence and Choisy (1845) also comprised *rhodorhiza* into *Convolvulus* L. Bentham and Hooker (1876) delimited the genus in their book Genera Plantarum. Hallier (1893) differentiated the *Convolvulus* L. from *Calystegia* L. by adding diagnostic characters of pollen grains. Austin (1998) positioned *Convolvulus* L., *Calystegia* L. and *Jacquemontia* L. under tribe Convolvuleae.

In tribe Convolvuleae, *Convolvulus* L. is closely related with *Calystegia*. Only pollen, stigmas and bracteoles morphology distinguished the *Convolvulus* L. from *Calystegia*. Molecular data also support the close relationship of both genus and nested *Calystegia* L. within *Convolvulus* L. (Carine *et al.*, 2004; Stefanovic *et al.*, 2002).

It is a challenging genus with a diverse and large number of species and several species has many synonymies (Williams *et al.*, 2014; wood *et al.*, 2015). Therefore, Physical comparison provides insufficient data for the specific result. To resolve this problem, molecular data provide a large amount of facts to examine hereditary molecular discrepancies and information about an organism's evolutionary relationships (Doebly, 1992; Vanderpoorten and Jonathan, 2014; Kousar *et al.*, 2016). Chloroplast DNA is widely used in phylogenetic studies at all taxonomic levels in plants.

It is easy to obtain sequencing data from polymerase chain reaction (PCR) products. Chloroplast *trnL-trnF* region is more variable and less homoplasious within the chloroplast genome and examined as a possible indicator for the phylogenetic relationship especially at lower taxonomic level (Kelchner, 2000; Bayer *et al.*, 2000; Hyosig and Susanne, 2005; Andreas *et al.*, 2007; Yang *et al.*, 2007). The aim of the present study is to delimit species and develop phylogeny for *Convolvulus* species using *trnL-trnF* sequence of chloroplast DNA.

Materials and methods

Plant Collection and Identification

The total of 15 *Convolvulus* L. specimens were included in the analysis, in which 11 species were collected as a herbarium material from Karachi University Herbarium, Centre for plant Conservation, and 7 fresh species (including outgroup).

Were collected from different areas of Sind and Karachi (Pakistan). All species were studied morphologically with the help of light and stereomicroscope and identified with the help of authentic literature and floras.

DNA Isolation, Purification and Amplification

The genomic DNA of Identified fresh specimens was isolated by using Cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1987).

With slight modifications as per requirement. Dried leaves from herbarium specimen were used to extract DNA by using Plant Direct PCR Kit (MOLEQULE-ON, NZ). The concentration of the isolated DNA was estimated at a wavelength of 260/280 nm using a NanoPhotometer (Implen, Germany).

The *trnL-trnF* region was amplified with the specifically designed genus specific primers 5'CAAATTCAGAGAAACC CTGGA3', and 5'AGATG CTGGGGTCTATGTCAA3'. Amplification reaction was performed in a final volume of 30µl, containing 1X PCR buffer, 1.5mM MgCl₂, 0.2mM dNTPs, 1µM of each forward and reverse primer and 1U of DNA Taq polymerase (MOLEQULE-ON, NZ), template DNA and Milli Q water in a thermocycler (Eppendorf Mastercycler, Germany).

Under following conditions; 94°C for 5 min, 35 cycles of 94°C for 30 sec, 55°C for 35sec, 72°C for 1min and a final extension at 72°C for 5min. Resulted PCR products were examined on 1% agarose gel stained with VisualaNA (MOLEQULE-ON, NZ) and after purification with PCR purification kit (MOLEQULE-ON, NZ), sent to direct sequencing to MOLEQULE-ON, New Zealand.

Sequence Editing and Alignment

The amplified DNA sequences were analyzed using NCBI BLAST Programme (Altschul *et al.*, 1990). To avoid any incongruity, sequences were manually edited. Multiple sequence alignment (MLA) was performed for all sequences using Multalin software (Corpet, 1988). The refined sequences were deposited to NCBI GenBank and accession numbers were recorded.

trnL-trnF sequences Data analysis

Phylogenetic relationship between different species of genus *Convolvulus* L. was conducted using Bayesian Inference (BI), a character and model based approach, which estimates probability value.

The different software of BEAST (v1.8.0) (Drummond and Rambaut, 2007) was used to reconstruct phylogenetic tree. Model selection for BI analysis was performed using default parameters of Jmodel Test (v 2.1.4) (Darriba *et al.*, 2012). Final trees were visualized in Fig Tree (v1.4.0). *Daturaalba* and *Solanum forskalii* from family Solanaceae were used as outgroup in the current analysis.

Result

A detail of deposited vouchers of studies materials and GenBank accession numbers is given in Table 1. The multiple alignment analysis result displayed as Fig. 1 demonstrated that various variable sites among species. *Convolvulus aitchisonii* exhibits most variations among all included species.

The variations in nucleotide found in the *Convolvulus* species were evident in the form of Indels and substitutions (both transitions and transversions). The transition of cytosine to thymine was most frequent among observed differences.

A phylogenetic tree was reconstructed based on the chloroplast *trnL-trnF* intergenic spacer sequence by Bayesian inference method. The tree topology was clear to a great extent (Fig. 2). Bayesian analysis supports a monophyletic clade of all ingroup taxa of genus *Convolvulus* L. with respect to outgroups from *Datura* and *Solanum* genus.

Table 1. Name of species with their voucher numbers and GenBank accession numbers.

Name of species	Voucher Number	Gen Bank Accession Number
<i>C. arvensis</i>	GH86577	MF621879
<i>C. prostratus</i>	GH86567	MF621880
<i>C. scindicus</i>	GH87719	MF621881
<i>C. glomeratus</i>	GH86572	MF621882
<i>C. spinosus</i>	GH86624	MF621883
<i>C. lineatus</i>	GH46811	MF621884
<i>C. fruticosus</i>	GH58159	MF621885
<i>C. virgatus</i>	GH61433	MF621886
<i>C. rhyniospermus</i>	GH61350	MF621887
<i>C. leiocalycinus</i>	GH46813	MF621888
<i>C. pseudocantabricus</i>	GH61485	MF621889
<i>C. cephalopodus</i>	GH92213	MF621890
<i>C. kotschyanus</i>	GH74622	MF621891
<i>C. acanthocladus</i>	GH46700	MF621893
<i>C. aitchisonii</i>	GH46701	MF621894
<i>Solanum forskalii</i>	GH88012	MF621895
<i>Datura alba</i>	GH88010	MF621896

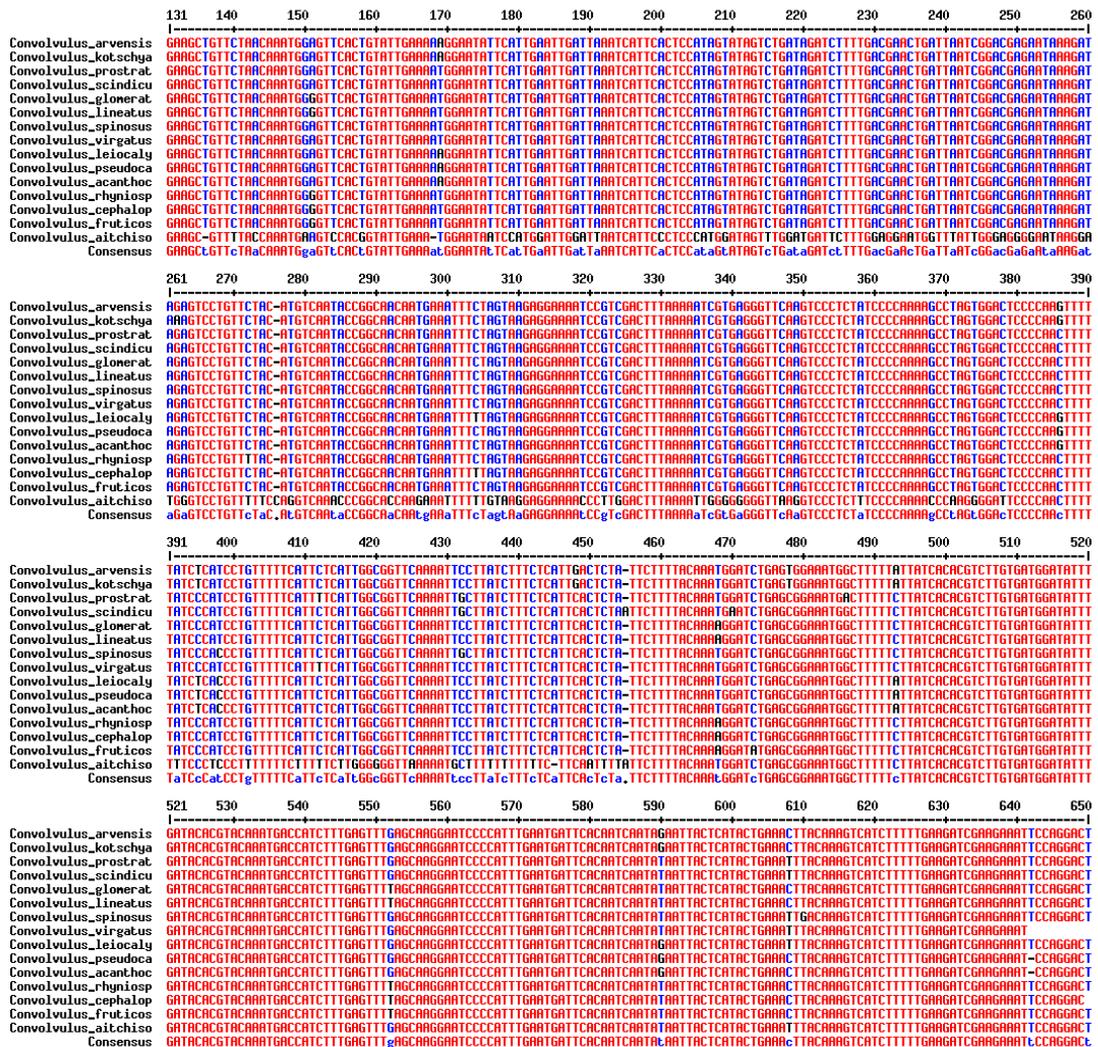


Fig. 1. The multiple alignment analysis of *Convulvulus* species. The red nucleotides in consensus represent conserved segments in all investigated species.

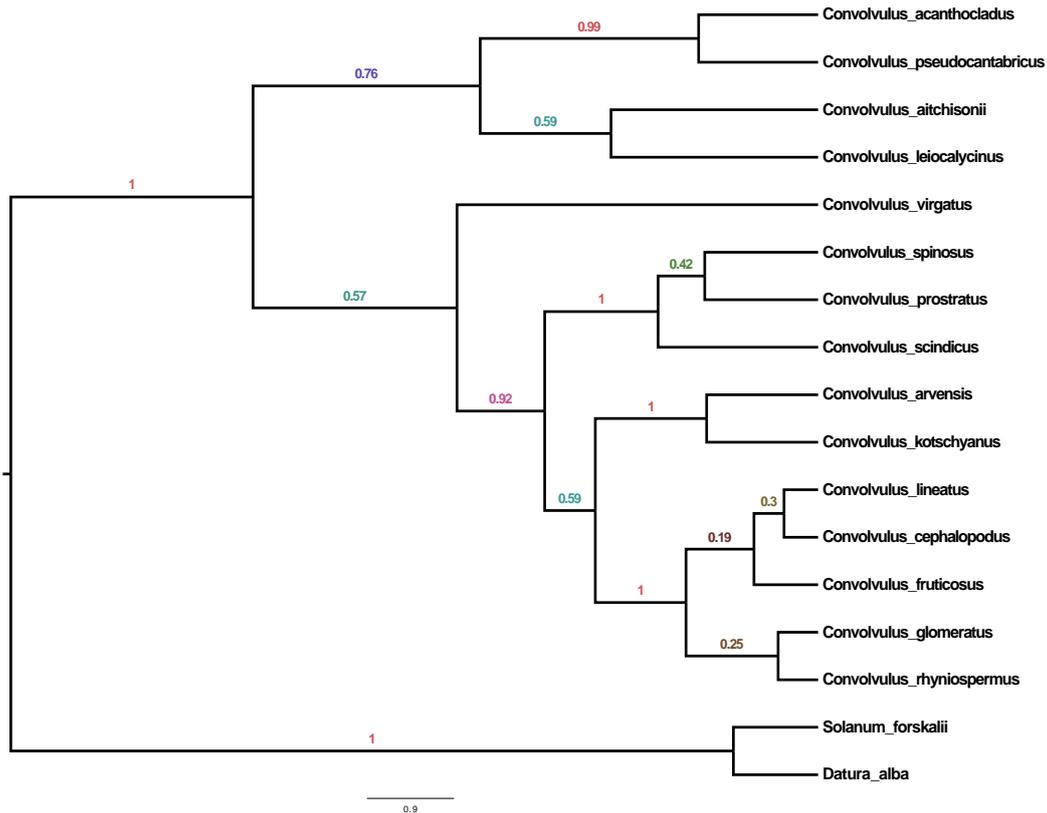


Fig. 2. Phylogenetic tree with mean branch lengths from a representative Bayesian analysis of Chloroplast *trnL-trnF* region data. Numbers on branches represent their respective posterior probabilities.

The *trnL-trnF* phylogenetic tree indicate that the monophyletic *Convolvulus* L. clade diverge into two lineages. The basal lineage comprises of eleven species of *Convolvulus* L. from which *C. rhyniospermus*, *C. glomeratus*, *C. fruticosus*, *C. cephalopodus* and *C. lineatus* are included in a single clade with 1PP (Posterior probability) within first nodal branch. Second nodal branch represents the sister association between *C. arvensis* and *C. kotschyanus* with 1PP (Posterior probability) while *C. prostratus*, *C. spinosus* and *C. scindicus* are weakly associated in a separate clade with 0.1PP (Posterior probability). *C. virgatus* shows weak association with all species with 0.57 PP.

The upper lineage comprises of four species with moderate support of 0.76 PP value further alienated into two nodes. The first nodal branch consists of *C. aitchisonii* and *C. leiocalycinus* with 0.59 PP. The second nodal branch represents the sister association of *C. acanthocladus* and *C. pseudocantabricus* with 0.99 PP.

Discussion

In the present study, the genus *Convolvulus* L. has been studied using *trnL-trnF* sequencing data by HKY with gamma distribution based Bayesian analysis. Although there is inconsistency between the resulted cladogram and the traditional taxonomy (based, to a large extent, on the spine, growth forms and ovary pubescence) of *Convolvulus* L. but similarities are also found to show the placement of different species.

Taxonomically, genus *Convolvulus* L. is very challenging. Linnaeus in 1735 described 31 species of *Convolvulus* L. in Species Plantarum with broad aspects (wood *et al.*, 2015). Boisser (1875) delimited *Convolvulus* L. into two series and ten sections. Sa'ad (1967) modified Boisser's classification and proposed 3 sections and 12 sub sections. In many phylogenetic studies including molecular studies, *Convolvulus* L. is treated as paraphyletic (Stefanovic *et al.*, 2002, Carine *et al.*, 2004). Carine *et al.*, (2003) found that the three infrageneric sections of *Convolvulus* L. are

polyphyletic because there is repeated convergence upon similar growth forms of species which are distantly related. Carine *et al.*, (2004) demonstrated that in Macaronesian and Mediterranean regions the genus *Convolvulus* L. exhibits a complex pattern of relationship. According to Wood *et al.*, (2015) it is difficult to correlate the DNA sequencing data of *Convolvulus* L. with morphology and geography to conclude infrageneric relationship.

The resultant tree diverged into two lineages. The first lineage contains the morphologically inconsistent species. It comprises three sections (sect. Scandentes, sect. Pannosi and sect. Acanthocladi) proposed by Boissier (1875) and all three sections (sect. Acanthocladi, sect. Inermes and sect. *Convolvulus*) modified by Sa'ad (1967) represented by *C. rhyniospermus*, *C. glomeratus*, *C. fruticosus*, *C. cephalopodus*, *C. lineatus*, *C. kotschyanus*, *C. arvensis*, *C. scindicus*, *C. prostratus*, *C. spinosus* and *C. virgatus*. Morphologically analogous species *C. rhyniospermus* and *C. glomeratus* illustrated sister association in first clade, both herbs acquired maximum morphological similarities, difficult to differentiate. Flower size is smaller and leaves are sub-sessile in *C. rhyniospermus*. *C. scindicus* and *C. spinosus* both are shrubs also similar morphologically to a great extent and traditionally included in a single section also showed sister association in a single clade. Geographically both species are found in different areas of sind region, in similar environmental and soil conditions.

Most of the species (*C. rhyniospermus*, *C. glomeratus*, *C. cephalopodus*, *C. lineatus*, *C. kotschyanus* and *C. prostratus*) included in this lineage are herbs belong to section Inermes except *C. arvensis* included separately in section *Convolvulus* based on twining habit and *C. fruticosus* and *C. virgatus* are spiny shrubs placed in Acanthocladi section.

The second lineage contains representative species of sect. Acanthocladii, sect. spinoscentes, sect. Inermes and sect. Diffusi proposed by Boissier (1875) and Sa'ad (1967). The four species anomalously associated in a

single clade. The two spiny shrubs *C. leiocalycinus* and *C. acanthocladus* (share morphological similarities and traditionally placed in same section Acanthocladus) discretely associated with nonspiny herbs *C. aitchisonii* and *C. pseudocantabricus* (share similar morphology included in the section Inermes). *C. leiocalycinus* associated with *C. aitchisonii* and *C. acanthocladus* showed association with *C. pseudocantabricus*. The species which showed sister association have maximum dissimilarity in their morphological characters.

In traditional classification spine is the basic feature used for the placement of *Convolvulus* L. species is dissimilar with molecular analysis. Carine *et al.*, (2014) described that the section Acanthocladii is polyphyletic, proposed by Sa'ad (1967) in which all spiny species are included. Kousar *et al.*, (2016) also demonstrated in their morphological study of *Convolvulus* L. that spiny species are also grouped together with non-spiny species.

From the four investigated spiny species, two species *C. fruticosus* and *C. scindicus* associated with non spiny herbs in basal lineage while *C. leiocalycinus* and *C. acanthocladus* also closely associated with non spiny herbs in upper lineage. Our results showed congruence with the earlier studies and confirmed the polyphyly of section Acanthocladii including other intraspecific sections of *Convolvulus* L. Further inclusion of samples from worldwide origin and use of different molecular markers could affect the associations between species.

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