PHYLOGENETIC RELATIONSHIPS AMONG SOME SPECIES OF THE GENUS *CONVOLVULUS* (CONVOLVULACEAE) IN KURDISTAN REGION – IRAQ

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Abstract

A molecular systematics analysis was conducted using sequence data of two nuclear ribosomal DNA ITS and 26SrRNA gene for the genus *Convolvulus* L., by character based phylogenetic methods. The aim of this study was to investigate phylogenetic relationships among the studied species as well as comparing them with other species. Thirty-seven representative representatives of the genus Convolvulus L., of which nine were sequenced and twenty-eight were collected from the NCBI gene bank, were included as in group, whilst one member of the sibling genus *Cressa* was used as an outgroup to root the tree. By using maximum parsimony (MP) as implemented in PAUP* and bayesian inference (BI) by using Mr. Bayes methods, intraspecific connections within Convolvulus were deduced. The results from molecular study showed that five major lineages of both regions with the minimum differences in the locations of the species. Its concluded that the maximum parsimony method was found to be the method of choice for establishing intraspecific associations between *Convolvulus* species using ITS and 26SrRNA data as it clearly defined the connections supported by bootstrap support (BS) values.

Keywords: Phylogenetic, ITS, 28SrRNA, *Convolvulus*.

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Introduction

One of the plant families present in Iraq is the Convolvulaceae (Morning Glory/Bindweed), which contains roughly 1900 species belonging to 59 genera globally (Simpson, 2019). With more than 250 species found in temperate and tropical parts of the world, Convolvulus L., the second-largest genus in the Convolvulaceae family, is one of the most diverse (Carine and Lavinia, 2010, Parnell et al., 2012). Plants are the unsung heroes of life. They contribute to our survival by supplying air, food, and shelter (Mojarradgandoukmolla and Akan 2022). The *Convolvulus* genus has a worldwide distribution since its species may be found in all or the majority of the world's favorable climatic conditions. Al-Farhan (1987) said that due to the occurrence of the greatest number of species, south-eastern Asia (Iraq, Iran, and Turkey) is most likely the center of diversity for *Convolvulus*. There were 32 species of plants in the flora of Turkey and the East Aegean Islands (Davis et al., 1988). As the seventh volume of the Iraqi Flora of the Convolvulaceae family has not been released, there is no accurate count of the species in the specified genus in Iraq. According to the most recent survey of this family, conducted more than 20 years ago by Al-Eadani (1998), there were 18 species of *Convolvulus* in Iraq. Many genera of the family Convolvulaceae, such as the genus *Convolvulus*, are challenging to categorize, and the location of some intraspecific taxa has also been disputably changed from one species to another by several authors (Wood et al., 2015). *Convolvulus* was challenging to categorize at an intraspecific level due to the high rate of variation in its phenotypic traits. As a species responds to environmental changes, differences can occasionally be detected within that species. Because just a small number of features were taken into consideration for categorization in earlier research, greater evidence for species delimitation and connection was not provided (Stefanovi et al., 2002; Khalik, 2008). Due to character overlap, the taxonomy of the genus *Convolvulus* has been updated from several

perspectives, leading to the identification of a few new species using phenetic characteristics (Alfarhan, 1993; Johnson, 2001; Aykurt & Sümbül, 2011; Mill, 2013). Unfortunately, fewer molecular investigations of *Convolvulus* have been carried out (Stefanovi et al., 2002; Carine et al., 2004). Thus, the main objective of the current research was to investigate the relationships between the species of *Convolvulus* based on nuclear ribosomal DNA ITS and 26SrRNA molecular data and reconstructing the phylogenetic tree.

MATERIALS AND METHODS

Taxon Sampling and Molecular Methods: Our approach involved sampling *Convolvulus* were gathered from several Kurdistan Region districts in Iraq as well as from conserved specimens kept in the Salahaddin University-College of Education Herbaria. The study used 38 different taxa, 37 in-group taxa and one out-group *Cressa cretica*. In this study sequenced 9 species of *Convolvulus* (Table 1), and other species were received from a gene bank (Table 2). Genomic DNA was extracted from fresh material, herbarium exsiccatae or silica-gel dried samples. For DNA extraction, different amounts of plant tissue and several extraction methods were tested. Two methods provided the best results in *Convolvulus*: a CTAB (cetyltrimethyl ammonium bromide) approach modified from Doyle and Doyle (1987, 1990) and the DNA Maxi Kit (Plant) (GeNetBio, Korea) following the manufacturer's protocol. For old and poorly preserved tissues from herbarium sheets the CTAB method, including 2% PVP40 (polyvinylpyroli-done), was used. No more than 10 mg of plant tissue was included per extraction to avoid decrease of DNA quality and yield. The two noncoding regions of nrDNA ITS and 26SrRNA were amplified by using the primers as shown in (Table 3). The primers were ordered from Macrogen Company, Seoul, Kore. The total volume of amplification reactions was 25 µL and Master Mix made up of 15 µL: 6 µL genomic DNA extract; 4 µL of forward and reverse primer; 5 µL free nuclease water. The PCR-Thermal cycler for 26SrRNA gene started with 5 min for initial denaturation at 94 C° followed by 35 cycles: denaturation at 94 C° for 30 sec.; annealing at 54 C° for 60 sec.; extension at 72 C° for 60 sec. and the final extension at 72 C° for 5 min. While, the PCR program for ITS gene started with 5 min for initial denaturation at 94 C° followed by 35 cycles: denaturation at 94 C° for 60 sec.; annealing at 58 C° for 60 sec.; extension at 72 C° for 120 sec. and the final extension at 72 C° for 5 min. The resultant PCR products were checked on 1.5% agarose gel run in TAE buffer. The gel was stained with Safe red dye and photographed under UV transilluminator. Cleaned amplification products were sent for sequencing to the National Science and Technology Development Agency (NSTDA) in Thailand. Forward and reverse sequences from each template were manually edited and combined into a single consensus sequences with Geneious v5.4 (Biomatters, available from www. geneious.com).

Sequence Alignment and Phylogenetic Analysis: All the DNA sequences were edited and aligned with Clastal W option available in BioEdit, Version 7.0.4.1 (Hall, 2001) and manual adjustment, there are 38 accessions for each ITS and 26SrRNA, including the out group species. Bayesian inference (BI) and Maximum parsimony (MP) analyses were conducted for each dataset separately built from the two markers that included 38 terminal taxa with all sequences available. For MP, PAUP_ 4.0a164 (Swofford, 2000) was also used. Using heuristic search with 100 replicates of random taxon additions, Tree-Bisection-Reconnection (TBR) branch swapping, MulTrees on, and steepest decent off was performed. The maximum numbers of saved trees were 100 for each replicate. The consistency index (CI), retention index (RI), rescaled consistency (RC), and homoplasy index (HI) were assessed, and the bootstrap values were computed from 100 repetitions (Felsenstein, 1985). Prior to performing BI, MrModeltest2 version 2.3's Akaike information criterion (AIC) was used to evaluate the best substitution models (Nylander et al., 2004). The estimated best-fit model for the ITS region and the 26SrRNA, were the general time reversible model of nucleotide substitution with gamma-shaped rate variation (GTR+I+G). MrBayes v.3.2 was utilized for BI analysis (Ronquist and Huelsenbeck, 2003). The software generated automated estimates of the priors for state frequencies, rates, and variance between sites. Two independent analyses were performed using two million generations for the ITS and 26SrRNA dataset, using four Markov chains (one cold and three heated) for each generation and a temperature parameter of 0.1. Every 100 generations, samples of trees were taken. A tree with a maximum of 50% (majority rule consensus tree) was then displayed after burn-in phase samples had deleted 25% of the initial tree

analyzed. The value of posterior probability (PP) was calculated and the final tree was visualized by using FigTree software version 1.4.3 (Rambaut, 2016).

Table 1: Specimen numbers of *Convolvulus* species which their DNA have been studied, and their preserved locations in the Herbarium of College of Education/ Salahaddin University with collection date.

Taxa	**Specimen number & Herbarium symbol**		**Locality in Kurdistan**	**Date of collection**
C. arvensi	8021	ESUH	Kory valley	7.11.2021
C. dorycnium	8028	ESUH	Halgurd M.	7.7.2011
C. major	8035	ESUH	Sakran M.	13.7.2018
C. pentapetaloides	8042	ESUH	Rowanduz	1.5.2005
C. pilosellaefolius	8049	ESUH	Hasarost M.	14.9.2021
C. reticulata	8056	ESUH	Halgurd M.	26.7.2018
C. scammonia	8064	ESUH	Haji Omran	20.7.2007
C. stachydifolius	8072	ESUH	Gara M.	7.11.2021
C. tricolor	8065	ESUH	Hasan Bag M.	7.7.2011

Table 2: list of primers and their sequences that have been used in the study.

Primer for regions	**Product size**	**Sequence 5'---- 3'**		**References**
		Foreword	**Reverse**	
26SrRNA	700 bp	TCTGACATGTGTGCGAGTCA	GATTCGGCAGGTGAGTTGTT	(Chen et al., 2010)
ITS	400 bp	ATGCGATACTTGGTGTGAAT	TCCTCCGCTTATTGATATGC	(Taberlet et al., 1991)

Table 3: Samples included in nrDNA ITS and 26SrRNA phylogenetic analyses obtained from NCBI genebank.

Taxa	**DNA source (location and Voucher)**	**Accession no**
C. althaeoides **nrDNA (ITS + 26SrRNA)**	Carine et al. 2004, Morocco	AY560272
C. cantabricus **nrDNA (ITS + 26SrRNA)**	Carine et al. 2004, Morocco	AY560278
C. commutatus **nrDNA (ITS + 26SrRNA)**	Mitchell et al. 103, 2016 unknown	KC528984
C. compactus **nrDNA (ITS + 26SrRNA)**	Mitchell et al. 140, 2016 unknown	KC528975
C. eremophilus **nrDNA (ITS + 26SrRNA)**	Mitchell et al. 113, 2016 unknown	KC528948
C. fatmensis **nrDNA (ITS + 26SrRNA)**	Mitchell et al. 176, 2016 unknown	KC528861
C. fruticosus **nrDNA (ITS + 26SrRNA)**	Mitchell et al. 116, 2016	KC528944

	unknown	
***C. galaticus* nrDNA (ITS + 26SrRNA)**	Mitchell et al. 58, 2016 unknown	KC528859
***C. holosericeus* nrDNA (ITS + 26SrRNA)**	Carine et al. 2004, Turkey	AH013866
***C. lanatus* nrDNA (ITS + 26SrRNA)**	Mitchell et al. 88, 2016 unknown	KC529002
***C. leiocalycinus* nrDNA (ITS + 26SrRNA)**	Carine et al. 2004, Iran	AH013868
***C. leptocladus* nrDNA (ITS + 26SrRNA)**	Mitchell et al. TM69, 2016 unknown	KC528941
***C. lineatus* nrDNA (ITS + 26SrRNA)**	Carine et al. 2004, Turkey	AH013871
***C. microphyllus* nrDNA (ITS + 26SrRNA)**	Sharma et al. 2018,India	MH260277
***C. oleifolius* nrDNA (ITS + 26SrRNA)**	Carine et al. 2004, Turland	AY560306
***C. oxyphyllus* nrDNA (ITS + 26SrRNA)**	Carine et al. 2004, Sudia Arabia	AH013878
***C. oxysepalus* nrDNA (ITS + 26SrRNA)**	Mitchell et al. 232, 2016 unknown	KC529010
***C. persicus* nrDNA (ITS + 26SrRNA)**	Mitchell et al. 172, 2016 unknown	KC528864
***C. pseudocantabrica* nrDNA (ITS + 26SrRNA)**	Mitchell et al. 237, 2016 unknown	KC528946
***C. pyrrhotrichus* nrDNA (ITS + 26SrRNA)**	Mitchell et al. 242, 2016 unknown	KC528991
***C. rectangularis* nrDNA (ITS + 26SrRNA)**	Mitchell et al. 239, 2016 unknown	KC528868
***C. rottlerianus* nrDNA (ITS + 26SrRNA)**	Mitchell et al. 133, 2016 unknown	KC528940
***C. scindicus* nrDNA (ITS + 26SrRNA)**	Mitchell et al. 143, 2016 unknown	KC529007
***C. siculus* nrDNA (ITS + 26SrRNA)**	Carine et al. 2004, Morocco	AY560312
***C. spinosus* nrDNA (ITS + 26SrRNA)**	Mitchell et al. 244, 2016 unknown	KC528988
***C. subhirsutus* nrDNA (ITS + 26SrRNA)**	Mitchell et al. 238, 2016 unknown	KC528960
***C. turrillianus* nrDNA (ITS + 26SrRNA)**	Mitchell et al. 233, 2016 unknown	KC528989
***C. virgatus* nrDNA (ITS + 26SrRNA)**	Mitchell et al. 107, 2016 unknown	KC528882
***Cressa cretica* nrDNA (ITS + 26SrRNA)**	Khan et al. 2013	KJ004289

RESULTS AND DISCUSSION

The PCR for ITS and 26SrRNA gene in all investigated species produced ~600 - 890 bp monomorphic fragment. The analogue of the sequenced products was recognized using the

BLAST on NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All the sequences showed 95 to 99% identity to available *Convolvulus* species. The ITS and 26SrRNA length of all sequences was 881 bp. The sequence lengths were different in the numbers of characters included because of ambiguity at the beginning and end of sequences. The ITS and 26SrRNA data set consisted of 37 ingroups and one outgroup taxa with 881 aligned DNA characters (including gaps) were used, from them 781 were parsimony informative. The maximum parsimony analysis showed 100 trees from which a single most parsimonious tree was retained with a tree length of 3892 steps. The CI, RI, RC and HI were 0. 503, 0.530, 0.267 and 0.497, respectively, with topology identical between MP and BI analyses. The summary of the analysis showed in (Table 4).

Table 4: A summary of alignment and tree statistics of nrDNA ITS and 26SrRNA region analyses.

Parameters/Regions	**ITS and 26SrRNA**
Aligned length	881
Number of parsimony informative characters	781
Number of variable parsimony uninformative characters	75
Number of constant characters	25
Tree length (steps)	3892
CI (Consistency Index)	0.503
RI (Retention Index)	0.530
RC (Rescaled Index)	0.267
HI (Homoplasy index)	0.497
Model	GTR+G

The maximum parsimony based phylogenetic tree was constructed based on the nuclear ribosomal DNA ITS and 26SrRNA gene sequence (Fig. 1). Total 881 characters were used in this analysis, out of which 25 characters were constant. 781 characters were considered informative for MP tree construction out of total 75 variable characters. The tree length was calculated as 3892 while measure of homoplasy were determined as CI (0.503) and RI (0.530). The overall topology of MP tree was not fully resolved. five major lineages with moderate support separate all studied nine species from others. The majority rule consensus tree gave moderate support (72%) to a clade that represented the eight studed species: *C. pilosellaefolius, C. reticulata, C. major, C. scammonia, C. tricolor, C. stachydifolius, C. arvensis* and *C. dorycnium from C. pentapetaloides*. while within this clade the sister association between the species was strongly supported by 93-100% bootstrap value.

Another tree using same data was reconstructed by Bayesian inference. This method relies on best nucleotide substitution model and for this dataset it was found to be General Time Reversable with gamma distribution (GTR+G). The tree topology was not clear compared to parsimony approach. A monophyletic clade of genus Convolvulus was observed against outgroup from the genus *Cressa* (Fig. 2). The Convolvulus clade diverges into five lineages as MP but with some variations between species nested in the clade. The upper lineage with strong support of posterior probability (PP) value combines nine species of Convolvulus (*C. stachydifolius*, *C. dorycnium*, *C. pilosellaefolius*, *C. reticulata*, *C. scammonia*, *C. tricolor*, *C. reticulata*, *C. arvensis* and *C. pentapetaloides*), while the other lineage with moderate to strong supportive value further separated additional species. The top nodal branch represents the sister association between *C. stachydifolius* and *C. dorycnium* with weak 0.58 PP. Inaddition, the second nodal branch represents the sister association between *C. pilosellaefolius* and *C. reticulata* with modearate 0.68 PP support. While, the third showing strongly supported sister relationship between *C. scammonia* and *C. tricolor* with strong 1.00 pp, while the other

strongly support paraphyletic species *C. reticulata* and *C. arvensis* separated from other sister species. In both MP and BI tree *C. pentapetaloides* was disjointed from them.

In order to identify intraspecific connections between Convolvulus species, the current study focused on several phylogenetic methods. Several phenetic, cluster, and phylogenetic techniques were used to clarify the connections between plant species since the species idea has long been an important subject in systematic biology. Also, a novel method for infraspecific categorization is the use of DNA sequence data for species delimitation (Williams et al., 2014). In MP and BI tree shows that the *C. pentapetaloides* was diverge from other species due to the *C. pentapetaloides* was a single species with annual plant duriation and the remain species were mostly perenial duriation. MP tree grouped *C. pilosellaefolius, C. reticulata* and *C. major* in a same clade caused by they were perenial and woody stem base.

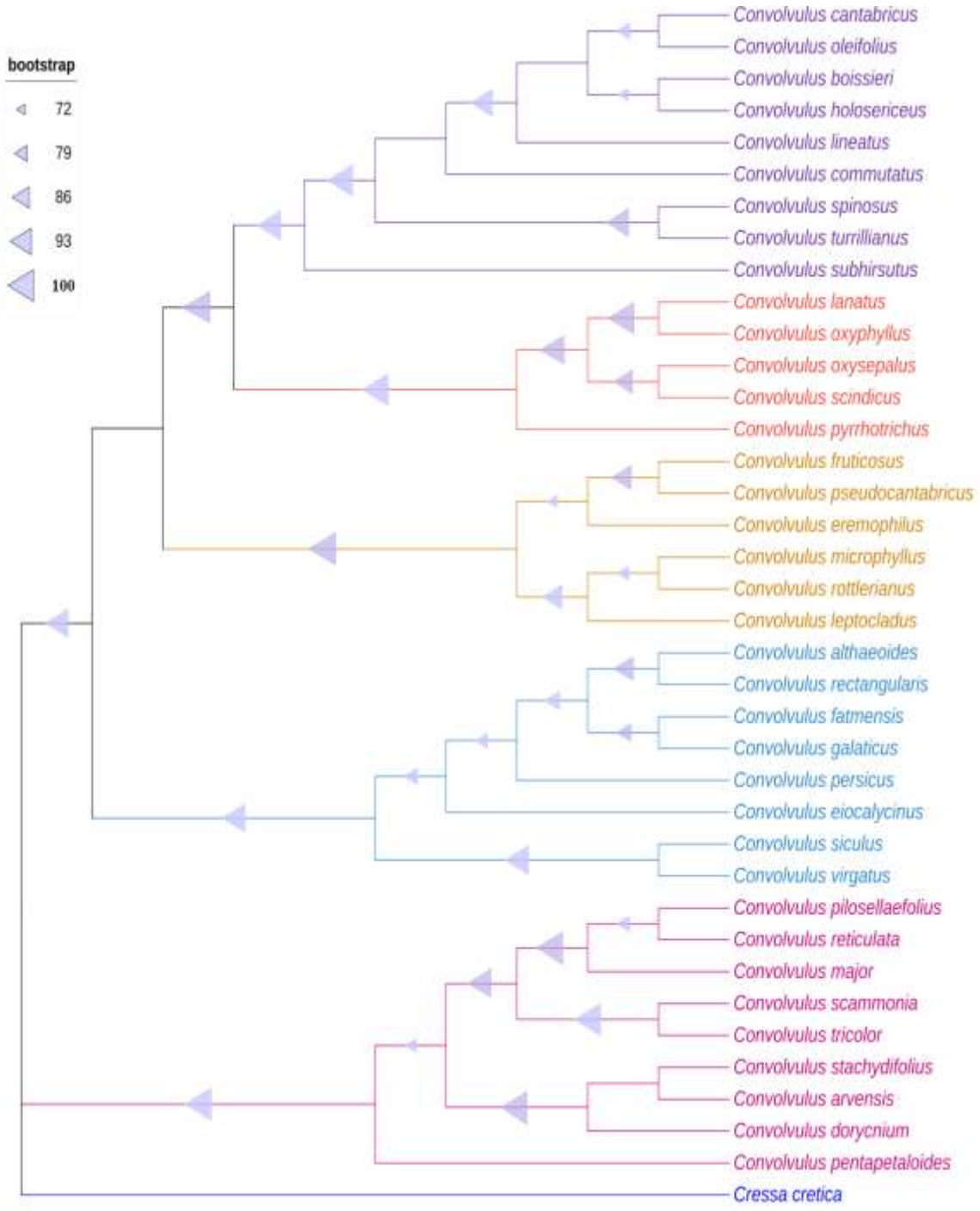

Figure 1: Majority rule consensus parsimonious tree resulting from the analysis of ITS and 26SrRNA gene sequence for Convolvulus. Bootstrap supports in percentages based on 1000 replication analysis are shown in the nodes. clades are separated by colour, the triangle on the branches indicate bootstrap support.

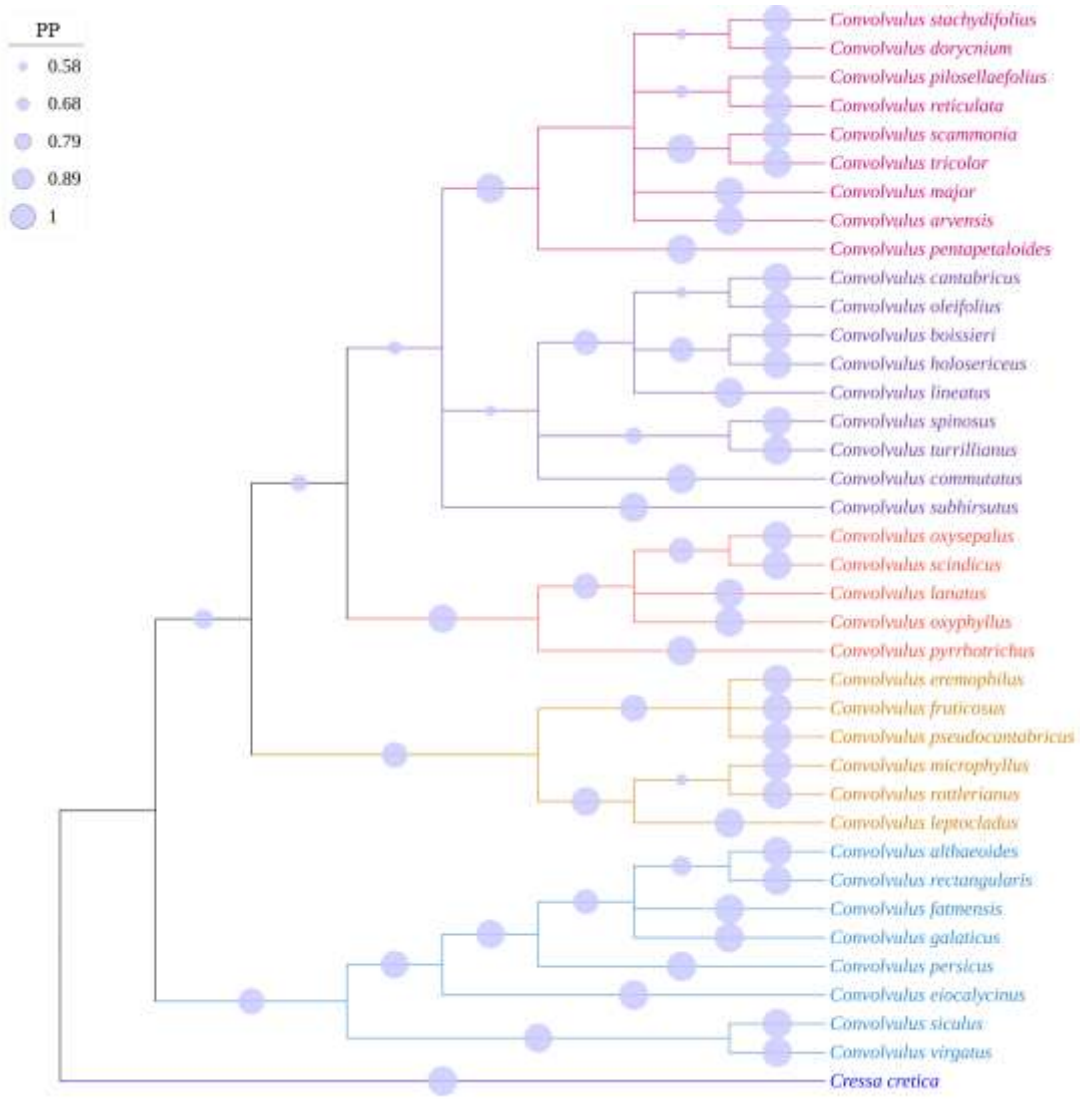

Figure 2: Phylogenetic tree with mean branch lengths from a representative Bayesian analysis of nrDNA ITS and 26SrRNA gene data. Circles on branches represent their respective posterior probabilities. Clades are separated by colour.

Stefanović et al. (2002), proposed that the glory family composed of eleven tribe including Convolvuleae tribe is congruent with the current finding. Nevertheless, The results from the present analyses are also quite similar, both in inferred patterns of evolution and support, as well as lack of support in certain regions, with the BI analysis based on a larger taxon sampling and nrDNA data (Eserman et al., 2014, Kousar et al., 2016, Simões and Staples, 2017, Wang et al., 2021).

CONCLUSION

This work is an exhaustive attempt to determine the phylogenetic position of a relatively small clade of *Convolvulus* species, both in terms of the quantity of DNA sequence data and the variety of analytical methods used. the inability to determine with certainty the precise number of *Convolvulus* species. Thus, the findings of this study have improved our knowledge of the evolutionary connections and periods of divergence among morning glory species and laid the groundwork for further research. The findings of earlier research are supported by the evolutionary intraspecific linkages provided here, which also point to the need for *Convolvulus* taxonomy revision and at the evolutionarily labile nature of complex morphological traits, including those of economic significance. Our finding showing the same tree topology based on MP and

BI with some variation in location of species nested within the clades. Also, MP tree displays better species separation, while the sequenced nine species in both tree were grouped together with minimal differences in position.

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