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PHYLOGENETIC RELATIONSHIP STUDIES ON THE GENUS *Limonium* MILL. PLUMBAGINACEAE FROM SAUDI ARABIA USING ITS SEQUENCES OF NUCLEAR RIBOSOMAL DNA

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Abstract- In Saudi Arabia, the family Plumbaginaceae is represented by two species: *Limonium* and *Plumbago*. The genus *Limonium* is represented by four species (i.e. *Limonium axillare, L. carnosum, L. cylindrifolium,* and *L. lobatum*). Out of these *L. carnosum* is endemic to Saudi Arabia. A perusal of taxonomic literature on *Limonium* reveals that the molecular evolutionary relationships of the species of *Limonium* distributed in Saudi Arabia is lacking. Owing to enormous phylogenetic significance of internal transcribed spacer sequence (ITS) of nuclear ribosomal DNA [-a gene which is now a day considered as better than its reputation, this study was undertaken with a aim to establish evolutionary relationships of the genus *Limonium* distributed in Saudi Arabia based on nrDNA ITS nucleotide sequences.

The combined length of the entire ITS region (ITS1, 5.8S and ITS2) from taxa sequenced in the present study ranged from 599-618 nucleotides. The length of the ITS1 region and GC contents ranged from 201-203 nucleotides and 45-50% respectively, the 5.8S gene was 161-163 nucleotides long, the length of the ITS2 region and the GC content ranged from 233-253 nucleotides and 48-51% respectively. The aligned data matrix has a total number of 670 characters of which 256 characters were constant, 255 characters were variable but parsimony-uninformative and 159 characters were parsimony-informative. Insertions and deletions (indels) were necessary to align the sequences. Indels ranged from 1 to 52 bp.

In the present study, *Limonium axillare, L. cylndrifolium, L. carnosum,* and *L. lobatum* were sequenced, and analysed together with the taxon from 1. Sect. Limonium Subsections Densiflorae, 2. Sect. Limonium Subsections Steirocladae, 3. Sect. Limonium Subsections Hyalolepidae, 4. Sect. Limonium Subsections Limonium, 5. Sect. Limonium Subsections Dissitiflorae, 6. Sect. Polyarthrion, 7. Sect. Schizhymenium. The parsimony analysis (using PAUP) of the entire ITS region resulted in to 1280 maximally parsimonious trees (MPTs) with a total length of 480 steps, a consistency index (CI) of 0.754 (0.730) excluding uninformative characters), a homoplasy index (HI) of 0.456 (0.432 HI excluding uninformative characters), rescaled consistency index (RC) of 0.579 and a retention index (RI) of 0.767.

The resulted phylogenetic tree clearly reveals that the largest section of the genus (section *Limonium*) does not constitute a monophyletic assemblage. *Limonium carnosum* clade with *Limonium narbonense* and *Limonium vulgare* (Sect. *Limonium,* Subsections *Limonium*); *L. cylindrifolium* and *L. axillare* which has been previously placed in Sect. *Limonium,* Subsections *Limonium* doesnot nested within its clade of own, rather were found base of the phylogenetic tree. *Limonium lobatum* which has been previously based on morphology have been placed in Section Pteroclados, subsection Pdontolepideae, occupied basal most position in the phylogenetic tree. A perusal of all trees (Strict Consensus Tree, Bootstrap Strict Consensus Tree and NJ tree) clearly indicates that *Limonium carnosum* included in this study from geographic region Saudi Arabia, phylogenetically very closely related with *Limonium narbonense* and *Limonium vulgare*. It is also clearly evident that *L. cylindrifolium* and *L. axillare* does not nested deeply within the phylogenetic tree, rather were found at the base of the phylogenetic tree and *L. lobatum* occupies most basal position in phylogenetic tree. *Limonium cylindrifolium* and *Limonium axillare* shows sister relationships; however, in ITS sequences both these two species differs in 5 base pairs addition in ITS2 region. Therefore, ITS2 secondary structure for these two species, and additionally all taxon included in the analysis were predicted to bring molecular morphological signature of the *Limonium* distributed in Saudi Arabia.

This is the first report of inferring the nrDNA ITS based phylogenetic relationships and establishment of molecular signature of the *Limonium* distributed in Saudi Arabia. Since, the majority of the species of the genus *Limonium* is yet to be sequence; the present study will nevertheless help in bringing the complete phylogeny of the genus.

Keywords- internal transcribed spacer (ITS), molecular phylogeny, ribosomal DNA (rDNA)

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Introduction

Plumbaginaceae is a family of flowering plants, with a cosmopolitan distribution. The family is sometimes referred to as the leadwort family or the plumbago family. Most species in this family are perennial herbaceous plants, but a few grow as lianas or shrubs. The plants have perfect flowers and are pollinated by insects. They are found in many different climatic regions, from arctic to tropical conditions, but are particularly associated with salt-rich steppes, marshes, and sea coasts. The family has been recognized by most taxonomists; however, [1] placed the family in a separate order Plumbaginales, which included no other families. [2] had segregated some of these plants as family Limoniaceae. The Angiosperm Phylogeny Group, APG II system of classification [3], recognizes this family and assigns it to the order Caryophyllales in the clade core eudicots. It includes 24 genera (*Acantholimon, Aegialitis, Armeria, Bamiana, Buciniczea, Cephalorhizum, Ceratostigma, Chaetolimon, Dictyolimon, Dyerophytum, Eremolimon, Ghasnianthus, Goniolimon, Ikonnikovia, Limoniastrum, Limoniopsis, Limonium, Muellerolimon, Neogontscharovia, Plumbagella, Plumbago, Popoviolimon, Psylliostachys, Vassilczenkoa*) and about 800 species.

The Genus *Limonium* in Brief

The genus *Limonium* is consists of 120 species. The members of *Limonium* are also known as Sea Lavender or Statice. *Limonium* is in Plumbaginaceae, the plumbago or leadwort family. Despite their common names, species are not related to the lavenders or to rosemary. The genus has a subcosmopolitan distribution in Europe, Asia, Africa, Australia and North America. By far the greatest diversity (over 100 species) is in the area stretching from Canary Islands east through the Mediterranean region to central Asia. Sea-lavenders normally grow as herbaceous perennial plants, growing 10-70 cm tall from a rhizome; a few (mainly from the Canary Islands) are woody shrubs up to 2 meter tall. Many species flourish in saline soils, and are therefore common near coasts and in salt marshes, and also on saline, gypsum and alkaline soils in continental interiors.

Morphological Characteristics of *Limonium*

The morphological characteristics of *Limonium* is: leaves simple, entire to lobed, and from 1-30 cm long and 0.5-10 cm broad; most of the leaves are produced in a dense basal rosette, with the flowering stems bearing only small brown scale-leaves (bracts). The flowers are produced on a branched panicle or corymb, the individual flowers small (4-10 mm long) with a five-lobed calyx and corolla, and five stamens; the flower color is pink, violet to purple in most species, white or yellow in a few. Many of the species are apomictic. The fruit is a small capsule containing a single seed, partly enclosed by the persistent calyx. Several species are popular garden flowers; they are generally known to gardeners as statices. They are grown both for their flowers, and for the appearance of the calyx, which remains on the plant after the true flowers have fallen, and are known as "everlasting flowers".

A Brief Review of Literature Taxonomy of *Limonium*

Limonium is the most species rich and widespread genus of Plumbaginaceae, although the number of species reported in the genus is rather speculative. A very high percentage of *Limonium* diversity is centered in the Mediterranean basin with nearly 300 taxa currently used in regional floras and checklists [4,5]. A significant portion of these taxa belong to section *Limonium*, one of the 12 sections in which the genus has been traditionally split [6]. In turn, section *Limonium* has been divided into six subsections [6]. Several of these sections were later grouped into subgeneric ranks. Thus, [7] included sections *Polyarthrion, Myriolepis, Siphonantha*, and *Psillyostachys* within subgenus *Siphonantha*. On the other hand, [8] raised section *Pteroclados* to the subgeneric level and excluded section *Myriolepis* from subgenus *Siphonantha* to create a new subgenus (subgenus Myriolepis). Other analytical treatments have dealt with some of the sections recognized by Boissier and Pax (section *Circinaria*, section *Schyzimenium*, section *Psillyostachis*, section *Schyzopethalum*, section *Pterolimon*) as separate genera [9]. Both sexual (diploid and tetraploid) and apomictic (spanning triploid to hexaploid cytotypes) species have been reported in section *Limonium*. Nevertheless, diploid species are few, and polyploid agamic species constitute the largest portion of the diversity currently known in this section. Several competing hypotheses, based mainly on karyological data, regarding the origin of polyploid *Limonium* species have been postulated [10]. Suggested that triploid taxa arose through hybridization between diploid and tetraploid species, the latter having originated from diploid ancestors [10]. In contrast, noted that within section *Limonium*, diploid species show two basic chromosome numbers (n= 58 and n= 59) and postulated that the complement of the polyploids arose through several combinations, involving reduced and unreduced gametes, of the n=58 and n=59 genomes. Hence, triploid (2n= 5, 24, 25, 26, and 27) and tetraploid (2n 5, 34, 35, and 36) taxa combine genomes of the two basic cytotypes. Although conflicting, both hypotheses agree that interspecific hybridization has played a substantial role in the evolution of section *Limonium*. However, this has not been tested by means of a rigorous phylogenetic analysis. Unfortunately, the very similar morphology exhibited by most members of section *Limonium* (with most characters showing continuous variation) has prevented the use of morphological characters in a phylogenetic (cladistic) context. Theoretically, molecular analyses could circumvent this drawback and offer robust hypotheses on the evolution of these species. Molecular approaches have been applied to some genera with a large apomictic element in order to detect species (microspecies) boundaries and to trace the origins of apomictics [11].

Taxonomic complexity has frequently been related to the mating system of plants. Hence, taxa having breeding systems favoring selfing or asexual reproduction (apomixis and clonality) are usually prone to taxonomical controversy. Apomictic plants circumventing sexuality, obligately or facultatively, defy classical species concepts and make the delimitation of taxa a difficult task [12].

Distribution of *Limonium* in Saudi Arabia

In Saudi Arabia, the family Plumbaginaceae is represented by two species: *Limonium* and *Plumbago*. The genus *Limonium* is (Sea Lavender, Statice, or Marsh-rosemary) is represented by four species (i.e. *Limonium axillare, L. carnosum, L. cylindrifolium,* and *L. lobatum*). Out of these *L. carnosum* is endemic to Saudi Arabia [Fig -1].

ITS Sequences of nrDNA and its Utility

The plant cell contains three different genomes: chloroplast, mitochondrion and nucleus. Nuclear ribosomal DNA is arranged in tandem repeats in one or a few chromosomal loci. Only among closely related species are the chromosomal locations similar. The nuclear genes that code for rRNA are repeated thousands of times within the typical plant genome. In fact they can comprise as much as

10% of the total plant DNA. The most remarkable feature of rDNA is the overall sequence homogeneity among members of the gene family in a given species. The process by which this pattern of intra-specific homogeneity and interspecific heterogeneity is maintained has been called concerted evolution [13]. In plants, the rDNA cistron encodes 18S, 26S and 5.8S rRNAs, which are separated by the two internal transcribed spacers (ITS1 and ITS2). The cistron is flanked by the 5' and 3' external transcribed spacers (5'-ETS and 3'-ETS). The nuclear ribosomal ITS region including the 5.8S gene [Fig-2] has been the most widely used molecular marker at the interspecific and intergeneric levels in plants. The region is relatively short to sequence, with ITS1 200-300 bases long, ITS2 180-240 bases, and 5.8S and 5.8S ca. 160 bp in flowering plants. The amplification and sequencing primers are highly universal [14].

Fig. 1- Genus Limonium distribution in Saudi Arabia

The nuclear ribosomal transcription unit (NRTU) is comprised of 18S, 5.8S and 28S genes, two internal transcribed spacers (ITS-1 and ITS-2), and an intergenic spacer (IGS). After transcription, the NRTU is processed to produce mature rRNAs that are key components of cytoplasmic ribosomes. NRTU are found in hundreds to thousands of tandem copies and usually several NRTU clusters are present within plant genomes. The conserved regions (18S and 28S genes) of NRTU are used to infer phylogenetic relationships at higher taxonomy levels, whereas the more rapidly evolving segments (ITS and IGS) are used for studies at the genic or population levels [15,16]. For over a decade, sequences of internal transcribed spacers (ITS) of NRTUs have been widely used to infer phylogenetic relationships, genetic diversity and to unravel evolution in a wide range of complexes in plants [15,16]. Although NRTUs are found in thousands of copies within a genome, intra-genomic diversity is generally low [17]. This homogeneity among NRTUs is attributed to concerted evolution [18], a process that acts through gene conversion and unequal crossing over. Despite the fact that homogenization is a norm among NRTUs in a genome, extensive intra-individual and intra-specific variation has been observed in various plant species [19]. Evidence is accumulating that suggests that intra-individual variation of nuclear ribosomal ITS regions should not be considered as exceptional [20]. Because of the influence of concerted evolution, the occurrence of ancestral polymorphisms is not the most likely ultimate cause for intra-genomic variability in this marker. Instead, a more frequent origin is the merging of different ITS copies within the same genome as a consequence of gene flow. Once the two copies meet, the fate of the polymorphism depends on genetic, reproductive and population-level factors: specifically, the number and location of ribosomal loci (on the same or different chromosomes), the occurrence of polyploidy and/or apomixes [21], and the relative abundance of different ITS copies in the breeding populations [20].

Sequences of the nuclear rDNA internal transcribed spacers (ITS region) have been widely applied to depict evolutionary relationships at lower taxonomic levels, notably at the intrageneric ones [18]. In addition, the ITS region has been a valuable tool for tracing the hybrid origin of diploid [22] and polyploid [16,23] species in flowering plants.

Since the first report of the utility of the cytochrome c oxidase subunit 1 (CO1) as a DNA barcode to identify animals, DNA barcoding has attracted worldwide attention [24.25]. Many loci such as ITS [26], *rbc*L [27], *psb*A-*trn*H [28], and *mat*K [28], combination of *rbc*L and *mat*K [29] etc. have earlier been proposed for plant DNA barcode. Nuclear genes can provide more information than barcoding based on organellar DNA which is inherited from only one parent [30]. It has been emphasized that an ideal barcode should possess sufficient sequence variation to discriminate the taxon at species level; however, it also need to have sufficiently conserved region so that there is less variability within species than between species [31]. The ITS2 shows significant sequence variability at the species level or lower [32,33]. The availability of structural information of ITS2 permits analysis even at higher taxonomic level too [32.34]. compared seven candidate DNA barcodes (*psb*A-*trn*H, *mat*K, *rbc*L, *rpo*C1, *ycf*5, ITS2, and ITS) and proposed that ITS2 has potential for use as a standard DNA barcode to identify medicinal plants[35]. The ITS2 region has also been shown to be applicable in discriminating among a wide range of plants genera and families e.g. Asteraceae, Rutaceae, Rosaceae, Araliaceae [36-38]. Besides plants, the ITS2 sequence also has potential for use in barcoding of animals. The secondary structure of ITS2 are conserved as well as possesses sufficient variation in primary sequences and secondary structure which also provides useful biological information for alignment; therefore, the ITS2 sequences is also used as molecular morphological characteristics for species identification [39].

Fig. 2- Internal Transcribed Spacer Region

Objective

While searching for the DNA sequence of *Limonium* in the GenBank, it was observed that the species of *Limonium* distributed in Saudi Arabia has earlier been sequenced (except *L. lobatum*) and detailed perusal of taxonomic literature on *Limonium* reveals that the molecular evolutionary relationships of the species of *Limonium* distributed in Saudi Arabia is lacking, thus molecular evolutionary studies on the genus *Limonium* from Saudi Arabia was very much needed. Hence, this study was undertaken to establish evolutionary relationships and molecular signature of the genus *Limonium* distributed in Saudi Arabia based on nrDNA ITS nucleotide sequences.

Materials and Methods

Plant Materials

Plant materials for sequencing of ITS sequences of nuclear ribosomal DNA were collected during field trip or from the herbarium specimens. Voucher specimens for the plant material used in the experiment are listed in [Table-1]. Leaves were dried in silica gel prior to DNA extraction.

Table 1- Voucher information and GenBank accession number for the taxon sequenced in the present study

Taxon	Locality	Collector
Limonium axillare	Farsan Islan, KSA	S. Choudhary 22.6.88, 13084 RIY
Limonium comosum	Wadi Sirhan, KSA	Al-Red, 13.11.65, 13082 RIY
Limonium cylindrifolium	Doshak Island, KSA	S. Chaudhary, 24.6.88, 15605 RIY
Limonium lobatum	Al-Taysiah, KSA	S. Chaudhary, 2.5.98, 16110 RIY

Total Genomic DNA Extraction

Total genomic DNA was extracted using the DNeasy Plant Mini kit (QIAGEN Inc., Crawley, West Sussex, UK). In brief, 20mg silica gel dried leaf tissue was taken in 1.5 ml eppendorf cup, and 5-6 tungsten carbide bead placed in eppendorf cup. The eppendorf cup (with leaf and tungsten carbide bead) placed in tissue lyser and grinded the leaves sample for 2-3 minutes. Eppendorf cup were taken out from tissue lyser after grinding, and 4 ul RNase stock solution (100 mg/ml) and 400ul Buffer AP1 was added and vortex. Buffer AP1 is the main extraction buffer which isolates DNA from the cells. The purpose of adding RNase (an enzyme) was to break the RNA into small pieces which remain in the cell, so that easy to pass from filter. After adding 4 ul RNase and 400 ul Buffer AP1 in to the eppendorf cup containing grinded leaf tissue, the eppendorf cup were place in the water bath or dry bath to incubate the mixture of 4 ul RNase, 400 ul Buffer AP1 and grinded leaf tissue for 10 minutes at 65ºC. It was mixed 2 or 3 times during the incubation period by inverting the tubes gently. After 10 minutes of incubation the eppendorf cup was taken out from water bath or dry bath, and 130 ul Buffer AP2 was added to the eppendorf cup containing Lysate and then mixed it properly by inverting the tubes and then incubated the eppendorf tube (containing mixture of 4 ul RNase, 400 ul Buffer AP1, grinded leaf tissue and Buffer AP2) for 5 minutes on ice. After cold incubation for 5 minute on ice, the eppendorf cup (containing mixture of 4 ul RNase, 400 ul Buffer AP1, grinded leaf tissue and Buffer AP2) were taken out and kept on the room temperature for 10 minutes. Now the eppendorf cup (containing cold incubated mixture of 4 ul RNase, 400 ul Buffer AP1, grinded leaf tissue and Buffer AP2) placed into centrifuge machine and centrifuge the eppendorf tube (containing cold incubated mixture of 4 ul RNase, 400 ul Buffer AP1, grinded leaf tissue and Buffer AP2) at 14000 rpm for 5 minutes. After centrifugation, the clear or colorless Lysate (supernatant, which contains DNA) transferred to QIAshredder mini spin column lilac- pink in color which is sitting or placed in a 2 ml collection tube), and centrifuge it for 2 minutes at 14000 rpm. The clear lysate (obtained into 2 ml collection tube after centrifuge transferred to the collection tube, and 1.5 volume Buffer AP3/E added to the clear lysate and mix it by pippeting. AP3/E is the binding buffer. Now 650ul of the mixture (mixture of clear lysate containing DNA and buffer AP3/E) was transferred into DNeasy mini spin colorless column (which are colorless and placed or sitting into 2 ml collection tube, supplied along with kit) and Centrifuge it for 1 minute at 8000 rpm. The flow through comes out into the collection tube after centrifuge was of no use therefore it was discarded. The process was repeated with the remaining lysate to get DNA on the filter. The filter was kept on room temperature before washing. For washing 500 ml Buffer AW was added to DNeasy mini spin column containing DNA and centrifuge 2 minutes at 14000 rpm. Flow through was discarded and the process of washing repeated again. After completion of washing, the DNeasy mini spin column containing DNA was kept on room temperature for 5 minutes, and then for elution, 100ul sterilized double distilled water (pyrogen free) was added to DNeasy mini spin column containing DNA and centrifuge for 2 minute at 14000 rpm. After elution, the DNA transferred to fresh 1.5 ml eppendorf tube and stored at -20 for further use of PCR.

Amplification of ITS Region

ITS sequences of nuclear ribosomal DNA were amplified using primers [Table-2] of via the polymerase chain reaction (PCR) 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 initial denaturation, denaturation, annealing, and extension, and a final extension [Table-3]. The PCR products were purified with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea) prior to sequencing [Fig-3].

Table 2- Primer sequence used in amplification of ITS region

ITS1	(Forward)	5'- G T C C A C T G A A C C T T A T C A T T T A G -3'
ITS4	(Reverse)	5'- T C C T C C G C T T A T T G A T A T G C -3'

Table 3- PCR Reaction condition USED FOR AMPLIFICrion of ITS region of nrDNA

1	Initial Denaturation		94°C for 5 minutes
2	Denaturation		94°C for 1 minute
	Annealing	Number of cycles: 40	49°C for 1 minute
	Extension		72°C for 1 minute
3	Final extension		72°C for 5 minutes

DNA Sequencing

The purified fragments were directly sequenced using dye terminator chemistry following the manufacturer's protocol. 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 minutes. Sequenced product was precipitated with 17µl of deionized sterile water, 3µl of 3 M NaOAc, and 70 µl of 95% EtOH. Polyacrylamide gel electrophoresis was conducted with Long Ranger Single packs (FMC BioProducts) and an ABI 3100 automated DNA sequencer (Perkin-Elmer, Applied Biosystems). Each sample was sequenced in the sense and antisense direction. The sequences were analyzed with ABI Sequence Navigator software (Perkin-Elmer/Applied Biosystems). Nucleotide sequences of both DNA strands were obtained and compared to ensure accuracy.

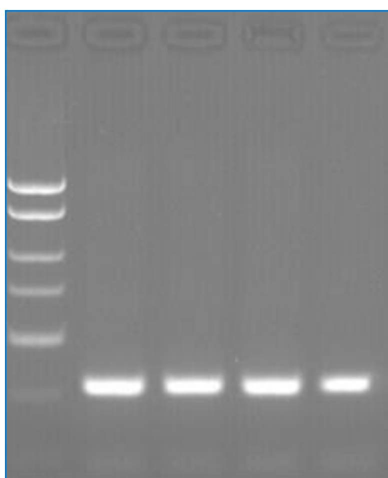

Fig. 3- Photos (Gel electrophoresis) to amplify the DNA by PCR technique

Sequence Alignment

Sequence alignment was performed using ClustalX version 1.81 [40] with gap opening penalty 10 and gap extension penality 3.0. Sequence alignments was subsequently adjusted manually using BioEdit [41]. Insertion-deletions (Indels) were scored as single characters when we had confidence in positional homology. Gaps were treated as missing data in phylogenetic analysis.

Sequence Retrieved from GenBank

ITS sequences of nrDNA of 19 species of *Limonium* and Outgroup [Table-4] were retrieved from GenBank database of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The generic classification of the *Limonium* has been present in the [Table-5]. The boundaries between the ITS1, 5.8S, and ITS2 gene for the data set (both the sequence generated for the present study and the sequences retrieved from the GenBank) were determined according to span mentioned in features of the nrDNA ITS sequences available in GenBank. ITS2 sequences were extracted from the complete set of the ITS sequence and used in the further analysis.

Phylogenetic Analyses

The Maximum Parsimony (MP), Maximum Likelihood (ML), NJ and Bayesian analyses were performed using PAUP* 4.0b10 [42].

Maximum Parsimony (MP) Analysis

Maximum Parsimony (MP) analysis was performed with the following settings: heuristic search algorithms with tree bisection reconnecting (TBR) branch swapping, MULPARS in effect, all characters equally weighted, gap treated as missing characters, zero length braches collapsed, random addition sequence set to 1000 replicates, and branch swapping limited to 10000000 rearrangements per replicate. When maximum parsimonious tree were saved, a strict consensus tree [43] was constructed. Bootstrap analysis [44] was performed using 1000 replicates, with the random addition sequence set to 10, and branch swapping limited to 10000000 rearrangements per replicates.

Table 4- ITS sequences of nrDNA retrieved from GenBank for phylogenetic analyses

Taxonomy	GenBank accession number
Ingroup	
Limonium dufourii	AJ222840
Limonium camposanum	AJ222841
Limonium gymnesicum	AJ222842
Limonium interjectum	AJ222843
Limonium girardianum	AJ222845
Limonium delicatulum	AJ222846
Limonium furfuraceum	AJ222856
Limonium dichotomum	AJ222858
Limonium echioides	AJ222861
Limonium narbonense	AJ222838
Limonium vulgare	AJ222839
Limonium tenuicaule	AJ222857
Limonium minutum	AJ132332
Limonium cavanillesii	AJ222852
Limonium angustebracteatum	AJ222853
Limonium virgatum	AJ222855
Limonium caesium	AJ222859
Limonium rigualii	AJ222854
Limonium aureum	JN187115
Outgroup	
Plumbago auriculata	JF831220

Table 5- Generic classification of *Limonium*

Taxon	Classification	
Limonium dufourii	Sect. Limonium,	Subsections Densiflorae
Limonium camposanum	Sect. Limonium,	Subsections Densiflorae
Limonium gymnesicum	Sect. Limonium,	Subsections Densiflorae
Limonium interjectum	Sect. Limonium,	Subsections Densiflorae
Limonium girardianum	Sect. Limonium,	Subsections Densiflorae
Limonium furfuraceum	Sect. Limonium,	Subsections Steirocladae
Limonium virgatum	Sect. Limonium,	Subsections Steirocladae
Limonium tenuicaule	Sect. Limonium,	Subsections Steirocladae
Limonium minutum	Sect. Limonium,	Subsections Steirocladae
Limonium dichotomum	Sect. Limonium,	Subsections Hyalolepidae
Limonium narbonense	Sect. Limonium,	Subsections Limonium
Limonium vulgare	Sect. Limonium,	Subsections Limonium
Limonium cavanillesii	Sect. Limonium,	Subsections Dissitiflorae
Limonium angustebracteatum	Sect. Limonium,	Subsections Dissitiflorae
Limonium rigualii	Sect. Limonium,	Subsections Dissitiflorae
Limonium delicatulum	Sect. Limonium,	Subsections Dissitiflorae
Limonium caesium	Sect. Polyarthrion	
Limonium echioides	Sect. Schizhymenium	

nrDNA ITS2 Secondary Structure Prediction

Secondary structure of nrDNA ITS2 region for each species sequenced in the present study was explored using the minimum free energy (MFE) program MFOLD (45) in GCG version 8.1 available at http://rna.tbi.univie.ac.at/.

Results

Sequence Characteristics

The combined length of the entire ITS region (ITS1, 5.8S and ITS2) from taxa sequenced in the present study ranged from 599-618 nucleotides (electrophenogram of one of he representative annexed). The length of the ITS1 region and GC contents ranged from 201-203 nucleotides and 45-50% respectively, the 5.8S gene was 161-163 nucleotides long, the length of the ITS2 region and the GC content ranged from 233-253 nucleotides and 48-51% respectively [Table-6]. The aligned data matrix has a total number of 670 characters (alignment annexed) of which 256 characters are constant, 255 characters were variable but parsimony-uninformative and 159 characters werre parsimony-informative. Insertions and deletions (indels) were necessary to align the sequences. Indels ranged from 1 to 52 bp.

Table 6- Characteristics of ITS sequences of nuclear ribosomal DNA in the taxon sequenced in the present study

Taxon	Total ITS length	ITS1		5.8		ITS2	
		Length	%GC	Length	%GC	Length	%GC
Limonium axillare	613	202	50	163	50	248	49
Limonium carnosum	615	201	45	161	48	247	51
Limonium cylindrifolium	618	202	50	163	50	253	48
Limonium lobatum	599	203	46	161	53	233	50

Phylogenetic Analyses

The nexus format of the aligned data matrix was imported to phylogenetic analysis software PAUP, and executed for the sequence data analyses. Strict consensus tree and bootstrap strict consensus tree [Fig-4], [Fig-5] resulted on completion of the analyses were saved for presentation. In addition with parsimony analyses, NJ analysis were also performed using PAUP to infer NJ tree [Fig-5] based on branch length.

The parsimony analysis (using PAUP) of the entire ITS region resulted in 1280 maximally parsimonious trees (MPTs) with a total length of 480 steps, a consistency index (CI) of 0.754 (0.730 CI excluding uninformative characters), a homoplasy index (HI) of 0.456 (0.432 HI excluding uninformative characters), rescaled consistency index (RC) of 0.579 and a retention index (RI) of 0.767. One of the MPTs has been shown in [Fig-5] in which number above the lines indicate the bootstrap support in 100 replicates.

A perusal of all trees (Strict Consensus Tree, Bootstrap Strict Consensus Tree, and NJ tree) clearly indicates that *Limonium carnosum* included in this study from geographic region Saudi Arabia phylogenetically very closely related with *Limonium narbonense* and *Limonium vulgare*. It is also clearly evident that *Limonium cylindrifolium* and *Limonium axillare* does not nested deeply within the phylogenetic tree, rather were found at the base of the phylogenetic tree and *L. lobatum* occupies most basal position in phylogenetic tree. *Limonium cylindrifolium* and *Limonium axillare* shows sister relationships; In ITS sequences both these two species differs in 5 base pairs in ITS2 region. Therefore, ITS2 secondary structure for these two species, and additionally all taxon included in the analysis were predicted to bring molecular morphological signature of the *Limonium* distributed in Saudi Arabia.

Average Evolutionary Divergence over all Sequence Pairs was estimated. The number of base substitutions per site from averaging over all sequence pairs was 0.129. The result was based on the pairwise analysis of 24 sequences included in the analyses. Analyses were conducted using the Maximum Composite Likelihood method in MEGA4 [46]. Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution [Table-7] was performed using MEGA4 [46].

Table 7- Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

	A	T	C	G
A	-	2.56	1.86	29
T	1.7	-	14.48	2.55
C	1.7	19.87	-	2.55
G	19.31	2.56	1.86	-

Each entry shows the probability of substitution from one base (row) to another base (column) instantaneously.

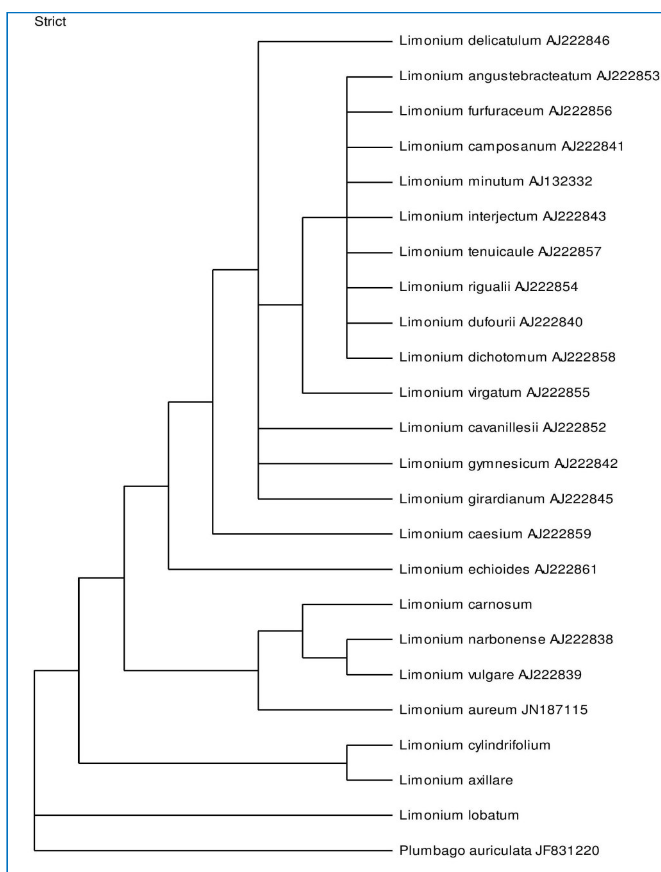

Fig. 4- Strict Consensus Tree inferred using PAUP 4.0b

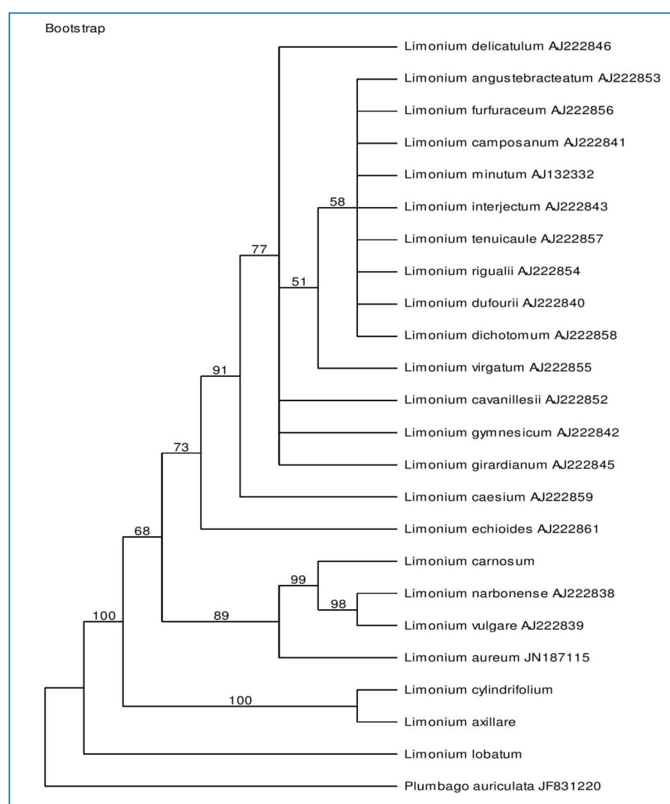

Fig. 5- Bootstrap Strict Consensus Tree inferred using PAUP 4.0b

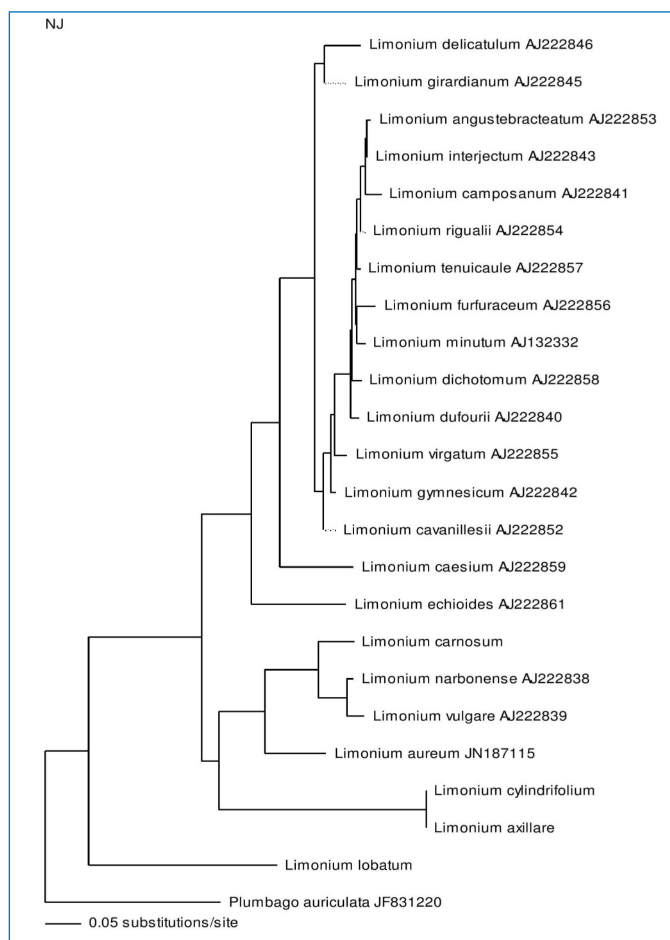

Fig. 6- NJ Tree inferred using PAUP 4.0b

Discussion

Plumbaginaceae are a cosmopolitan family well represented in temperate zones of the Northern Hemisphere and showing preferences for arid or saline, often coastal, environments [47]. The Angiosperm Phylogeny Group classification of flowering plants [3] included them in a broadly defined order Caryophyllales, together with other families adapted to extreme environments including oligotrophic soils (e.g., Droseraceae), arid zones (e.g., Cactaceae and Portulacaceae), and soils with high salt content (e.g., Amaranthaceae and Tamaricaceae). Phylogenetic studies of Plumbaginaceae based on plastid DNA sequences and morphological data have been produced recently [48]. These studies confirmed the classification of Plumbaginaceae into two subfamilies, Plumbaginoideae and Staticoideae, well differentiated by morphological, chemical, and molecular characters. Plumbaginoideae comprise four genera, of which *Plumbago*, with approximately 20 species, is the largest. Staticoideae are morphologically more diverse. More than 85% of the species are in three genera, *Limonium*, *Armeria*, and *Acantholimon*, and the remaining species belong to monotypic or small genera split from *Limonium* and *Acantholimon*, although the status of these genera is not clear yet, as [47] pointed out. Recent systematic and phylogenetic studies of *Limonium* lack a global perspective, having been based on specific geographic areas [5,49] or individual sections or groups that are putatively monophyletic [50]. The latest comprehensive accounts for the family and *Limonium* were published by [6], and later authors have followed his system with only small alterations. Boissier divided *Limonium* former *Statice*, *nom. rej.* vs. *Armeria*; [51] into 13 sections belonging to two main groups: "*corolla polypetala*" with eight sections and "*corolla gamopetala*" with four sections plus two others published at a later date. Only two sections of the original *corolla gamopetala*, *L.* sect. *Polyarthrion* and *L.* sect. *Siphonantha*, are still included in *Limonium*, although phylogenetic relationships of these new genera to other members of Staticoideae have not yet been assessed. Within the *corolla polypetala* group, only *L.* sect. *Circinaria* is currently separated from *Limonium* as genus *Afrolimon*.

The most recent attempt to clarify the taxonomy of *Limonium* was made by [52] who rearranged the genus for *Flora Europaea.* Pignatti divided the European taxa in three subgenera. *Limonium* subgenus *Pteroclados* (equivalent to the section with the same name) has approximately 18 species mostly endemic to the Canary Islands. *Limonium* subgenus *Myriolepis* was created to include three sections (*L.* sects. *Myriolepis*, *Siphonantha*, and *Polyarthrion*), all of them members of Boissier's *corolla gamopetala* group. Finally, placed the remainder of the European sections in a large unstructured *L.* subgenus *Limonium*, leaving African, American, and Asiatic species out of his system. In addition, nomenclature of the genus is also obsolete. Most of the sectional names were originally published under the former generic name *Statice*. Although some names have been consequently combined under *Limonium* (e.g., for European groups), a large number of sections are still based on dubious nomenclature.

Despite the systematic and taxonomic confusion around *Limonium* and Plumbaginaceae in general, two aspects of the family, breeding systems and phytochemistry, are better known. Phytochemical studies [53,54] have confirmed the differences between subfamilies and among Southern and Northern Hemisphere groups. Nevertheless, the lack of a robust systematic and phylogenetic framework makes interpretation of these results speculative. Breeding systems

were extensively studied by [55,56], who has been influential in the development of evolutionary and biogeographical hypotheses for the family, including the dispersal of genera related to *Limonium* to the southern continents (*Afrolimon*, *Bakerolimon*, and *Muellerolimon*). Using all available information (morphology, breeding systems, and karyology), [56]) made the first attempt to clarify relationships among genera and tribes in the family. [56] results were obviously limited by the amount and types of data available at that time, and in the light of new molecular data these have been shown to be only partially correct [48]. [56] also studied the evolution of *Limonium* in more detail. Heterostyly, although typical in Plumbaginoideae, is also present in *L.* subsect. *Genuinae* [55], whereas pollen/stigma dimorphism and self-incompatibility, common within Staticoideae, are widespread in *Limonium*. Facultative apomixis is common in *Limonium*, whereas truly sexual species are infrequent (e.g., *L.* sects. *Polyarthrion* and *Pteroclados*); sexual groups usually display fewer species than the apomictic ones. Hybridization, apomixis, and polyploidy in *L.* subsects. *Densiflorae*, *Dissitiflorae*, and *Steirocladae* are putatively key factors in the radiation of *Limonium* in areas such as the western Mediterranean basin. As a consequence, a large number of microspecies have been described, making difficult attempts to clarify the taxonomy of the group [57]. One of the advantages of constructing molecular trees is the possibility of estimating the ages of lineages. The calibration of molecular trees is usually based on the fossil record or specific events that can be dated e.g., presence of lineages on oceanic islands [58]. Plumbaginaceae have not been the focus of any extensive fossil research, and even pollen records are restricted to Quaternary strata [59]. Despite the lack of fossil information, the presence of phylogenetically isolated lineages on a volcanic archipelago, the Canary Islands, can be used as a calibration point. The Canary Islands are too close to the African continent to behave as a truly oceanic archipelago (110 km for the closest island, Fuerteventura, and 460 km for the most distant island, La Palma). The geomorphology of the Canary Islands has been extensively studied, as well as biogeography and colonization patterns [60]. The archipelago was formed by a series of volcanic periods that started approximately 20 million years ago (mya). There are three groups of *Limonium* present in the island flora, of which *L.* sect. *Pteroclados* and *L.* sect. *Ctenostachys* are not exclusive to the Islands. The monotypic and morphologically atypical *L.* sect. *Limoniodendron* (*L. dendroides*), which is a small tree or shrub only found on La Gomera, does not seem to be closely related to any other taxa. This island, formed approximately 10 mya [60], is the only island in the archipelago that has been without major volcanic activity for the last 4 million years. The age of the island can be used as a reasonable minimal age for *L. dendroides* to calibrate the tree, assuming that the intervening volcanic activity did not eliminate the flora.

In *Limonium,* polyploidy and apomixis are common, numerous hybrids occur naturally, and, as a consequence, reticulate evolution seems to be the rule rather than the exception. Sizes of ITS-1 and ITS-2 in the *Limonium* species studied were similar to those reported for other flowering plants, with ITS-1 longer than ITS-2 [18]. In *Limonium,* the ITS region has evolved primarily by point mutations, which conforms to other studies on closely related plants [18]. The conservation of ITS sequences is presumably due to their role in the production of mature rRNA, and this functionality depends on evolutionarily conserved secondary structural motifs. Inference of nonindependence at directly opposing sites in these secondary structures can be determined empirically [61]. However; in the present study, differential character weights for stem vs loop positions did not lead to different results in the analysis of ITS sequences from *Limonium.* It has been demonstrated that rRNA processing mechanisms could be labile enough to allow readjustments of intrastrand RNA pairing, which could imply mutations at nonpaired positions [62]. This pattern of substitutions could have important implications for phylogenetic analysis but it could also mean that selection for compensatory mutations might be weaker for these spacers than for nrDNA coding regions, alleviating the concern about nonindependence of characters [63]. The analysis of ITS using parsimony methods of phylogenetic reconstruction has revealed a relatively stable phylogenetic structure [64,65]. In the present study, the relationships obtained among *Limonium* ITS sequences using ML, NJ, and parsimony approaches are, in general, congruent for well-supported groups.

Palacios et al [66] have detected intraspecific polymorphism in species of *Limonium* with two molecular markers, RFLPs of chloroplast DNA and nrDNA ITS sequences. *L. furfuraceum,* a sexual species with a very conspicuous morphology but no variability was found in its rDNA. *L. delicatulum* represents another case in which intraspecific variability has been detected but with an opposite rDNA-cpDNA pattern of variability. A similar explanation has also earlier been suggested for ITS polymorphism in other plant species [67]. In some cases, conspecific samples rendered identical genotypes; in others, intraspecific variability has been detected through pooled DNA samples from different individuals of the same population. These phenomena should prevent reporting these ITS sequences as unique sequences, representative of the corresponding species.

In the present analyses taxon were included from 1. Sect. Limonium Subsections Densiflorae, 2. Sect. Limonium Subsections Steirocladae, 3. Sect. Limonium Subsections Hyalolepidae, 4. Sect. Limonium Subsections Limonium, 5. Sect. Limonium Subsections Dissitiflorae, 6. Sect. Polyarthrion, 7. Sect. Schizhymenium. In the present study, *Limonium axillare, L. cylndrifolium, L. carnosum,* and *L. lobatum* were sequenced as well as studied morphologically also. *Limonium axillare* (Forssk.) O. Kuntze, [Revisio Generum Plantarum 2: 395. 1891. (5 Nov 1891), Synonym= *Statice axillaris* Forssk. Peltier, M. 1981. Plombaginacées. Fl. Madagasc. 163: 15-23; *Statice bovei* Jaub. & Spach. Peltier, M. 1981. Plombaginacées. Fl. Madagasc. 163: 15-23], is in Arabic known as Qataf, is distributed in Tropical Africa, Egypt, and Costal region on the red sea in Saudi Arabia; which have been characterized as: a costal small shrub up to c. 50 cm tall; leaves fleshy, alternate, lamina oblanceolate to oblanceolate-spathulate, 3-8 mm wide, tapering to amplexicaul base, inflorescence a panicle of one sided spikes bearing few flowered cymes; bracts and bracteoles reddish, the bracteoles c. 2.5 mm long; calyx tube c. 2 mm long, the limb c. 4 mm across, calyx tube plicate, papery membranous, white with reddish ribs, the ribs pubescent at base, corolla purplish red, crumpling.

Limonium cylndrifolium (Forssk) O. Kuntze, [rev Gen. Pl 2: 395 (1891); Synonym= *Stice cylindrifolium* Forssk (1753)], is distributed in Ethiopia, Yemen, and Red sea coast and island, Farsan Island near Ras Hassis in Saudi Arabia, have been morphologically characterized as: acostal perennial herb up to c. 50 cm tall, old branches covered with whitish, spirally arranged remnants of bases of fallen-off leaves; leaves cylindrical, up to c. 6 cm long, 1.5-2 mm across, mucronate; flowers in few flowered cymes densely arranged in one sided spikes forming racemes or panicles on leafless space; bracteoles up to c. 3.75 mm long, reddish brown; calyx up to c. 3.25

mm long, hardly projecting above the bracteoles; calyx tube c. 2 mm long, glabrous, not pubescent along the ribs; corolla deep reddish projecting about 1 mm beyond calyx, crumpled within calyx on drying up, stamens exserted.

Limonium carnosum (Boiss.) O. Kuntze, [Rev. Gen. Pl. 2 : 394 (1891); synonym= *Statice carnosa* Boiss (1848)], is in Arabic known as Qataf or Awaidhan, and distributed in Eastern region or at the margins of costal marshes of Saudi Arabia, and Iran; have been morphologically characterized as: perennial, ascending to erect, much branched undershrub up to c. 40 cm tall; branches thin, lower leafy, the scapose leafless with only small bracts, mostly warty with foviate pustular glands; leaves densely foveolate-pustular, alternate, rather densely arranged, linear spathulate, up to 3 cm long and up to 3 mm at the top, tapering downwards into thin petiole like basal parts which expand to form membranous edged, amplexicaul bases; flowers pink, in few to several flowered, dense, cymose, shortly pedunculate or sessile, axillary clusters arranged on leafless branches of paniculate spaces; bracts foveolate pustular at the least in the median region; bracteoles up to c. 2 mm long; calyx c. 3 mm long, pink, glabrous or sparsely pubescent basally along some of the ribs; corolla c. 4.5 mm long, pink or white, becoming crumped and inconspicuous at the base of calyx.

Limonium lobatum (L. f.) Chaz. [Dict. Jard. 2:36(1790) plate 172; synonym= *Staice thouinii* Viv., (1802); *S. lobata* L. f. (1782); *Limonium thouinii* Kuntze (1891)] is patchily seen in silty soils of Saudi Arabia, known in Arabic as Kitaah sibsab, have been characterized as: annual, stiff herb up to c. 25 cm tall with several erect scapes from a basal rosette of leaves; scapes simple or branched, 3 winged in the upper nodes; leaves sessile or subsessile, oblanceolate to spathuate, up to 8 x 2 cm, branched, pinnately lobed into usually 3-4 pairs; the lobes rounded, usually 3-4 pairs; flowers in few-several flowered dense cymes subtended by broadly 3-winged obconical branches of the scape, the whole acting as a disseminule and falling off together; bracteoles 2-keeled at back, up to c. 6 mm long; calyx silvery white to pale blue c. 4 mm long, lobes 5, about as long as the tube, alternating with 5, prominent bristles, coyellowish, crumpling up.

The analyses clearly reveals that the largest section of the genus (section *Limonium*) does not constitute a monophyletic assemblage. *Limonium carnosum* clade with *Limonium narbonense* and *Limonium vulgare* (Sect. *Limonium,* Subsections *Limonium*); L. cylindrifolium and L. axillare which has been previously placed in Sect. *Limonium,* Subsections *Limonium* doesnot nested within its clade of own, rather were found base of the phylogenetic tree. *Limonium lobatum* which has been previously based on morphology have been placed in Section Pteroclados, subsection Pdontolepideae, occupied basal most position in the pylogenetic tree.

In the absence of a previous phylogenetic analysis of section *Limonium* based on other independent characters [48], [66] discussed the results only in relation to the classification of the group, which is based mainly on morphological and karyological characters [6,5], and find out that the splitting of section *Limonium* does not agree with the results of the phylogenetic analyses of nuclear and organellar markers. suggested that the largest section of the genus (section *Limonium*) does not constitute a monophyletic assemblage. The basal position of the two analyzed species of subsection *Limonium* (*L. vulgare* and *L. narbonense*) was strongly supported in all analyses. The same results were obtained using *rbcL* sequences with five *Limonium* species. Levels of sequence divergence suggest that molecular differentiation between taxa of subsection *Limonium* and the other species from section *Limonium* is larger than expected based on exomorphic features. In addition, morphological and anatomical data suggest that subsection *Limonium* is a group of well-knit species not linked by intermediates to the remaining section *Limonium.* This agrees with results based on anatomical and morphological proposed that subsections *Densiflorae* and *Dissitiflorae* should be merged. Nevertheless, *Limonium* taxa belonging to these four subsections are apomictic and, presumably, have a hybrid origin from ancestors belonging to either subsection [67].

Conflicts of Interest: None declared.

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