

MOLECULAR PHYLOGENY OF ROCHELIA (BORAGINACEAE) BASED ON NRDNA ITS AND CPDNA TRNL-F SEQUENCES

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We here present molecular phylogeny of the genus *Rochelia* (*Boraginaceae-Eritrichieae*). A total of 8 species of *Rochelia* and 2 species of *Lappula* as outgroups were included in analyses using nrDNA ITS and cpDNA *trnL-F* separately and in combination. To examine evolutionary trend of morphological characters, we mapped six diagnostic characters on the combined tree using MacClade 4. The analyses revealed that sect. *Rochelia* due to inclusion of the monotypic section *Cryptocarpa* (*Rochelia cardiosepala*) is not monophyletic. Likewise, its subsections, *Rochelia* and *Pedunculares* are paraphyletic. *Rochelia persica* and *R. disperma* along with *R. cancellata* of the monospecific subgenus *Neo-Rochelia*, as unresolved branches, were sisters to the remaining species. One of six diagnostic characters examined (non-hamate tip of calyx hairs) had evolved as reversal in both *R. persica* and *R. bungei* and the other one (nutlets completely clasping the adaxial part of gynobase) had undergone parallel evolution between *R. cancellata* plus *R. peduncularis* and *R. cardiosepala*. Based on the present molecular analyses, the current infrageneric classification of *Rochelia*, at least at the sectional and subsectional level based upon traditional morphological characters is artificial.

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Key words. *Boraginaceae*, cpDNA *trnL-F*, *Eritrichieae*, Molecular Phylogeny, nrDNA ITS, *Rochelia*.

فیلوژنی مولکولی جنس *Rochelia* (Boraginaceae) بر اساس توالی های nrDNA ITS و cpDNA *trnL-F*

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تعداد ۸ گونه از جنس *Rochelia* و دو گونه از *Lappula* (به عنوان برون گروه) با استفاده از توالی هسته‌ای ITS و توالی کلروپلاستی *trnL-F* به طور جداگانه و نیز به صورت ترکیبی آنالیز شدند. برای مشخص کردن روند تکامل صفات مورفولوژیکی، شش صفت تشخیصی بر روی درخت ترکیبی با استفاده از برنامه MacClade 4 نشان داده شد. آنالیزها آشکار کرد که بخشه *Rochelia* به دلیل قرار گرفتن بخشه مونوتیپیک *Cryptocarpa* (*Rochelia cardiosepala*) در میان آن، تک تبار نیست. همچنین زیر بخشه های مربوط به این بخشه (*Rochelia* و *Pedunculares*) تک تبار نیستند. *Rochelia persica* و *R. disperma* همراه با *R. cancellata* از زیرجنس تک گونه ای *Neo-Rochelia*، در شاخه‌هایی با روابط حل نشده، به عنوان گروه خواهری با بقیه گونه‌ها قرار می‌گیرند. یکی از شش صفت تشخیصی (کرک های کاسه با راس غیر قلابی) به عنوان یک صفت برگشت در هر دو گونه *R. persica* و *R. bungei* مشاهده شد و صفت دیگر (فندقه‌ها به طور کامل متصل به بخش شکمی ژینوباز) متحمل تکامل موازی در بین سه گونه *Rochelia cancellata*، *R. peduncularis* و *R. cardiosepala* شده بود. بر اساس آنالیز مولکولی حاضر، رده بندی موجود در سطوح تحت جنس در *Rochelia*، حداقل در سطح بخشه و زیر بخشه با استفاده از صفات مورفولوژیکی، مصنوعی است.

INTRODUCTION

Rochelia Reichenb. is a cosmopolitan genus with its dispersal centre in S.W. and central Asia, extending to the Mediterranean area and Australia, comprising 15-20 species (Nasir 1989, Mabberley 1990, Luque 1992). It is represented in Iran by 8 species (Khatamsaz 2002). *Rochelia* is characterized by 2-nutlet fruits mostly ornamented by stellate papillae (Khatamsaz 2002, Riedl 1967, Hilger 1984, Kazempour Osaloo 1993). De Candolle first (1846) classified *Rochelia* in the monotypic tribe *Rochelieae* with the two species *R. stellulata* Reichenb. and *R. leiocarpa* Ledeb. Popov (1953) while keeping the genus in the tribe, following Zakirov's treatment (1941, cited therein) divided it into two sections, *Eurochelia* Zak. and *Cryptocarpa* Zak. Section *Eurochelia* was further divided into two series: *Stellulatae* Zak. and *Pedunculares* Zak. *Cryptocarpa* is a monotypic section (*R. cardiosepala* Bge.). Later on, Riedl (1967) in the Flora Iranica reduced the tribe to the subtribal level, *Rocheliinae*, within the tribe *Eritrichieae*. Based on the number of sepals, he reclassified the genus into two subgenera, *Rochelia* (with 5 sepals) and the monotypic *Neo-Rochelia* H. Riedl (with 9-10 sepals). The former subgenus was, in turn, divided into two sections, *Rochelia* (= *Eurochelia*) and *Cryptocarpa*. The section *Rochelia*, as a multi-species taxon, is characterized by linear or lanceolate sepals converging/recurving fruit but not enclosing it. Whereas, *Cryptocarpa* is distinguished by its cordate calyx completely enclosing fruit. Riedl (1967) substituted series *Stellulatae* and *Pedunculares* of section *Eurochelia* as subsections *Rochelia* and *Pedunculares* Zak., respectively. Several works were performed on the genus using non-molecular data. On the basis of nutlet micromorphology, Hilger (1984) tried to group the species of the genus. Pollen morphological and karyological studies (Diez & Benito 1991, Kazempour Osaloo 1993, Khatamsaz 2001, Luque 1992) implied that *Rochelia* is related to *Lappula* Gilib. Hitherto, no relatively comprehensive study on the molecular phylogeny of the genus has been conducted. Our preliminary phylogenetic analyses using either nrDNA ITS or chloroplast *trnL* intron and *trnL-trnF* intergenic spacer (hereafter abbreviated as *trnL-F*) sequences for 40 species of the tribe *Eritrichieae* and related tribes showed that *Rochelia* with three species sampled, formed a monophyletic group as sister to *Lappula* (Khoshokhan et al. 2008, Khoshokhan & Kazempour Osaloo 2008). The internal transcribed spacer (ITS) is the region of the 18S- 5.8S- 26S nuclear ribosomal cistron. The spacers contain the signals needed to process the rRNA transcript (Baldwin 1992, Baldwin et al. 1995) and have often been used for inferring phylogeny at the

Table 1. Taxa included in the present mtDNA ITS and cpDNA *trnL-F* phylogenetic analyses.

Taxa	DNA source (Location, voucher)	GenBank Accession No. mtDNA ITS	cpDNA <i>trnL-F</i>
<i>Rochelia</i>			
subgen. <i>Rochelia</i>			
sect. <i>Rochelia</i>			
subsect. <i>Rochelia</i>			
<i>R. bungei</i> Trautv.	Iran: Assadi & Massoumi 55785 (TARI)	AB564695	AB564705
<i>R. mirheydari</i> Riedl & Esfandari	Iran: Faghmina & Zangooei 23477 (TMUH)	AB564696	AB564706
<i>R. persica</i> Bge. ex Boiss.	Iran: Kazempour Osaloo 2007-1 (TMUH)	AB564697	AB564707
<i>R. dispersma</i> (L. F.) Koch.	Iran: Kazempour Osaloo 2007-2 (TMUH)	AB564698	AB564708
subsect. <i>Pedunculares</i>			
<i>R. peduncularis</i> Boiss.	Iran: Abdolzadeh 20447 (FUMH)	AB564699	AB564709
<i>R. macrocalyx</i> Bge.	Iran: Freitag & Jaldidi 29088 (TARI)	AB564700	AB564710
sect. <i>Cryptocarpa</i>			
<i>R. cardiosepala</i> Bge.	Iran: Kazempour Osaloo 2006-1 (TMUH)	AB564701	AB564711
subgen. <i>Neo-Rochelia</i>			
<i>R. cancellata</i> Boiss. & Bal.	Turkey: Bani 4971 (TMUH)	AB564702	AB564712
outgroups			
<i>Lappula barbata</i> (M. B.) Gurke	Iran: Kazempour Osaloo 2008-1 (TMUH)	AB564703	AB564713
<i>Lappula sessiliflora</i> (Boiss.) Gurke	Iran: Kazempour Osaloo 2007-3 (TMUH)	AB564704	AB564714

Abbreviations used in accession information: FUMH, Ferdowsi University of Mashhad Herbarium; Mashhad; TARI, Herbarium of the Research Institute of Forests and Rangelands, Tehran; TMUH, Tarbiat Modares University Herbarium, Tehran.

Table 2. Selected morphological characters for evaluation of their evolutionary trend in *Rochelia*.

1. Tip of calyx hairs: non hamate (0), hamate (1).
2. Fruit pedicel to sepal ratio : <1 (0), =1 (1), >1 (2).
3. Shape of calyx lobes: narrowly linear (0), rectangular/ lanceolate (1), cordate (2).
4. Sepal midrib: non-prominent midrib (0). prominent midrib (1).
5. Nutlet size: medium (>3 mm) (0), small (\leq 3 mm) (1).
6. Attachment of nutlets to gynobase: not clasping adaxial part of gynobase (0), clasping completely adaxial part of gynobase (1).

generic and infrageneric levels in plants (e.g., Baldwin 1992, Baldwin et al. 1995, Kazempour Osaloo et al. 2003 & 2005, Ahangarian et al. 2007). *trnL-F*, is the chloroplast DNA (cpDNA) sequence that is now widely used to investigate interspecific/generic relationships among angiosperms and other plants using the universal primers of Taberlet et al. (Taberlet et al. 1991, Shaw et al. 2005).

In this study, we attempt to infer infra-generic relationships in *Rochelia* and evaluate character evolution among its species in the context of the combined nrDNA ITS-*trnL-F* phylogeny.

MATERIALS AND METHODS

A total of seven species representing two sections of the subgenus *Rochelia* and a single species of subgenus *Neo-Rochelia* plus 2 *Lappula* species as outgroups, were included in molecular studies (Table 1). Total genomic DNA was extracted from fresh and dried leaves, using a modification of the 2X CTAB protocol of Doyle and Doyle (1987). The nrDNA ITS region was amplified as a sharp single fragment using the primer pair ITS5/ITS5m and ITS4 in all cases (White et al. 1990, Sang et al. 1995). The *trnL-F* region was amplified using primers c and f as one fragment (Taberlet et al. 1991). Each fragment was directly sequenced using the Big dye terminator cycle sequencing ready reaction kit with the same primers. Sequencing of the fragments was done in an ABI Prism 3730xl DNA Analyzer (Applied Biosystems, USA).

Sequence alignment

Sequences were edited using BioEdit ver. 7.0.9.0 (Hall 1999) and aligned using ClustalX (Larkin et al. 2007) followed by manual adjustment. Alignment of each dataset required the introduction of several single and multiple-base indels (insertions/deletions). Positions of indels were treated as missing data for all datasets.

Phylogenetic analyses

Phylogenetic analyses were performed on the aligned nrDNA ITS and *trnL-F* data matrices separately and in combination. These datasets were analyzed using Maximum parsimony (MP) criterion as implemented in PAUP* (Swofford 2002). Heuristic searches were performed with 100 replicates of random addition

sequence, tree-bisection-reconnection (TBR) branch-swapping with MulTrees on and steepest descent off. Bootstrap values (Felsenstein 1985) with 1000 replications were calculated using the heuristic search option, simple sequence addition and TBR branch swapping. To assess combinability of these datasets, incongruent length difference (ILD, Farris et al. 1995) test was conducted using PAUP*. ILD test suggested that the both datasets were not incongruent (P=1). To examine evolutionary trend of morphological characters, we mapped six diagnostic ones on the combined nrDNA ITS- *trnL-F* tree using MacClade 4 (Maddison & Maddison 2005) (Table 2). These characters compiled from different sources (Khatamsaz 2002, Riedl 1967, Hilger 1984, Kazempour Osaloo 1993, Khatamsaz 2002) and studying the living materials and herbarium specimens deposited at TARI, TUH and FUMH (see Holmgren & Holmgren 1998, for the herbaria abbreviations) as well as Tarbiat Modares University Herbarium.

RESULTS

Phylogenetic analyses

Length of nrDNA ITS1-5.8S-ITS2 sequences for *Rochelia* was 632 nucleotide sites, of which 22 sites were parsimony informative. MP analysis of the dataset resulted in 4 equally most parsimonious trees having a length of 35 steps and consistency index (CI)= 0.714 and a retention index (RI)= 0.697 (excluding uninformative characters). The aligned *trnL-F* dataset comprised of 866 nucleotide sites, of which 5 were informative. MP analysis of this dataset resulted in a single most parsimonious tree with a length of 5 steps (CI= 1, RI= 1). nrDNA ITS and *trnL-F* phylogenies are conflicting on the position of *R. macrocalyx* Bge. On the former, the species is moderately allied (bootstrap value of 71%) with a subclade of *R. cardiosepala* and *R. peduncularis* Boiss.; whereas, on the latter tree, it is an unresolved branch. The low resolution in *trnL-F* phylogeny is mainly due to the low number of parsimony informative characters. As noted in the material and methods section, ILD test suggested the congruency of both datasets. The combined nrDNA ITS-*trnL-F* dataset was composed of 1498 nucleotide

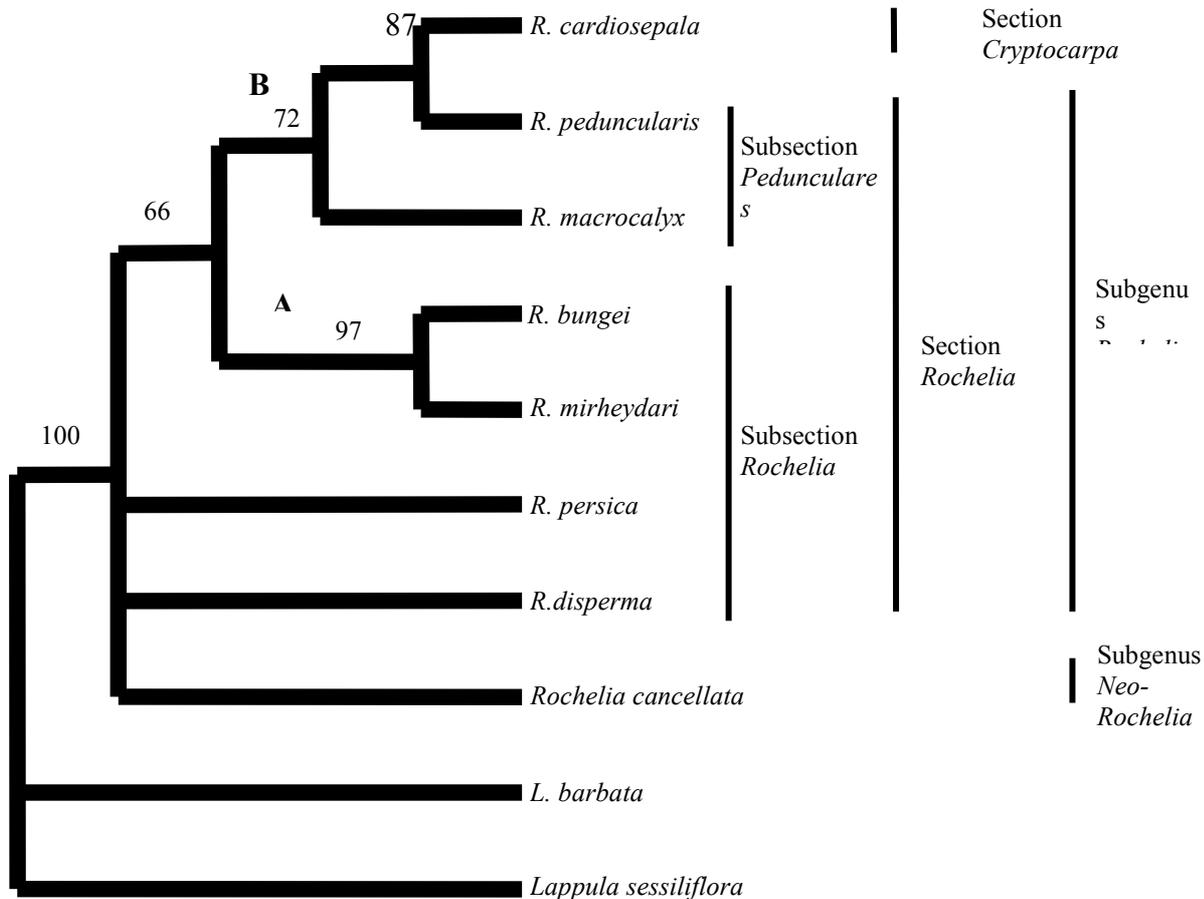


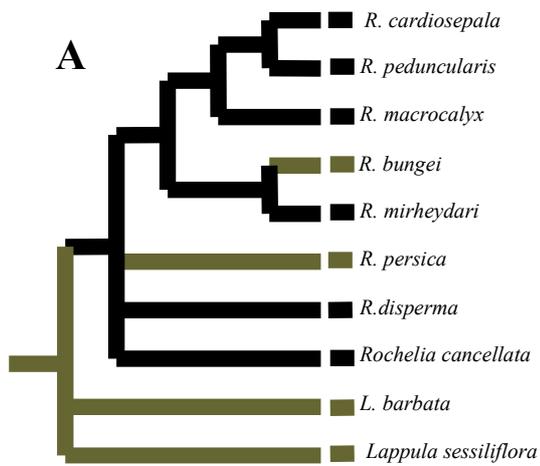
Fig. 1. Strict consensus tree of 4 most parsimonious trees resulting from phylogenetic analysis of the combined nrDNA ITS - cpDNA *trnL-F* sequences for *Rochelia* and two outgroup taxa (Length= 40 steps, CI= 0.750 , RI= 0.737) . Numbers above branches are bootstrap values for 1000 replicates analyses; values < 50% are not indicated.

sites, of which 27 were parsimony informative. MP analysis of the dataset resulted in 4 equally most parsimonious trees with a length of 40 steps with a CI= 0.750 and an RI= 0.737. The strict consensus tree of these trees, the same as that of nrDNA ITS tree, with accompanying bootstrap values was presented in Fig. 1. On this tree, *Rochelia persica* Bge., *R. disperma* (L. f.) C. Koch. and *R. cancellata* are unresolved branches as sisters to a clade of the remaining five species examined. This clade was, in turn, composed of two subclades (A and B). The first subclade (A) contained well allied *R. bungei* Trautv. and *R. mirheydari* Riedl & Esfandiari (bootstrap value of 97%) and the second subclade (B) constituted a relatively weakly supported group (72% bootstrap) comprising *R. macrocalyx* plus *R. cardiosepala* and *R. peduncularis*.

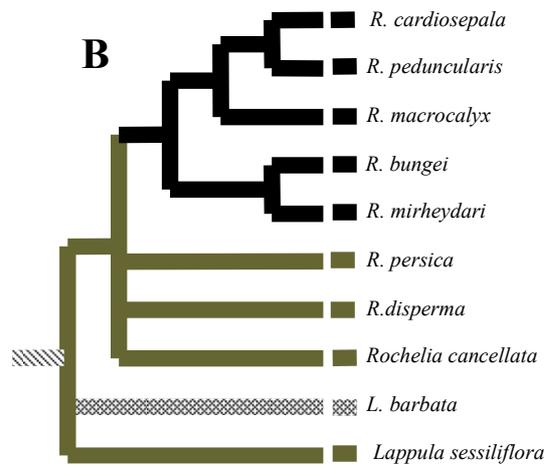
Character evolution

We here mapped six diagnostic characters on the strict consensus tree resulting from the combined nrDNA

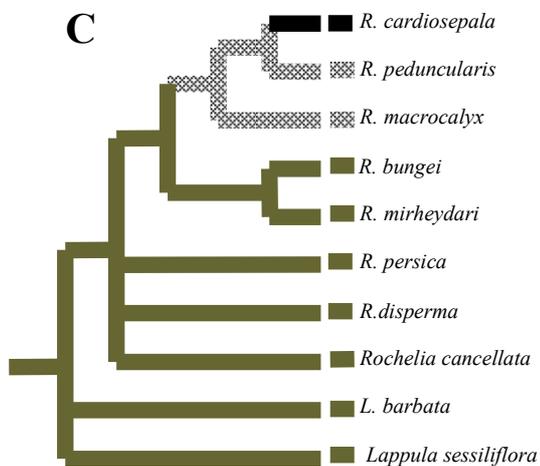
ITS-*trnL-F* dataset (Figs 2A-F). Character 1, tip of calyx hairs, has been undergone reversal evolution from hamate hairs to non-hamate ones in both *Rochelia persica* and *R. bungei* (Fig. 2A). The second character, fruit pedicel to sepal ratio [longer pedicel (>1)], is a synapomorphy for the clade comprising *R. bungei*, *R. mirheydari*, *R. macrocalyx*, *R. peduncularis* and *R. cardiosepala*. This character state is evolved from shorter pedicel (<1) (Fig. 2B). Character 3, shape of calyx lobes, have been changed from narrowly linear calyx through wide lanceolate/rectangular in *R. macrocalyx* and *R. peduncularis* to cordate one only in *R. cardiosepala* (Fig. 2C). Sepal with prominent midrib is a synapomorphy for *R. macrocalyx*, *R. peduncularis* and *R. cardiosepala*, that has been derived from non-prominent midrib (character 4) (Fig. 2D). Character 5, medium-sized nutlets, > 3mm in length, is unique to *R. peduncularis* and *R. cardiosepala*, that is in fact a reversal from small-sized nutlets (≤ 3 mm) (Fig. 2E). Nutlets completely clasping the gynobase, character 6,



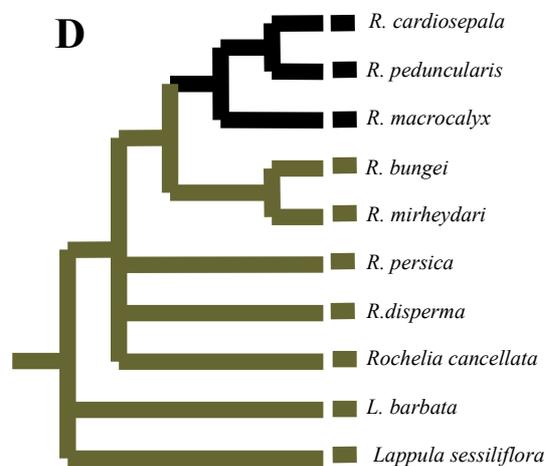
1. Tip of calyx hairs
 ■ Non-hamate
 ■ Hamate



2. Fruit pedicel to sepal ratio
 ■ < 1
 ■ = 1
 ■ > 1
 ■ equivocal



3. Shape of calyx lobes
 ■ Narrowly linear
 ■ Rectangular/ lanceolate
 ■ Cordate



4. Sepal midrib
 ■ Non-prominent
 ■ Prominent

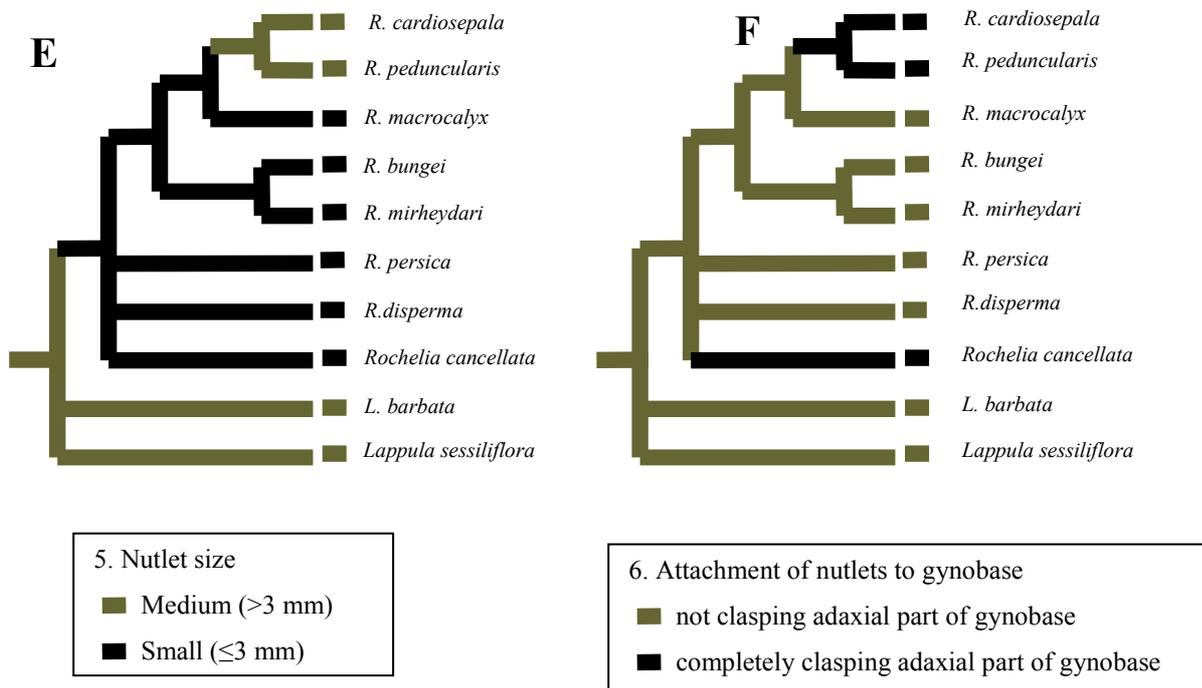


Fig. 2. MacClade reconstruction of the evolution of 6 characters mapped on the combined nrDNA ITS - cpDNA *trnL-F* tree (A-F) for *Rochelia* and two outgroup taxa.

is evolved in parallel between *R. cancellata* plus *R. peduncularis* and *R. cardiosepala* (Fig. 2F).

DISCUSSION

The present data did not support the monophyly of the subgenus *Rochelia*. Likewise, its species rich section, *Rochelia*, due to inclusion of the monotypic section *Cryptocarpa*, is paraphyletic. Furthermore, the two subsections of *Rochelia*, viz. *Rochelia* and *Pedunculares* appeared to be non-monophyletic. The subsection *Rochelia* was represented herein by 4 species, two of which, *R. disperma* and *R. persica*, along with *R. cancellata* of the subgenus *Neo-Rochelia* are unresolved branches. The two others, *R. bungei* and *R. mirheydari* are the closest sister taxa (subclade A), as united with a subclade of subsection *Pedunculares* and the monotypic section *Cryptocarpa* (subclade B, Fig.1). *Pedunculares* is a small subsection with 5 species, 2 of which, *R. peduncularis* and *R. macrocalyx*, were included herein. They were not united as sister species, but the former species is well allied with *R. cardiosepala* of the section *Cryptocarpa* (see also Hilger 1984). The derived position of *R. cardiosepala* within the section *Rochelia* indicates that this species should be classified within it, and thus, the sectional status of *Cryptocarpa* is no longer tenable. The species is characterized by specialized

autapomorphies including cordate calyx lobes and completely invisible nutlets in calyx (Fig. 2C).

All six diagnostic characters examined herein but non-hamate tip of calyx hairs and nutlets completely clasping the gynobase, were not homoplasious for *Rochelia* species (see Fig. 2). The shorter pedicel is evolved once to longer pedicel in the clade of five species comprising the *R. mirheydari* through *R. cardiosepala* (Fig. 2B). The character 3, shape of calyx lobes underwent evolutionary changes twice terminating to cordate calyx in *R. cardiosepala*. These character states were evolved from narrowly linear calyx to wide lanceolate/rectangular one in both *R. macrocalyx* and *R. peduncularis* (Fig. 2C). The next character is sepal with prominent midrib which is a synapomorphy of the subclade of *R. macrocalyx*, *R. peduncularis* and *R. cardiosepala* (Fig. 2D). In the regional Floras, e.g., USSR, Iranica, Iran and Pakistan (Popov 1953, Khatamsaz 2002, Riedl 1967 and Nasir 1989), this feature along with the two later ones, were mostly used a key characters to separate them from other species. The two other characters, nutlets size, (medium) and attachment of nutlets to the adaxial part of gynobase (completely clasping) are shared by *R. peduncularis* and *R. cardiosepala*. The latter character state is also found in the other unrelated species, *R. cancellata* (Fig. 2F). In terms of this feature (nutlets

clasping the gynobase), Hilger (1984) suggested, however, that these three species are closely related.

CONCLUDING REMARKS

The present phylogenetic hypothesis showed that the infrageneric classification of *Rochelia*, at least at the sectional and subsectional level based upon traditional morphological characters is artificial. To treat a comprehensive circumscription of the genus at the infrageneric level, more taxa and other fast evolving DNA fragments are definitely necessary.

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