

A NEW COLLECTION OF *ROCHELIA RETROSEPALA* (BORAGINACEAE), A RARE ENDEMIC SPECIES FROM IRAN; ITS PHYLOGENETIC POSITION AND CONSERVATION STATUS

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Recent botanical expeditions in Isfahan province led to the recollection of *Rochelia retrosepala* as a very rare and endemic species. Molecular data obtained from ITS nuclear sequences and *trnL-F* chloroplast sequences are presented to indicate its phylogenetic position in the genus. Based on the molecular results, *R. persica* is the closest relative taxon to the *R. retrosepala*. Some notes about the distribution and habitat of this rare species are presented. Furthermore, according to IUCN criteria and categories the conservation status of the species was assessed as Critically Endangered (CE).

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Keywords: *Rochelia*; Boraginaceae; Flora of Iran; Zagros; phylogeny; threatened species

جمع‌آوری جدید *Rochelia retrosepala* (تیره گاوزبانیان)، یک گونه نادر و انحصاری از ایران، موقعیت فیلوژنتیکی و جایگاه حفاظتی آن

آزاده اخوان روفیگر: استادیار پژوهش، بخش تحقیقات منابع طبیعی، مرکز تحقیقات و آموزش کشاورزی و منابع طبیعی استان اصفهان، سازمان تحقیقات، آموزش و ترویج کشاورزی، اصفهان، ایران

انجام مطالعات گیاه‌شناسی اخیر در استان اصفهان منجر به جمع‌آوری مجدد *Rochelia retrosepala*، گونه‌ای بسیار نادر و انحصاری شد. داده‌های مولکولی بدست آمده از توالی‌های هسته‌ای ITS و توالی‌های کلروپلاستی *trnL-F* برای نشان دادن موقعیت فیلوژنتیک این گونه در جنس ارائه می‌شود. براساس نتایج مولکولی *R. persica*، نزدیک‌ترین خویشاوند به *R. retrosepala* است. برخی یادداشت‌ها در خصوص پراکنش و زیستگاه این گونه نادر ارائه شده است. همچنین، وضعیت حفاظتی آن بر اساس معیارها و دسته‌بندی‌های IUCN با درجه "در بحران انقراض" ارزیابی شد.

INTRODUCTION

The flora of Iran is rich in the number of endemic plant species. Thirty-two percent of Iranian vascular plant species are endemics and limited to this area (Noroozi & al., 2019). About eighty-eight percent of endemic species of Iran are restricted to the Irano-Turanian region. Isfahan province is one of the largest and vital endemism centers of Iran and represents an area with high species richness with numerous endemic species, particularly in the mountainous regions and the alpine zones (Noroozi & al., 2008). Among these, local

endemics that are restricted to a small distinct area are considered as the most important species due to habitat limitation and extinction threat. Therefore, the narrow endemic species are in the first place and priority for botanical conservation studies. Some of these species are very rare as no specimens have been collected to date since the first discovery.

Rochelia Rchb. with c. 15- 20 species is distributed mainly in southwest Asia and central Asia, extending to the Mediterranean area and Australia (Nasir, 1989; Mabberley, 1990; Luque, 1992). The genus is

represented in Iran by 8 species (Khatamsaz, 2002). *Rochelia retrosepala* Khatamsaz is a rare endemic species of the Boraginaceae family in the Isfahan province of Iran. There is only one collection of this species (holotypus) collected by Nowroozi and Shams in 1998. My floristic studies in Isfahan province during the last five years made a collection of rare and endangered species from this region. I collected a small population of *R. retrosepala* in Fereidunshahr, 23 years after its primary collection in 1998. It is currently known from a population in Isfahan province. Between 2017 and 2021, I visited the location of type species and the other neighborhood sites of *R. retrosepala*, where it might potentially occur. My surveys have pointed out that the species has not been collected since the first report. Until now, a number of studies have been done on the different species of the genus but phylogenetic

studies (Khoshshokhan & al., 2010; Khoshshokhan-Mozaffar & al., 2018) including *R. retrosepala* have not yet been investigated. Accordingly, the main goal of the current study is to assess the conservation status of this rare species and to clarify the phylogenetic placement of *R. retrosepala* within the genus using sequences of ITS and *trnL-F*.

MATERIAL AND METHODS

Taxon sampling

My field exploration for finding *Rochelia retrosepala* started in June 2015, focusing on the type locality in the Cheshme Langan area in Fereidunshahr and adjacent areas in the western parts of Isfahan province. Finally, in July 2018, I found and collected some individuals of this species around 20 km far from the type locality in Tange Duzan area (fig. 1).

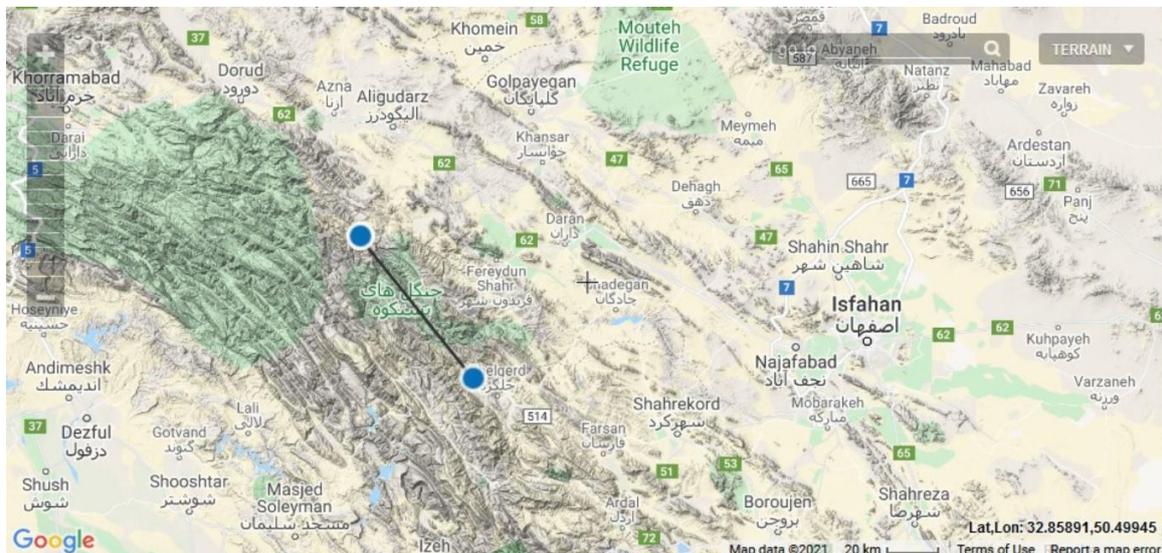


Fig. 1. Distribution map of *Rochelia retrosepala*.

Specimens examined

IRAN. Isfahan province: Fereidunshahr, Cheshme Langan, 2550 m, 11 June 1998, *Nowroozi & Shams 11685* (holotype TARI!); Isfahan province: Fereidunshahr, Tange Duzan, 33° 02' 29"N, 49° 56' 19"E, 2670 m, 9 June 2018, *Akhavan & Bagheri 16641 SFAHAN*, (fig. 3).

Conservation status

The conservation status of *Rochelia retrosepala* was estimated using the guideline of IUCN (IUCN 2017). The area of occupancy (AOO), the extent of occurrence (EOO), and other criteria such as population size, habitat quality, natural regeneration, and the number of mature individuals were used to determine the species threat status.

Phylogenetic analysis

The total DNA of the newly rediscovered species *Rochelia retrosepala* was extracted from dried leaf material using the DNAeasy Plant Mini Kit (Qiagen). According to the manufacturer's instructions. For the molecular phylogenetic analyses, I used ITS and *trnL-F* in 12 taxa (table 1). The ITS region was PCR amplified using the primers ITS-A and ITS-B (Blattner, 1999), and *trnL-F* spacer was PCR amplified using the primers provided in (Taberlet & al., 1991). Both regions were directly Sanger sequenced on an ABI 3730 XL using the amplification primers. Forward and reverse sequences from each individual were manually checked and assembled into individual contigs using Chromas (<https://technelysium.com.au/wp/>). Other sequences except the *Rochelia retrosepala* were obtained from the GenBank nucleotide database. A list

of all the taxa and voucher specimens used for the molecular analysis, as well as GenBank accession numbers, are given in table 1. The sequences of the nine *Rochelia* spp. together with three sequences of *Myosotis* and two *Lappula* species as outgroups were aligned with ClustalW multiple alignments and analyzed using BioEdit program (Hall 1999). Potential conflict between the ITS and *trnL-F* loci was tested prior to combination by the partition-homogeneity test (ILD test) in PAUP*. Modeltest 3.7 (Posada & Crandall, 1998) was used to test different models of sequence evolution and the GTR+I model was selected by Akaike information criterion (AIC). Molecular phylogenetic analyses were executed using the

Bayesian inference (BI) and Maximum Parsimony (MP). The Bayesian analyses were performed using MrBayes v.3.1 (Ronquist & Huelsenbeck, 2003). The analyses were done for 2 million generations, with a sampling frequency of one in every 1000 generations. The initial 25% of the trees were discarded as burn-in. The remained tree was used to build a 50% majority of consensus trees. FigTree (<http://tree.bio.ed.ac.uk/software/figtree>) was used for tree visualization. A maximum parsimony (MP) analysis was conducted in PAUP* 4.0a163 (Swofford 2002) using the heuristic search algorithm with TBR branch swapping. To test clade, a bootstrap analysis with 1000 bootstrap resamples was conducted.

Table 1. The examined taxa and their gene bank accession numbers.

Species	DNA source	accession No. of ITS	accession No. of <i>trnL-F</i>
<i>Lappula barbata</i> (M.Bieb.) Gürke	GenBank	AB564703.1	AB564713.1
<i>Lappula microcarpa</i> (Ledeb.) Gürke	GenBank	AB758303.1	AB758332.1
<i>Myosotis asiatica</i> (Vestergr.) Schischk. & Serg.	GenBank	AB758315.1	AB758341.1
<i>R. bungei</i> Trautv.	GenBank	AB564695.1	AB564705.1
<i>R. cancellata</i> Boiss. & Balansa	GenBank	AB564702.1	AB564712.1
<i>R. cardiosepala</i> Bunge	GenBank	AB564701.1	AB564711.1
<i>R. disperma</i> (L.f.) K.Koch	GenBank	AB564698.1	AB564708.1
<i>R. macrocalyx</i> Bunge	GenBank	AB564700.1	AB564710.1
<i>R. mirheydari</i> Riedl & Esfand.	GenBank	AB564696.1	AB564706.1
<i>R. peduncularis</i> Boiss.	GenBank	AB564699.1	AB564709.1
<i>R. persica</i> Bunge ex Boiss.	GenBank	AB564697.1	AB564707.1
<i>R. retrosepala</i> Khat.	16641, SFAHAN	OK490278.1	OK483323.1

RESULTS

Molecular phylogenetic data

After preliminary distinct analyses of ITS and *trnL-F* sequences that did not provide main differences in an ILD test, I comprised the ITS and *trnL-F* sequences in a combined dataset for my molecular phylogenetic analyses. These combined data produced an alignment with the length of 1582 base pairs. The topology of the combined tree with ITS and *trnL-F* is almost identical, so that, the Bayesian tree is shown in fig. 2. In this tree *Rochelia* is a monophyletic clade, also, *Lappula* and *Myosotis* are the sister taxa to the *Rochelia*.

Conservation Status

The conservation status of *Rochelia retrosepala* was defined, calculating its Area of Occupancy (AOO), by surveying its habitats in the field. An area of 600 m² was measured. The geographical distribution of this species is very limited, there are only two known localities for it at a distance of about 20 km from each other (fig. 1). Some other criteria including habitat quality, natural regeneration, and the number of mature

individuals in the Tange Duzan population were checked. As a result, drought and overgrazing in this area have negative impacts on habitat quality. The population size estimated for this plant was twenty mature individuals, mostly resulting from natural regeneration. Based on field studies, resulting in data and IUCN threat categories (IUCN, 2017), the conservation status of *Rochelia retrosepala* is assessed as Critically Endangered (CR).

Distribution and habitat

The distribution of *Rochelia retrosepala* is restricted to a small area in the central Zagros Mountain Range in the Fereidunshahr County (fig. 1). It belongs to the high mountain steppe zone and is a rare local endemic growing on rocky slopes of Tange Duzan Mountain in Isfahan province. The most commonly associated species in the observed localities of the *Rochelia retrosepala* are *Dorema aucheri* Boiss., *Ferula haussknechtii* H.Wolff ex Rech.f., *Astragalus verus* Olivier, *Eryngium billardieri* Delile, *Silene* sp. and *Bromus* sp.

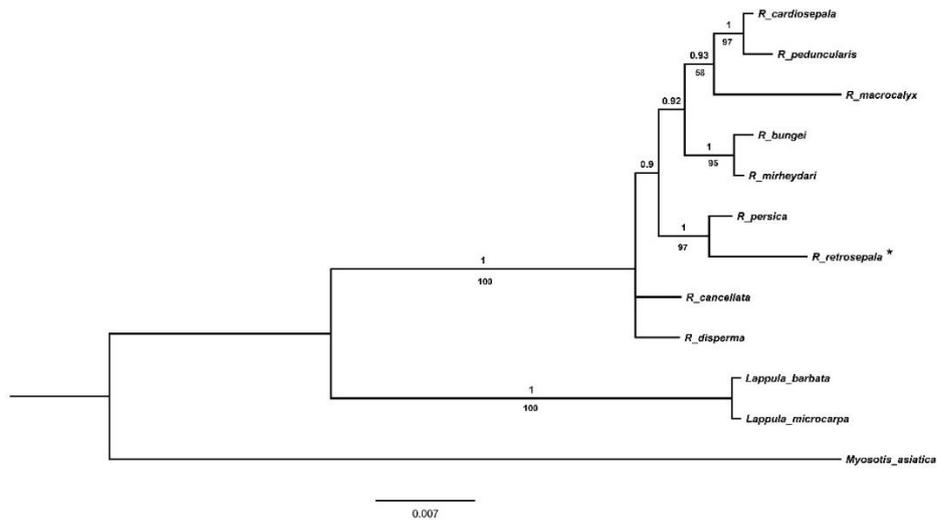


Fig. 2. Phylogenetic tree obtained from a Bayesian inference (BI) analysis of the combined ITS and *trnL*-F sequences. Numbers above branches indicate posterior probabilities of the Bayesian analysis, below the bootstrap values (%) for the parsimony analysis are given. An asterisk indicates *R. retrosepala*.

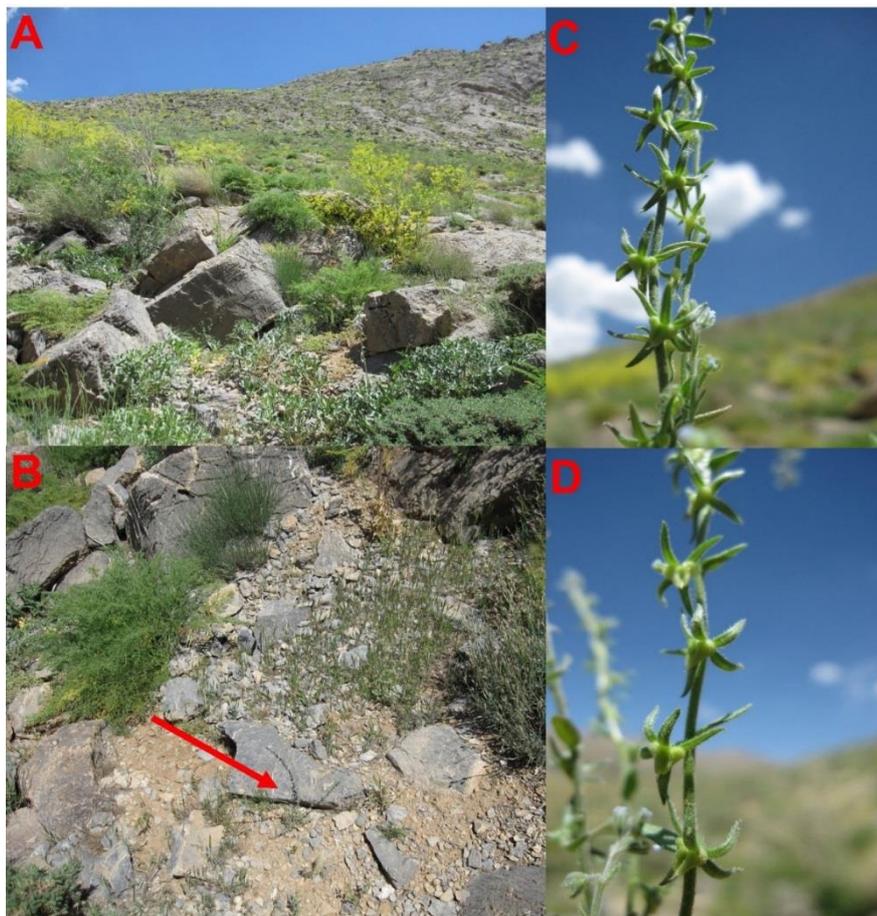


Fig. 3. *Rochelia retrosepala*, recollected specimen by Akhavan & Bagheri. A & B, Habitat; C-D, close up of fruits and inflorescence.

DISCUSSION

The phylogenetic relationships of the genus *Rochelia* and its allies in Boraginaceae was previously studied by Khoshokhan & al. (2010) and Khoshokhan- Mozaffar & al. (2018), using a nrDNA ITS and cpDNA trnL-F analysis. According to their studies, *Rochelia* is a monophyletic genus among the tribe *Rochelieae*. Here the *R. retrosepala* sequences were added to previous molecular datasets to define its phylogenetic relationships within the genus. In both Maximum parsimony (MP) and Bayesian inference (BI) analyses recovered *R. retrosepala* as a sister clade to *R. persica* as sister taxa. Morphologically, *R. retrosepala* is easily distinguishable from all the other *Rochelia* species of Iran, by having reflexed sepals. Also, this species is recognized from its closely related taxon (*R. persica*), by having longer stems (up to 35 cm vs. 5-20 cm), branching arrangement (from middle stem vs. the stem base), long calyx at the fruiting time (6-7 mm vs. up to 5 mm) and long nutlets (4 mm vs. 2.5 mm). In terms of geographical distribution, *R. retrosepala* is a very rare, locally endemic species found in a small area close to Fereidunshahr in central Zagros, Isfahan province, while the most similar taxon, *R. persica*, have a much wider distribution, as it occurs in different regions of Iran, as well as Central Asia, Caucasus, Iraq, and Turkmenistan. Due to its topographic condition, western parts of Isfahan province, are species-rich areas with numerous endemics and rare plant species. Some other narrow endemic species have also been reported from the western part of Isfahan, especially in Fereidunshahr, including *Astragalus lignipes* Akhavan & Maassoumi (Akhavan & Maassoumi 2020) and *Scrophularia isphahanica* Attar & Nowrouzi (Attar & al., 2006). Fereidunshahr is an area with high species diversity in central Zagros of Iran. Unfortunately, the area is at high risk due to overgrazing and human activities (road construction and mining activities). To protect this species, it is recommended that its habitat be declared protected.

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