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Molecular phylogeny of the genus *Sanguisorba* from Iran: Evidence based on cpDNA and nrDNA sequencing analysis

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Abstract

In this research, the molecular phylogeny of the genus *Sanguisorba* including two species (*S. officinalis* and *S. minor*), and the three subspecies (*S. minor* subsp. *muricata*, *S. minor* subsp. *lasiocarpa*, and *S. minor* subsp. *minor*) were studied from Iran using nrDNA ITS and cpDNA *rpl*32-*trn*L$_{(UAG)}$. For this purpose, 26 taxa, comprising four Iranian samples plus 22 previously sequenced data received from GenBank were analyzed. The phylogenetic relationships were reconstructed within *Sanguisorba* using maximum parsimony and Bayesian analyses. The results of nuclear sequence analysis showed separation of two subfamilies (*Agrimoniinae* and *Sanguisorbinae*), monophyly of *Sanguisorba*, complete separation of *S. officinalis* (in Sanguisorba clade) from *S. minor* and the three subspecies (in Poterium clade). Although, the intraspecific relationship remained unresolved, but it was found that, the use of micro- and macromorphological criteria could be used as an important tool in different taxonomic ranks, especially in intraspecific identification. In addition, average sequence divergence, genetic differentiation, morphological, and micromorphological evidence are discussed.

Keywords: Bayesian, delimitation, maximum parsimony, sequences divergence, taxonomy

مطالعه فیلوژنی جنس *Sanguisorba* در ایران: شواهدی مبتنی بر تجزیه و تحلیل توالی DNA کلروپلاستی و هسته‌ای*

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خلاصه

در بررسی حاضر، فیلوژنی مولکولی جنس *Sanguisorba* متعلق به گل‌سرخیان (شامل دو گونه *S. officinalis* و *S. minor*) و سه زیرگونه (*S. minor* subsp. *muricata*, *S. minor* subsp. *lasiocarpa* و *S. minor* subsp. *minor*) از ایران با استفاده از nrDNA ITS و *rpl*32-*trn*L$_{(UAG)}$ مورد مطالعه قرار گرفت. به این منظور، ۲۶ آرایه، شامل چهار نمونه از ایران به همراه ۲۲ توالی از بانک ژن مورد آنالیز قرار گرفت. روابط فیلوژنتیکی گونه‌های *Sanguisorba* با استفاده از آنالیزهای ماکزیمم پارسیمونی و بایزین بازسازی شدند. نتایج آنالیز توالی هسته‌ای منجر به جدایی دو زیرطایفه (*Agrimoniinae* و *Sanguisorbinae*)، تک‌تباری جنس *Sanguisorba* و جدایی کامل *S. officinalis* (روی شاخه *Sanguisorba*) از *S. minor* و سه زیرگونه آن (روی شاخه Poterium) شد. با وجودی که طی بررسی حاضر، روابط فروگونه به طور حل نشده باقی ماند، اما مشخص گردید که استفاده از معیارهای ریخت‌شناسی و ریزریخت‌شناسی می‌تواند به عنوان ابزار مهمی برای تفکیک سطوح مختلف تاکسونومیک به ویژه، شناسایی فروگونه‌ایی استفاده شود. به علاوه، در این تحقیق، میانگین درصد واگرایی نوکلئوتیدی، تفاوت توالی‌ها، شواهد ریخت‌شناسی و ریزریخت‌شناسی مورد بحث قرار گرفته است.

واژه‌های کلیدی: بایزین، تاکسونومی، تعیین حدود، ماکزیمم پارسیمونی، واگرایی نوکلئوتیدها

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Introduction

Sanguisorba L. (*Rosaceae*) is a complex genus, classified in the subtribe *Sanguisorbinae* (Schulze-Menz 1964, Potter *et al.* 2007, Zhang *et al.* 2017), tribe *Sanguisorbeae* DC. (Schulze-Menz 1964, Nordborg 1966, Takhtajan 1997, Mabberley 1997, Li *et al.* 2003, Kalkman 2004, Potter *et al.* 2007, IPNI 2021, Park *et al*. 2021), or *Agrimonieae* (Zhang *et al.* 2017), and *Poterieae* Dumort. (Hutchinson 1964). The circumscription of *Sanguisorba* has been reconsidered many times. Primarily, Linnaeus (1753) introduced the genus based on its unisexual flowers, single carpel, and style. Often, its species integrated into *Poterium* (e.g., Scopoli 1772, Bertoloni 1835, Spach 1846, Bentham & Hooker 1865, Brown & Bouche 1867, Hutchinson 1964, Nordborg 1966). However, Schulze-Menz (1964), Takhtajan (1997), and Kalkman (2004) supported the distinction between the two genera. Molecular studies have demonstrated that, *Sanguisorba* sensu stricto (including *S. officinalis* L.) comprises monophyletic groups within the clade *Sanguisorbeae*, while subclades *Sanguisorba* and *Poterium* showed sister group relationship with *Cliffortia* L., *Acaena* Mutis ex L., and *Polylepis* Ruiz & Pav. (Kerr 2004).

The genus *Sanguisorba* includes ca. 33 species, mainly distributed in northern hemisphere from Europe to southwest Asia, as well as North and South America, Australia, and New Zealand (Nordborg 1966, 1967, Kerr 2004, Park *et al*. 2021). The genus comprises two species (*S. officinalis* and *S. minor* Scopoli) and four subspecies in the Flora Iranica (Nordborg 1966). Khatamsaz (1993) introduced one representative (*S. minor*) and its three subspecies [*S. minor* subsp. *minor* and subsp. *lasiocarpa* (Boss. et Hausskn.) Nordborg, and *S. minor* subsp. *muricata* (Spach) Briq.], which are especially distributed from N to NW, NE, W, C, and S of Iran. Deylami Moezi *et al.* (2019) collected *S. officinalis* from N and NE, especially Gilan and Khorasan provinces. The most outstanding studies conducted in this genus are palynological (Hebda & Chinnappa 1990, Chung *et al.* 2010), flower and fruit micro- and macromorphological characters (Tantawy & Naseri 2003, Deylami Moezi *et al.* 2019), chromosome number (Mishima *et al*. 2002), and medicinal properties (Thomas 1998, Wu *et al.* 2005, Zhang *et al.* 2012, Yang *et al.* 2015). Previous phylogenetic studies of the genus were largely limited to family (Morgan *et al.* 1994, Eriksson *et al*. 1998, Potter 2007), subfamily (Eriksson *et al.* 2003), and related genera (Kerr 2004). Recently, Park *et al*. (2021) studied the floral micromorphology, palynology and plastome analysis of this genus. In the present study, both the nrDNA ITS region and the plastid intergenic space [*rpl*32-*trn*L$_{(UAG)}$] were used to reconstruct the phylogenetic relationships between species of this genus in Iran. The following questions are answered herewith: 1. Do all species belong to the genus *Sanguisorba*, 2. Are there representatives of *Poterium* among them, 3. To what extent can this study clarify the intraspecific relationship, and 4. Do the previous micro- and macromorphological features support the results of phylogenetic analyses?

Materials and Methods

In the current study, both dried and freshly collected specimens were used. The herbarium specimens were obtained from Research Institute of Forests and Rangelands (TARI), Faculty of Pharmacy, Tehran University of Medical Sciences (THE), Tehran University (TUH), and Guilan University (GUH) herbaria (Iran) (Table 1). The fresh specimens were collected during 2015–16 from different parts of Iran (Table 1). The voucher specimens of newly collected samples were deposited at Guilan University Herbarium (GUH). For identification purpose, the following references were used: Juzepczuk (1941), Nordborg (1969), Schönbech-Temesy (1969), and Khatamsaz (1993). A total of 26 taxa were included in this study for nrDNA ITS, cpDNA *rpl*32-*trn*L$_{(UAG)}$, and combined analyses (four newly and 22 sequenced data from GenBank) (Tables 1–2). Out group species were selected based on previous studies (Eriksson *et al.* 1998, 2003, Kerr 2004, Faghir *et al.* 2014, 2017).

Table 1. Newly sequenced plant samples used in the current study

Taxon	Locality and collector	GenBank Accession No. *rpl*32-*trn*L$_{(UAG)}$/ITS
Sanguisorba officinalis	Gilan prov.: Asalem to Khalkhal road, Faghir & Dailamy, 5303 (GUH)	LC581500/LC581496
S. minor subsp. *minor*	Gilan prov.: Asalem to Khalkhal road, Faghir & Dailamy 5300 (GUH)	LC581501/LC581497
S. minor subsp. *lasiocarpa*	Qazvin prov.: Alamoot, Faghir & Dailamy, 5301 (GUH)	LC581502/LC581498
S. minor subsp. *muricata*	Kerman prov.: Koe-ghar, Mirtajedini, 33151 (THE)	LC581503/LC581499

Table 2. Samples form GenBank which included in cpDNA *rpl*32-*trn*L$_{(UAG)}$ and nrDNA ITS in phylogenetic analyses

Taxon	DNA source	Accession No. *rpl*32-*trn*L$_{(UAG)}$/ITS
Acaena cylindristachya	Eriksson *et al.* 2003, Stockholm, Sweden	-/AJ512780.1
A. laevigata	Eriksson *et al.* 2003, Stockholm, Sweden	-/ AJ512781.1
Agrimonia eupatoria	Eriksson *et al.* 1998, Uppland, Sweden	-/U90798
Alchemilla alpina	Eriksson *et al.* 1998, Uppland, Sweden	-/U90817
A. mollis	Eriksson *et al.* 1997, Uppland, Sweden	-/AJ511769
Aphanes arvensis	Eriksson *et al.* 1998, Uppland, Sweden	-/AJ511770
Aremonia agrimonioides	Eriksson *et al.* 1998, Uppland, Sweden	-/U90799
Clliortia odorata	Kerr 2004, University of Maryland, USA	-/AY634874
Hagenia abyssinica	Eriksson *et al.* 1998, Harvard University, USA	-/U90800
Leucosidea sericea	Helfgott 2000, Austin, Texas, USA	-/AF183547
Polylepis hieronymi	Eriksson *et al.* 2003, Stockholm, Sweden	-/AJ512779
P. tarapacana	Eriksson *et al.* 2003, Stockholm, Sweden	-/AJ512778
Poteridium annuum	Kerr 2004, University of Maryland, USA	-/AY635032
Poterium sp.	Kerr 2004, University of Maryland, USA	-/AY635038
Potentilla kurdica	Faghir *et al.* 2014, Tehran, Iran	-/AB894153
P. pannosa	Faghir *et al.* 2014, Tehran, Iran	-/AB894155
Tetraglochin cristatum	Eriksson *et al.* 2003, Stockholm, Sweden	-/AJ512782
Alchemilla roccatii	Gehrke *et al.* 2015, Mainz, Germany	KT322066.1/-
A. stuhlmannii	Gehrke *et al.* 2015, Mainz, Germany	KT322072.1/-
Sanguisorba officinalis	Helfgott *et al.* 2000, Austin, Texas, USA	-/AF183556
S. parviflora	Eriksson *et al.* 1998, Uppland, Sweden	-/U90797.1
S. minor	Helfgott *et al.* 2000, Austin, Texas, USA	-/AF183555.1

- DNA extraction, amplification, and sequencing analysis

Total genomic DNA was isolated by Qiagen herb extraction kit from dried specimen and fresh leaves. The nrDNA ITS region and cpDNA *rpl*32-*trn*L$_{(UAG)}$ were amplified by symmetric PCR, using ITS5-m (Forward: 5′-GGAAGTAAAAGTCGTAACAAGG-3′) (Sang *et al.* 1995), ITS4 (Revers: 5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.* 1990) and *rpl*32 (Forward: 5′-CAGTTCCAAAAAAACGTACTTC-3′) and *trn*L$_{(UAG)}$ (Revers: 5′-CTGCTTCCTAAGAGCAGCGT-3′) (Shaw *et al.* 2007) primers. The total volume of amplification reactions was 20 µL. The PCR cycles started with 2 min 30 s at 94 °C, followed by 40 cycles of 94 °C for 30 s; annealing at 48 °C for 1 min, extension at 72 °C for 1 min 30 s; and final extension at 72 °C for 7 min. Nucleotide sequences of PCR products were determined using cycle sequencing and an automated DNA sequencer by Gen. Fanavaran Co.

To edit sequences of nrDNA ITS and cpDNA *rpl*32-*trn*L$_{(UAG)}$ datasets, BioEdit ver. 7.0.9.0 (Hall 2001) was used, and the alignment process was carried out using ClustalX (Larkin *et al.* 2007) analyzed with Muscle ver. 4.0 (Edgar 2004). Maximum parsimony analyses were conducted using the PAUP* 4.0b10 program (Swofford 2002). The heuristic search option was selected using 1000 replications of random addition sequence with 10 trees held at each step and tree bisection reconnection (TBR) branch swapping, with Mul-Trees on and steepest

descent off. Branch support was assessed by 1000 bootstrap replicates, yielding bootstrap percentages, and bootstrap support (Felsenstein 1985) with the same settings as for heuristic searches.

Bayesian analyses were run with MrBayes ver. 3.2 (Ronquist *et al.* 2012) as implemented in the CIPRES Science Gateway (http://www.phylo.org/, Miller *et al.* 2010) with the following settings: Four Markov chain Monte Carlo heuristic searches of 10 million generations were performed in four independent runs. It was verified that, convergence of parameter estimates, and effective sample sizes were > 200 for all parameters using Tracer ver. 1.6 (Drummond & Rambaut 2007). The first 25% trees were discarded as burn in. Posterior probabilities (PP) were used to illustrate the support of nodes. In this analysis, *Potentilla kurdica* Boiss. & Hohen., *P. pannosa* Boiss. & Hausskn. ex Boiss., *Alchemilla roccatii* Cort., *A. stuhlmannii* Engler, *A. mollis* (Buser) Rothm., and *A. alpina* L. were considered as out groups in the nrDNA ITS (first and second species), cpDNA (third and fourth), and combine analyses (fifth and sixth species), respectively. Finally, the mean distances between sequences were calculated on a p-distance matrix with complete deletion of gaps, using MEGA ver. 7 (Kumar *et al.* 2016).

Results

The nrDNA ITS datasets contained 19 taxa, 703 aligned DNA characters. In parsimony analysis, 224 characters were parsimony-informative while 479 were parsimony-uninformative. The results led to the formation of a strict consensus tree with a length of 489 [Consistency Index (CI) = 0.6864, Retention Index (RI) = 0.8146, Homoplasy Index (HI) = 0.3136, and Rescaled Consistency Index (RC) = 0.5591]. The Bayesian analysis resulted a tree with fully supported clade with PP = 1. Since both maximum parsimony (MP) and Bayesian (BA) trees were topologically similar, therefore, only the Bayesian tree was presented here (Fig. 2).

In this analysis, two species of *P. pannosa* and *P. kurdica* (as out groups) formed a monophyletic group at the base of the tree while other 19 studied species assembled on the main strongly supported clade [with posterior probability (PP) = 1 in BA tree, bootstrap value (BP) = 100% in MP tree] of which two clades A and B are derived. From clade A, two clades (A1 and A2) were originated, forming two monophyletic groups: A1 a well-supported clade [with posterior probability (PP) = 0.92 in BA tree and bootstrap value (BP) = 76% in MP tree], comprising two species viz. *Polylepis tarapacana* and *P. hieronymi* and A2 strongly supported clade [with posterior probability (PP) = 1 in BA tree and bootstrap value (BP) = 98% in MP tree] of which A2a and A2b were derived. The former clade consisted of six species viz. *Poterium* sp. and *Poteridium annuum* (forming a monophyletic group), and four representatives of *Sanguisorba* (*S. minor* subsp. *minor*, subsp. *muricata*, and subsp. *lasiocarpa*) plus *Acaena cylindristachya*, *A. laevigata*, and *Tetraglochin cristatum* in the independent branches. A2b comprising three species viz. two taxa of *S. officinalis*, one forming a monophyletic group with *S. parviflora* and the other placed below on a paraphyletic independent branch. Clade B consisted of three species viz. *Leucosidea sericea* and *Agrimonia eupatoria* (forming a monophyletic group), and *Aremonia agrimonioides* (Fig. 1).

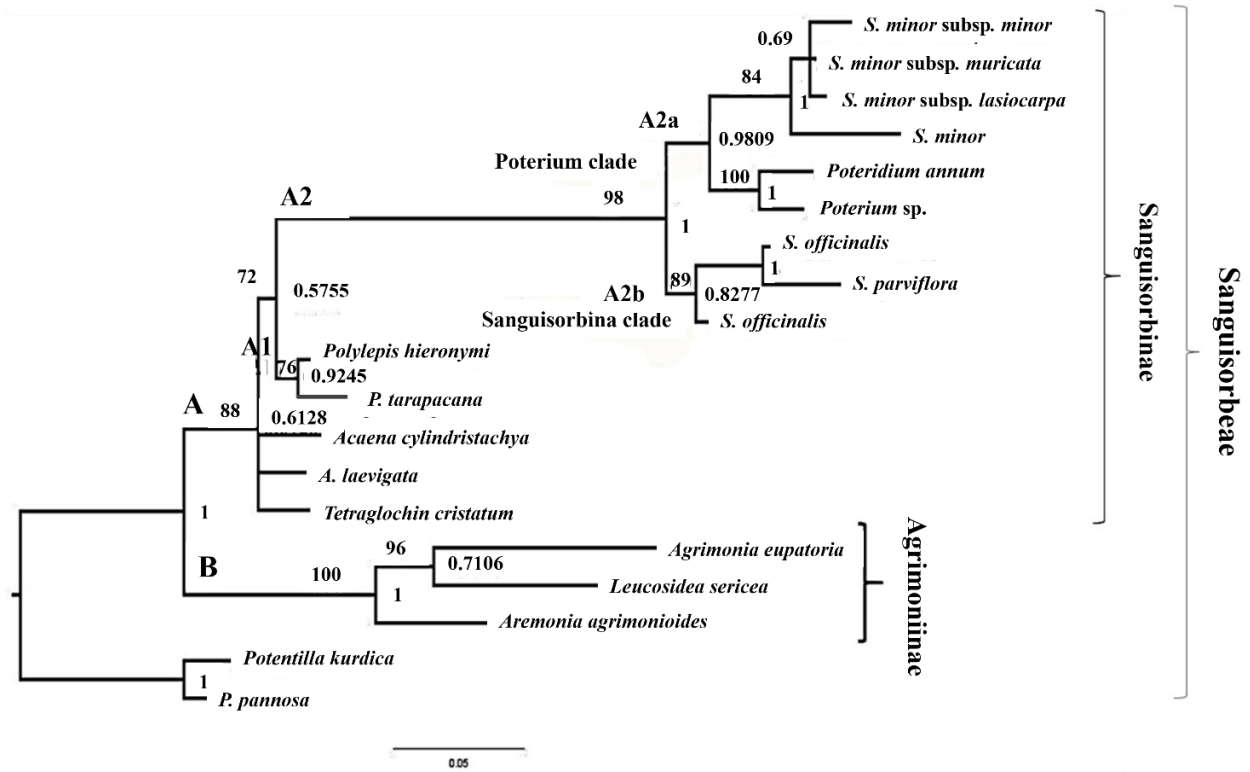

Fig. 1. Bayesian 50% majority-rule consensus tree of the nrDNA ITS sequence data of the genus *Sanguisorba*. Posterior probabilities (PP) are indicated adjacent and bootstrap values BP are shown above the branches.

The *rpl*32-*trn*L$_{(UAG)}$ dataset consisted of six species viz. four *sanguisorba* and two *Alchemilla* species (*A. roccatii* and *A. stuhlmannii*). The MP analysis resulted in a strict consensus tree with a length of 102 (Fig. 2A) [Consistency Index (CI) = 0.9608, Retention Index (RI) = 0.9600, Homoplasy Index (HI) = 0.0392, and Rescaled Consistency Index (RC) = 0.9224]. In the MP trees *S. officinalis* and *S. minor* subsp. *minor* formed a small monophyletic group (BP = 61%) while *S. minor* subsp. *muricata* and subsp. *lasiocarpa* situated in two independent branches. In the Bayesian analysis, *Sanguisorba* species formed a monophyletic group in a well-supported clade (PP = 1) but, exhibiting polytomy relationship (Fig. 2B).

The combined data matrix, consisted of six species with 2072 DNA characters of which, 303 characters were parsimony informative. The single most parsimonious tree is presented in figure 3A [Consistency Index (CI) = 0.9637, Retention Index (RI) = 0.9626, Homoplasy Index (HI) = 0.0363, and Rescaled Consistency Index (RC) = 0.9276]. The Bayesian analysis of the combined dataset resulted in 235, 275 trees, after discarding 7875 initial trees as burn in. In both combined MP and Bayesian trees the out-group species [*A. mollis* (Buser) Rothm. and *A. alpina* Linnaeus] situated in the basal clades either as two independent branches (in MP tree) or as basal monophyletic group (in Bayesian tree). These two trees also showed polyphyletic origin of *S. minor* subspecies and *S. officinalis.* The later species in both combined trees of MP and Bayesian analysis (Fig. 3B) placed in the paraphyletic independent clades below the *S. minor* subspecies. Among the three subspecies, *S. minor* subsp. *lasiocarpa* and subsp. *muricata* formed a monophyletic group on a strongly supported clade (BP = 100%), and *S. minor* subsp. *minor* located on a paraphyletic independent branch below them. In combined Bayesian trees, three subspecies of *S. minor* formed monophyletic group in tritomy condition.

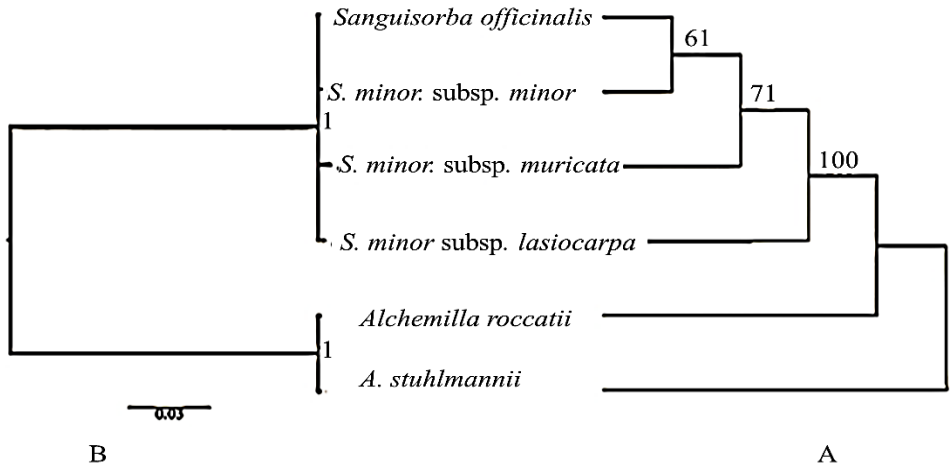

Fig. 2. A 50% majority consensus tree derived from maximum parsimony (A) and Bayesian analyses (B) of *rpl*32-*trn*L$_{(UAG)}$ sequence data of the genus *Sanguisorba*. Numbers above and adjacent to the branches in figures A and B are bootstrap values and posterior probabilities, respectively.

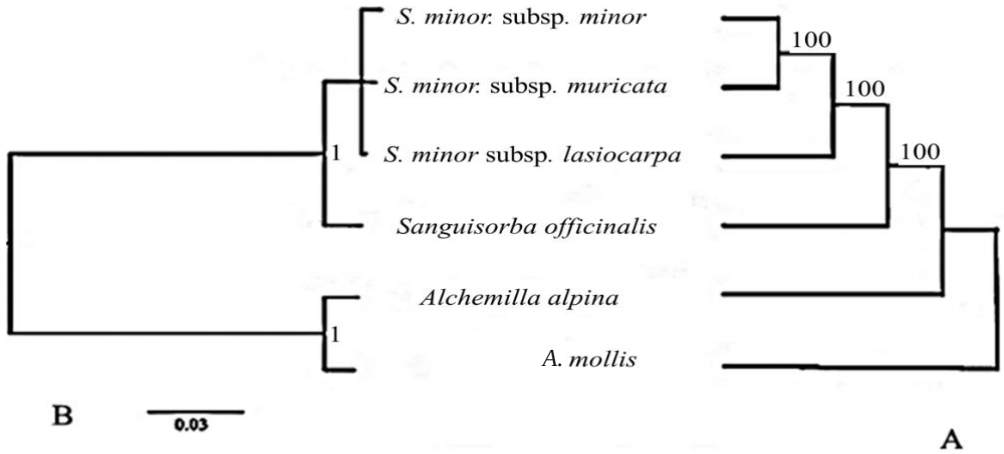

Fig. 3. A 50% majority consensus tree derived from maximum parsimony (A) and Bayesian analyses (B) of the combined plastid and ITS sequences. Numbers above branches are bootstrap values (clades are identified by letters). In Bayesian tree posterior probabilities are indicated adjacent to the branches.

In the DNA sequence characteristics and statistics data (Table 3), authors of the paper examined 19 ITS (of 19 species), 6 *rpl*32-*trn*L $_{(UAG)}$ and 6 ITS+ *rpl*32-*trn*L $_{(UAG)}$ (of each six species) sequences of *Sanguisorba* species and its subspecies. The alignment includes 703, 1369 and 2072 characters for ITS, *rpl*32-*trn*L$_{(UAG)}$ and combined sequences. Maximum parsimony analysis of the aligned ITS, *rpl*32-*trn*L $_{(UAG)}$ and combined sequences produced 186, 98, and 273 informative characters, respectively.

The mean G+C content varied from 27.9 in *rpl*32-*trn*L $_{(UAG)}$, to 41.3% in combined ITS+ *rpl*32-*trn*L$_{(UAG)}$ and 63.9 in ITS datasets. The combined ITS+ *rpl*32-*trn*L$_{(UAG)}$ dataset possessed the highest nucleotide divergence (0.13%), followed by cpDNA (0.12%) and nrDNA ITS (0.08). The sequence divergence between *S. officinalis* and the three subspecies varied from 0.055% in ITS, 0.01% in *rpl*32-*trn*L$_{(UAG)}$ and 0.03% in combined dataset. The average sequence divergence (0.08%) between *S. officinalis* and subsp. *minor* were recognized in ITS data, which is eight times higher than *rpl*32-*trn*L$_{(UAG)}$ and about 2.7 times higher than combined sequence variations. The nucleotide divergence (0.09%) between *S. officinalis* and subsp. *muricata* were recorded in ITS data, which is about 4.6 times higher than *rpl*32-*trn*L$_{(UAG)}$ and about 2.3 times higher than combined sequence variations. The maximum nucleotide divergence of nrDNA ITS (0.1%) between *S. officinalis* and *S. minor* subsp. *lasiocarpa* recorded, that is five times higher than *rpl*32-*trn*L$_{(UAG)}$ and two times higher than combined sequence variations. Within all samples of the three subspecies the average

sequence divergence (%), changed from 0.058 in ITS, 0.025 in *rpl*32-*trn*L$_{(UAG)}$ and 0.022 in combined datasets. The nucleotide sequences showed least divergence (0.01%) between *S. minor* subsp. *muricata* and *S. minor* subsp. *lasiocarpa.* The average sequence divergence between *S. minor* subsp. *minor*, subsp. *muricata*, and subsp. *lasiocarpa* (0.02 to 0.03 ITS, 0.02 for *rpl*32-*trn*L $_{(UAG)}$ and ITS+*rpl*32-*trn*L$_{(UAG)}$) were almost identical for the three sequences data.

Table 3. DNA sequence characteristics and statistics for each data partition

Primer used	ITS	*rpl*32-*trn*L$_{(UAG)}$	ITS+*rpl*32-*trn*L$_{(UAG)}$
Number of sequences	19	6	6
Number of characters	703	1369	2072
G+C content (%)	63.9	27.9	41.3
Number of parsimony-informative characters	186	98	273
Average sequence divergence (%) in all sequences	0.08	0.12	0.13
Average sequence divergence (%) between *Sanguisorba officinalis* and the three subspecies	0.055	0.01	0.03
Average sequence divergence (%) between *S. officinalis* and *S. minor* subsp. *minor*	0.08	0.01	0.03
Average sequence divergence (%) between *S. officinalis* and *S. minor* subsp. *muricata*	0.09	0.02	0.04
Average sequence divergence (%) between *S. officinalis* and *S. minor* subsp. *lasiocarpa*	0.1	0.02	0.05
Average sequence divergence (%) between three subspecies	0.058	0.025	0.022
Average sequence divergence (%) between *S. minor* subsp. *minor* and subsp. *muricata*	0.02	0.02	0.02
Average sequence divergence (%) between *S. minor* subsp. *minor* and subsp. *lasiocarpa*	0.03	0.02	0.02
Average sequence divergence (%) between *S. minor* subsp. *muricata* and subsp. *lasiocarpa*	0.01	0.03	0.02
Number of MPTs	489	100	302
Average sequence divergence (%) between *Sanguisorba* and *Poterium*	0.053	-	-
Length of MPTs	349	102	303
C.I. of MPT	0.6864	0.9608	0.9637
R.I of MPT	0.8146	0.9600	0.9626
Evolutionary model selected (under AIC)	GTR+G	GTR+G	HKY+G

For determining sequence statistics 703 and 1369 characters were aligned for ITS and *rpl*32-*trn*L$_{(UAG)}$ and combined ITS + *rpl*32-*trn*L$_{(UAG)}$ regions, respectively. Portions of ITS and *rpl*32-*trn*L$_{(UAG)}$ alignments are presented in tables 5–6. Both regions' variations (transitions, transversions and number of indels) were identified. ITS nucleotide displays variations at 10 position numbers 36–40, 75–80, 143–146, 153–155, 240–243, 545–549, 575–567, 657–666, 670–675, and 680–685 between *S. minor* subsp. *minor* and *S. officinalis.* This is reduced from six position numbers (58–62, 509–512, 577, 658–659,662–663, and 672–673) between *S. minor* subsp. *minor* and *Poterium*; to three position numbers (77–78, 145–146, and 241–244) among *S. minor* subsp. *minor* and both subsp. *muricata* and subsp. *lasiocarpa* and only one position number (688–692) between *S. minor* subsp. *muricata* and *S. minor* subsp. *lasiocarpa* (Tables 5–6). In the investigated taxa, sequence analysis of *rpl*32-*trn*L$_{(UAG)}$ region revealed variations within four regions (76–90; 934–936; 1083–1103, and 1326–69) which have been indicated in table 5. On comparing *S. officinalis* and *S. minor* subsp. *minor*, nucleotide base transversions at

position numbers 82 (A to T), 88 (T to A); insertion at position number 936 (CA to CAT); and variations and deletions at position numbers 1353–1369 (GGA-to CTGTTAGGGGG-TCG) were recorded. The result of comparing nucleotides base variations between *S. minor* subsp. *minor* and subsp. *muricata* also revealed changes at two position numbers 79–80 (CT to AC) and 83–90 (ACCAAT- to GACTATTT); a deletion (CAT- to CA -) at position numbers 936; 1337–1343 (GGAAAA- to TGGGAAC), 1349–1359 (AACCTCTGTTA to GGTTATTTAGT), 1365–1366 (- T to TT). Authors of the paper recognized the following nucleotides base variations between *S. minor* subsp. *muricata* and *S. minor* subsp. *lasiocarpa* at position numbers 79–90 (ACTAGACTATTT to TTCCTAAACC-TT -), 1083–1085 (- AA to AA), 1326–1327 (TA to T -), 1338–1339 (TG to GG), 1340–1341 (GG to GA), 1344–1346 (ACT-AGG) 1351–13621 (GGTTATTTAGT to AATTTCAAAGG) and 1365–1367 (TTT to -).

Table 4. Motifs identified within ITS

nrDNA ITS nucleotide No.	**36–40**	**75–80**	**143–146**	**153–155**	**240–243**	**545–549**	**576–567**	**657–666**	**670–675**	**680–685**
Sanguisorba officinalis	GT**AT**	C**C**TC	GC**CT**	CCC	GT**CT**	**T**CC	C**T**	T**T**TCAC**T**G	GCG**C**GT	TCC**G**T
S. minor subsp. *minor*	GT**TT**	C**T**TC	GC**TT**	C**T**C	GT**TT**	**C**CC	C**C**	T**C**TCAC**A**C	GCG**T**GT	TCC**TT**
nrDNA ITS nucleotide No.	58–62	509–512	577	658–659	662–663	672–673				
S. minor subsp. *minor*	G**AG**GC	GGGG**T**	TCC**C**C	**GTCT**	**CA**	G**T**				
Poterium sp.	G**GT**GC	GGGG**C**	TCC**T**C	**TTTC**	**CG**	G**C**				
nrDNA ITS nucleotide No.	77–78	145–146	241–244							
S. minor subsp. *minor*	C**T**T	C**TT**	GT**T**T							
S. minor subsp. *muricata*	C**C**T	C**C**T	GT**C**T							
S. minor subsp. *lasiocarpa*	C**C**T	C**C**T	GT**C**T							
nrDNA ITS nucleotide No.	688–692									
S. minor subsp. *muricata*	GC**T**TT									
S. minor subsp. *lasiocarpa*	CG**C**TT									

Table 5. Motifs identified within cpDNA *rpl*32-*trn*L$_{(UAG)}$ region

Taxon	**cpDNA nucleotide No.**			
	76–90	**934–936**	**1083–1103**	**1326–1369**
Sanguisorba officinalis	TTCC**TAA**CCA**TT**-	CA-	-AAAAAAAAAAATTCTA	TACGTTTTTTTGGAAAA-TGGAAAACCT-T**GGA**-
S. minor subsp. *minor*	TTCCT**T**AACCA**AT**-	CA**T**	-AAAAAAAAAAATTCTA	TACGTTTTTTTGGAAAA-TGGAAAACCT**CTGTTA**GGGGG-**T**CG
S. minor subsp. *muricata*	TTC**ACT**A**GACT**A**TTT**	CA-	-AAAAAAAAAAATTCTA	TACGTTTTTTTT**T**GGGAA**C**TGGAA**GGTTATTTAGT**GGGGG**TTT**CG
S. minor subsp. *lasiocarpa*	TTC-CTA**AA**CC**TT**-	CA-	-AAAAAAAAAAATTCTA	TCGTTTTTTT**G**GGAA**AGG**GGAA**ATTTC**A**AA**G**G**GGGG-**C**CG

Table 6. Comparative micro- and macromorphological characters between the studied species and subspecies (based on Nordborg 1966, Khatamsaz 1993, Faghir *et al.* 2017, and Deylami Moezi *et al.* 2019)

Character	***Sanguisorba officinalis***	***S. minor* subsp. *minor* = *Poterium sanguisorba* subsp. *sanguisorba***	***S. minor* subsp. *muricata* = *Poterium sanguisorba* subsp. *muricata***	***S. minor* subsp. *lasiocarpa* = *Poterium sanguisorba* subsp. *lasiocarpa***
Plant height (mm)	25–100	30–60	15–90	30–60
*+** Stem type	Erect	Erect and ascending	Erect and ascending	Erect
*+** Stem hair types	Glabrous	Glabrous	Glabrous below, crispate short, and long hairs	Scarcely hairy, hairs crispate
*+** Leaf surface	Scattered hairs on both surfaces	Glabrous or scarcely hairy	Glabrous	Glabrous
* Flower sexuality	Bisexual, upper female	Either male or female, bisexual, upper female, middle bisexual, and lower male	Either male or female, bisexual, upper female, middle bisexual, and lower male	Either male or female, bisexual, upper female, middle bisexual, and lower male
* Number of Bract(s)	1	2	2	2
* Number of stamens	4	20–30	20–30	20–30
* Stamen length/calyx length	Stamen shorter than calyx	Stamen longer than calyx	Stamen longer than calyx	Stamen longer than calyx
* Number of carpel(s)	1	2	2	2
*+** Stigma length	+	++	++++	+++
* Stigma shape	Papillate or with short villi	Penicillate	Penicillate	Penicillate
* Hypanthium shape	Tetragonal	Turbinate tapering at summit	Ovoid, tapering at summit	Ovoid, tapering at summit
Leaf micromorphology				
* Leaf lower side	Hairy	Scarcely hairy	Glabrous	Glabrous
* Hair types	curved and straight, flexuous	curved and flexuous	–	–
* Trichome surface	Having alternate linear warts	Trichome surface is not veruccose	Trichome surface is not veruccose	Trichome surface is not veruccose
*+** Wax type of upper surface of the leaf	Smooth layer-granulate	Crust	Crust-granulate -platelets	Smooth layer-granulate
* Wax type of lower surface of the leaf	Smooth layer	Crust-granule	Crust-granule	Crust-granule
** Anticlinal layers of the leaf upper surface	Depressed	Depressed undulate	Raised	Raised
*+** Anticlinal layers of the leaf lower surface	Depressed undulate	Depressed	Depressed	Raised-oblate
*+** Outer periclinal layers of the lower surface	Raised undulate	Raised	Raised	Depressed-oblate
*+** Outer stromal rim	Raised / raised	Overlapping	Overlapping	Overlapping
* Peristomatal rim	Overlapping-stout	Overlapping	Overlapping	Overlapping-stout
* Inner stomatal rim	Sinuolate-erose	Smooth	Smooth	Smooth
* Wax distribution on the stomata rims, pore, and epidermal cells	Stomata rims and pore free, guard cell covered by wax	Stomata rim and guard cell not completely covered by wax; pore free	Stomata rim and guard cell completely covered by wax, pore free	Stomata rim and guard cell not completely covered by wax; pore free

Table 6 (contd.)

Achene morphology				
* Achene shape	Conical	Ovoid to tetragonal	Ovoid to tetragonal	Ovoid to tetragonal
*+** Achene upper 1/3 thickness (µm)	1.02	1.52	1.13	1.67
* Number of wings	2	4	4	4
*+** Achene surface	Brain shape	Reticulate (finely netted-veined)	Reticulate	Deeply alveolate faces
*+** Papilla density	+	+	++	+++
Achene micromorphology				
* Epicuticular wax types	Granule-Crust	Granule-smooth layer and platelets	Granule-smooth layer and platelets	Granule-smooth layer and platelets
*+** Achene sculpturing types	Reticulate	Reticulate	Reticulate-foveate	Reticulate
** Papilla intervals (µm)	16.83–85.33	19.5–73.91	8.29–58.33	6.47–25.9
* Papilla sculptures	Regulate-striate	Regulate-striate	Regulate-striate	Regulate-psilate
* Lumen length (mm)	0.05–0.11	0.10–0.75	0.13–0.37	0.08–0.32
* Lumen width (mm)	0.07–0.21	0.1–0.27	0.11–0.31	0.07–0.17
* Hair types on the wing	Appressed flexuous	Erect-suberect straight	Erect-suberect straight	Erect-suberect straight
* Hair types of the achene surfaces	Erect	Erect-suberect	Erect-suberect	Erect-suberect
* Wing thickness (mm)	±0.23.4	±0.15.3	±0.18.4	±0.14.2

Discussion

The present study is the first attempt to infer the phylogeny of the genus *Sanguisorba* in Iran. The genus has been often used in molecular analyzes of the several members of tribe *Rosoideae*, e.g., *Potentilla* L. and *Geum* L. as out group species (Eriksson *et al.* 1998, 2003, Faghir *et al.* 2014, 2017). The current results support the monophyly of the tribe *Sanguisorbeae*, which is phylogenetically divided into two subtribes: *Sanguisorbinae* (clade A in Fig. 1) comprising apetalous inflorescences and large fimbrillate stigmas and *Agrimoniinae* (clade B in Fig. 1) including petalous members, yellow or cream flowers. The result is in agreement with previous studies (Kerr 2004, Xiang *et al.* 2017, Zhang *et al.* 2017, Park *et al.* 2021).

The present findings represent resolution of phylogenetic relationships at inter and intraspecific levels, suggesting taxonomic solutions within the genus *Sanguisorba*. The nrDNA ITS MP and Bayesian trees showed clear segregation of *S. officinalis* (in subclade "Sanguisorba", A2b in Fig. 1) from *S. minor* (in subclade "Poterium" (A2b in Fig. 1). The results are supported by highest ITS sequence divergent (0.08, 0.09, and 0.1%) and maximum genetic differentiation (at 10 position numbers) of cpDNA region between *S. officinalis* and *S. minor*. In contrast, according to our findings, the ITS sequence divergent (0.053%) between *S. minor* and *Poterium* (obtained from GenBank) is about 1.7 times lower than of *S. minor* and *S. officinalis.* In addition, lower genetic differentiation (at six position numbers 58–62, 509–512, 577, 658–659, 662–663, and 672–673) was observed when cpDNA of *S. minor* and *Poterium* was examined. The present result is consistent with Kerr's (2004) molecular analysis and is supported by the differences in morphological characters (Linnaeus 1753, Spach 1846, Rydberg 1908, Juzepczuk, 1941, Faghir *et al.* 2017, Deylami Moezi *et al.* 2019).

The genus *Poterium* is characterized by its greenish, unisexual, monoecious, often perfect flowers with two pistils penicillate stigma; fruits narrowly winged along ribs or wingless, finely netted/pitted or nearly smooth, were distinguishing characters in *sanguisorba* (Juzepczuk 1941). Based on this evidence, *S. officinalis* represents the only species of *Sanguisorba* in Iran, hence, it is recommended to include *S. minor* in the genus *Poterium.*

The nrDNA ITS MP and Bayesian trees showed that, the three subspecies are also nested within the subclade Poterium (including *Poterium* and *Poteridium*).

The validity of three subspecies can also be discussed based upon the distinction between their sequences and nucleotide divergence. The three subspecies showed generally low sequence divergence (0.01–0.03% in ITS, 0.02–0.03% in cpDNA, and 0.02% in combined data), and little genetic differentiation. When the ITS region was analyzed, it was found that, *S. minor* subsp. *minor* displays three motifs (CTT, CTT, and GTTT) at position numbers 77–78, 145–146, and 241–244, which do not occur in other two subspecies, Whereas *S. minor* subsp. *muricata* and *S. minor* subsp. *lasiocarpa* differ genetically only at the single position (692–688). Intraspecific genetic differentiation became more apparent when maternally inherited cpDNA was analyzed and it was shown that, *rpl*32-*trn*L$_{(UAG)}$ intergenic spacer is the most informative for the low-level phylogenetic studies (Shaw *et al.* 2007, 2014).

Genetic differentiation showed that, similar chloroplast nucleotide variation between the three subspecies. *S. minor* subsp. *minor* is genetically identified by its TTCACTAGACTATTT repetition at position numbers 76–90 and GGTTATTTAGTGGGGGTTTCG repetition at position numbers 1326–1369. According to our findings, maximum genetic similarity was observed between *S. minor* subsp. *minor* (by having -AAAAAAAAAAATTCTA repetition at position numbers 1083–1103) and *S. minor* subsp. *lasiocarpa* (by having CA- repetition at position numbers 934–936). The two unique genetic differentiations: TTCACTAGACTATTT repetition, that show no deletion on the last two T (TT) nucleotides and changes at position numbers 1326–1369, explain genetic divergence among *S. minor* subsp. *muricata* and other two subspecies. This result showed little genetic differentiation of chloroplast nucleotide. Nordborg (1966) also reported that, there is no clear discontinuity in terms of morphological characters between these subspecies. However, recent micro and macromorphological studies revealed useful diagnostic characters that can be used for subspecies identification. (Khatamsaz 1993, Faghir *et al.* 2017, Deylami Moezi *et al.* 2019). This includes stem type, stem and leaf hair types, wax type of upper leaf surface, anticlinal layers of the leaf upper and lower surfaces, outer periclinal layers of the lower surfaces, outer stromal rim type: achene upper 1/3 thickness, surface and sculpturing types, papilla intervals and density (Table 6). Several micro- and macro-morphological evidence supports the separation of the three subspecies and therefore, authors of the paper propose to consider them as a subspecies of *Poterium sanguisorba* based on the diagnostic features shown below:

Sanguisorba minor subsp. *minor* = *Poterium sanguisorba* subsp. *sanguisorba* L., is recognized by its glabrous stem, glabrous or scarcely hairy leaf surface, shorter stigma length, turbinate tapering at summit hypanthium shape, scarcely hairy leaf lower side, curved and flexuous hair type, crust wax type of upper surface of the leaf, depressed undulate and depressed anticlinal layers of the leaf lower and upper surface, raised outer periclinal layers of the lower surface, reticulate, finely netted-veined achene surface, highest papilla interval (19.5–73.91 µm). *S. minor* subsp. *lasiocarpa* = *P. sanguisorba* subsp. *lasiocarpa* Boiss. & Hausskn. were identified based on their erect stem, smooth layer-granulate wax type of upper and lower surface of the leaf, raised and raised-oblate lower, depressed upper and lower surfaces, anticlinal layers of the leaf, overlapping-stout peristomatal rim, deeply alveolate faces achene surface, densely papillate, and regulate-psilate achene sculpturing. Stem glabrous below or with crispate short, and long hairs, hypanthium ovoid, tapering at summit, leaf lower side glabrous; crust and crust-granule wax type of upper and lower surface of the leaf; raised and depressed anticlinal layers of the leaf upper and lower surfaces, reticulate achene surface and reticulate-foveate achene sculpturing are characteristics features of *Sanguisorba minor* subsp. *muricatum* = *P. sanguisorba* subsp. *muricatum* Bonnier & Layens.

Conclusions

Phylogenetic reconstruction based on nrDNA ITS and *rpl*32-*trn*L$_{(UAG)}$ resulted in resolution of phylogenetic

relationships at inter generic and specific taxonomic ranks. The results revealed genetic similarities between *S. minor* and *Poterium* which is supported by previous morphological evidence. Upon this evidence, authors of the paper suggest to include *S. minor* in the genus *Poterium.* The phylogenetic relationships of the three subspecies (nested within Poterium clade), remained unresolved. They also showed generally low sequence divergence and little genetic differentiation. However, micro- and macromorphological evidence supports separation of the three subspecies.

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