The phylogeny of *Calligonum* and *Pteropyrum* (Polygonaceae) based on nuclear ribosomal DNA ITS and chloroplast *trn*L-F sequences

Solmaz Tavakkoli[1], Shahrokh Kazempour Osaloo[1*], Ali Asghar Maassoumi[2]

[1]Department of Plant Biology, Faculty of Biological Sciences, Tarbiat Modares University, P.O. Box 14115-175,Tehran, Iran [2]Department of Botany, Research Institute of Forests and Rangelands, P.O. Box 13185-116, Tehran, Iran

Abstract
This study represents phylogenetic analyses of two woody polygonaceous genera *Calligonum* and *Pteropyrum* using both chloroplast fragment (*trnL-F*) and the nuclear ribosomal internal transcribed spacer (nrDNA ITS) sequence data. All inferred phylogenies using parsimony and Bayesian methods showed that *Calligonum* and *Pteropyrum* are both monophyletic and closely related taxa. They have no affinity with *Atraphaxis,* instead allied with a clade in which the genus is nested. Infrageneric relationships in *Calligonum,* due to the paucity of informative nucleotide sites in both DNA regions are not resolved.
Keywords: *Calligonum*; cpDNA *trn*L-F; Molecular phylogeny; nrDNA ITS; *Pteropyrum*; Polygonaceae

INTRODUCTION

Calligonum possesses 80 species with xeromorphic shrubby characteristics distributed throughout Southern Europe, North Africa and Western and Central Asia as its main biodiversity center (Brandbyge, 1993; Mabberely, 1990). This genus is well distinguished from other genera of the family by the higher number of stamens (12-15) and four carpels/stigmas as well as C4 photosynthesis (Sage, 2004; Pyankov *et al*., 2000; Brandbyge, 1993). Eighteen species including six endemic ones have been identified among the flora of Iran (Mozaffarian, 2004; Rechinger and Schiman-Czaika, 1968). According to fruit morphology the genus has been divided into three sections: *Calligonum* (with bristled fruit), *Pterococcus* (with winged fruit) and *Calliphysa* (with membranous saccate fruit) (Rechinger and Schiman-Czaika, 1968). The genus *Pteropyrum* has 4 or 5 species in south west Asia and the Middle East, of which 3 species are distributed in Iran. The members of this genus like that of *Calligonum* are shrubs but have achenes with only 3 membranous wings. *Pteropyrum* in particular *Calligonum* are typical arid and hot desert plants in active sand dunes and playing the key role in the stability of desert natural vegetation ecosystem (Ren and Tao, 2004; and personal observations).

The climatic distribution pattern of *Calligonum* is similar to that of C4 large shrubby chenopod species (*Haloxylon persicum*, *H. ammodendron* and *Salsola richteri*) with NADP-ME metabolism and salsoloid assimilation organ anatomy (Pyankov *et al*., 2000).

Tavakkoli *et al*. (2008) conducted a morphology based-phylogenetic analysis of these taxa to test their relationships and monophyly. Sanchez and Kron (2008) using cpDNA sequences (*rbc*L and *mat*K), and then Sanchez *et al*. (2009) using those genes as well as *ndh*F plus nrDNA ITS regions showed that monophyly of *Calligonum* is controversial. Their analyses revealed that *Calligonum*, *Pteropyrum* and *Pteroxygonum* form a weakly to moderately supported clade.

In this study, therefore, phylogenetic analyses were performed using both nrDNA ITS (ITS1, 5.8S and ITS2) and cpDNA *trn*L-F sequence data to address the

*Correspondence to: **Shahrokh Kazempour Osaloo,** Ph.D.
Tel: + 98 21 82884404; Fax: +98 21 82883463
E-mail: skosaloo@modares.ac.ir and skosaloo@yahoo.com

following questions:
1) Are *Calligonum* and *Pteropyrum* both monophyletic? 2) Are *Calligonum* and *Pteropyrum* closely related taxa? 3) Is the current sectional classification of *Calligonum* supported? and 4) are these genera related to *Atraphaxis*?

MATERIALS AND METHODS

Taxon sampling: A total of 27 accessions representing 26 species were included in phylogenetic analyses using nrDNA ITS region and 28 accessions representing 27 species were analyzed for cpDNA *trn*L-F regions. Eleven species including *Calligonum* (6 species), *Pteropyrum* (3 species) and *Atraphaxis* (2 species) were sequenced for both regions newly. The remaining sequences were obtained from GenBank. The leaf/branchlet material was taken mostly from herbarium specimens deposited at the herbarium of the Research Institute of Forests and Rangelands (TARI). In some cases, the materials were collected from the field. Information on the accessions used in this study is presented in Table 1. According to Lamb Frye and Kron. (2003), *Triplaris americana* was chosen as an outgroup.

DNA extraction, amplification, and sequencing:

Table 1. Taxa included in nrDNA ITS and cpDNA *trnL-F* phylogenetic analyses.

Species	Voucher	GenBank Accession No.	
		nrDNA ITS	*trn*L-F
Aconogonon molle (D. Don) H. Hara.		EF653687*	EF653790*
Atraphaxis spinosa Jaub & Spach.	Iran: Tavakkoli *et al*. 2006-1 (TMUPC)	AB542772	AB542783
A. suaedifolia L.	Iran: Mozaffarian 87202 (TARI)	AB542773	AB542784
Calligonum arborescens Litvinov.		-	EU109591*
C. bungei Boiss.	Iran: Tavakkoli *et al*. 2006-2 (TMUPC)	AB542775	AB542786
C. comosum L.	Iran: Assadi & Sardabi 42047 (TARI)	AB542778	AB542789
C. crinitum Boiss.	Iran: Tavakkoli *et al*. 2006-3 (TMUPC)	AB542776	AB542787
C. junceum (Fisch & Mey) Litw.	Iran: Mozaffarian 83955 (TARI)	AB542774	AB542785
C. junceum (Fisch & Mey) Litw.		-	EU109590*
C. persicum (Boiss & Buhse) Boiss.	Iran: Tavakkoli *et al*. 2006-4 (TMUPC)	AB542777	AB542788
C. pologonoides L.	Iran: Tavakkoli *et al*. 2006-5 (TMUPC)	AB542779	AB542790
C. pumilum L.		-	EU109592*
Fagopyrum esculentum Moench.		F653685*	EF653788*
F. callianthum Ohnishi.		AB000322*	-
Fallopia convolvulus (L.) A. Love.		EU580715*	EU586189*
F. dentatoalata (*F. Schmidt*) Holub.		EU580726*	EU586186*
F. multiflora var.ciliinervis (Nakai) Yonekura & Ohashi.		EU580713*	EU024783*
Parapteropyrum tibeticum Li.		EU718499*	EU109589*
Persicaria posumbu (Buch.-Ham. ex D. Don) H. Gross.		EF653701*	EF653804*
P. hydropyper (L.) Spach.		EF653702*	EF653805*
P. filiformis (Thunb.) Nakai ex W. T. Lee.		EF653697*	EF653800*
Polygonum aviculare L.		EF653684*	EF653787*
Pteropyrum aucheri Jaub & Spach.	Iran: Tavakkoli *et al*. 2006-7 (TMUPC)	AB542780	AB542791
Pt. naufelum Al-khayat.	Iran: Assadi & Nikchehreh 76316 (TARI)	AB542781	AB542792
Pt. olivierii Jaub & Spach.	Iran: Dini & Bazargan 30675 (TARI)	AB542782	AB542793
Pterozygonum giraldii Dammer & Diels.		EU580725*	EU402464*
Pt. giraldii Dammer & Diels.		DQ406627*	-
Rumex acetosella L.		AJ580776*, AJ580792*	AJ583842*,AJ583855*
R . japonicus Houtt.		AF338220*	AJ810936*,AJ810946*
Triplaris americana L.		FJ154486*	AJ312251*

Abbreviations used in accession information: TARI, Herbarium of the Research Institute of Forests and Rangelands, Tehran; TMUPC, Tarbiat Modares University Plant Collection, Tehran. *Sequences from GenBank. The two accession numbers for nrDNA ITS of *Rumex acetosella* represent ITS1 and ITS2, respectively. The two accession numbers for *trn*L-F of *Rumex acetosella* and *R. Japonicus* represent *trn*L intron and *trn*L-*trn*F intergenic spacer, respectively.

Total genomic DNA was isolated from dried leaf or branchlet (only for *Calligonum*) of samples using the modified cetyl trimethylammonium bromide (CTAB) method of Doyle and Doyle (1987). The amplification of nrDNA ITS and *trn*L-F regions by polymerase chain reaction (PCR) were performed in a 25 µl reaction mixture, containing 16 µl of sterile water, 2 µl of 2.5 mM $MgCl_2$, 2.5 µl of 10X Gene Taq universal buffer (Cinnagen, Iran), 2.5 µl of 2.5 mM dNTPs mixture (Wako Nippon Gene, Japan), 0.5 µl of each primer (5 pmol/µl), 0.2 µl (4 Units) of Taq DNA polymerase (Cinnagen, Iran), and 1-1.3 µl of genomic DNA template (approximately 20 ng) using primer pair "ITS1F" (Navajas-P´erez *et al*., 2005) and ITS4 (White *et al*., 1990). The *trn*L-F region was amplified using the universal "c" and "d" primers of Taberlet *et al*. (1991). The PCR condition, performed in a DNA thermal cycler (Primus 96, MWG, Germany), was 2.5 min at 95°C for initial denaturation followed by 38 cycles of 1 min at 95°C, 45 sec at 53°C for annealing, 2 min at 72°C for extension, followed by a final 7 min incubation at 72°C. The quality of PCR products were checked by electrophoresis on an 0.8% (w/v) agarose gel (using 1X TAE as the gel buffer) stained with ethidium bromide and then visualized under UV light. Each region was sequenced using the 'Big dye terminator cycle sequencing ready reaction kit' (Applied Biosystems, USA). with the appropriate primers in an ABI Prism 377 DNA sequencer (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 numerous single and multiple-base indels (insertions/deletions). Positions of indels were treated as missing data for all datasets.

Phylogenetic analyses

Maximum parsimony method: Maximum Parsimony (MP) analyses were conducted using the PAUP* program version 4.0b10 (Swofford, 2002) for phylogenetic analyses. The heuristic search option was employed for each of the datasets, using tree bisection-reconnection (TBR) branch swapping, with 1000 replications of random addition sequence and an automatic increase in the maximum number of trees. Uninformative characters were excluded from the analyses. Branch support values were calculated using a full heuristic search with 1000 bootstrap replicates (Felsenstein, 1985) each with simple addition sequence. Combinability of these two datasets was assessed using the partition homogeneity test (the incongruence length difference (ILD) test of Farris *et al*.,1995) as implemented in PAUP*. The test was conducted with exclusion of invariant characters (Cunningham, 1997) using the heuristic search option involving simple addition sequence and TBR branch swapping with 1000 homogeneity replicates.

Bayesian method: Models of sequence evolution were selected using the program MrModeltest version 2.3 (Nylander, 2004) as implemented in MrMTgui (Nuin, 2005) based on the Akaike information criterion (AIC) (Posada and Buckley, 2004). On the basis of this analysis, datasets were analyzed using the GTR+I+G and GTR+G models for nrDNA ITS and *trnL-F* sequences, respectively. The combined sequences for 25 taxa were analyzed as a single partition with the GTR+I+G model. The program MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) was used for the Bayesian phylogenetic analyses. Posteriors on the model parameters were estimated from the data, using the default priors. The analysis was carried out with 2 million generations, using the Markov chain Monte Carlo search. MrBayes performed two simultaneous analyses starting from different random trees (Nruns=2) each with four Markov chains and trees sampled at every 100 generations. The first 25% of trees were discarded as the burn-in. The remaining trees were then used to build a 50% majority rule consensus tree accompanied with posterior probability (PP) values using. Tree visualization was carried out using Tree View version1.6.6 (Page, 2001).

RESULTS

Individual sequence data: The nr|DNA ITS dataset is 749 nucleotide sites long, of which 305 sites are potentially parsimony informative. The length of the nrDNA ITS ranges from 507 bp in *Atraphaxis spinosa* to 607bp in 6 studied species of *Calligonum*. A 50% majority-rule consensus tree obtained form Bayesian analysis with posterior probabilities and bootstrap values is presented in Figure 1. This tree is, topologically, almost the same as the single most parsimonious tree resulting from the MP method (data not shown), except that the *Fagopyrum/Parapteropyrum* clade is part of a weakly supported trichotomy comprising the *Calligonum*/*Pteropyrum* and *Persicaria* clades (Fig. 1).

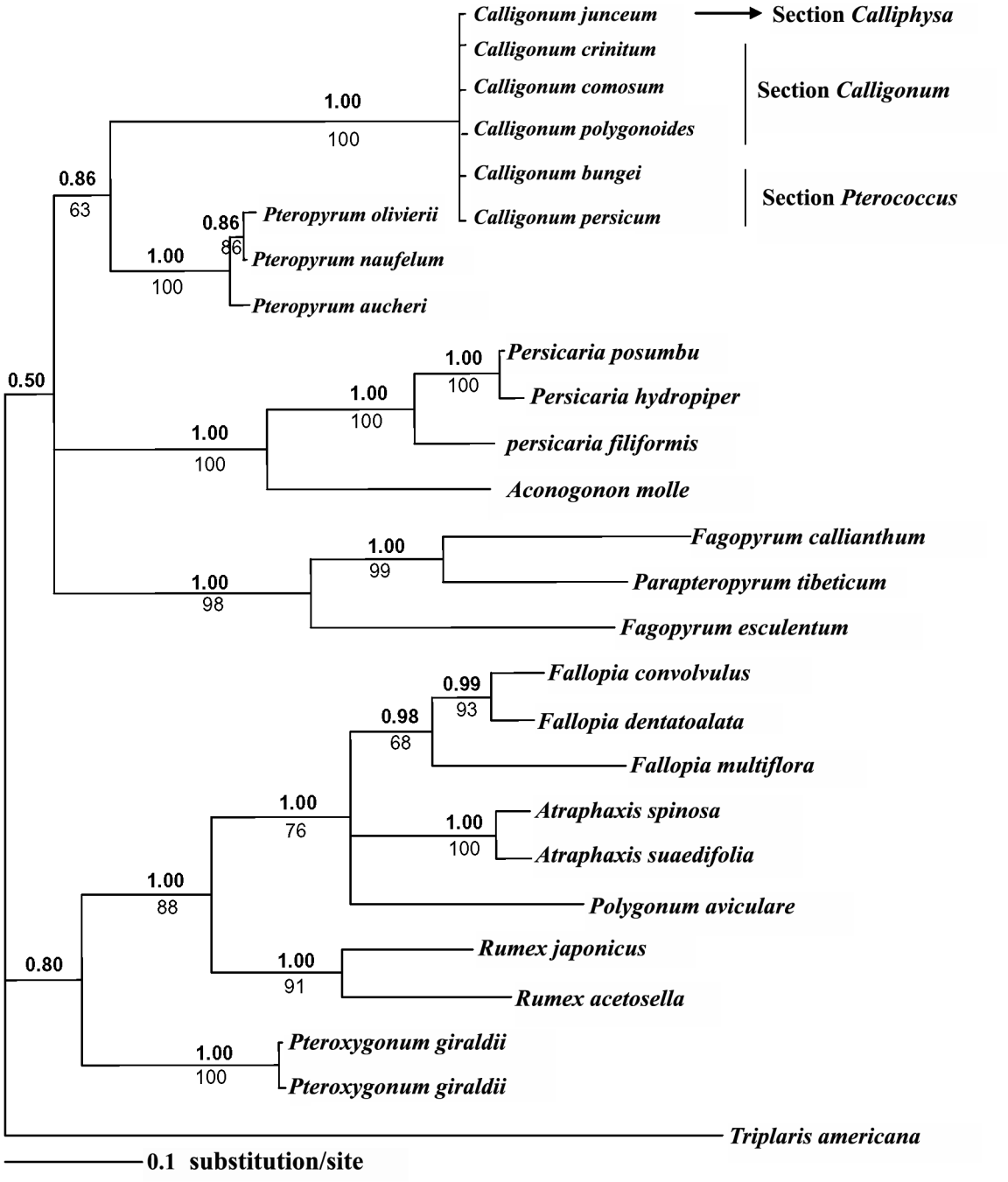

Figure 1. Fifty percent majority rule consensus tree resulting from Bayesian analysis of the nrDNA ITS data set. Numbers above branches are posterior probabilities and the numbers below them indicate MP bootstrap values. Values < 50% were not shown.

The *trn*L-F dataset consisted of 28 accessions with 1201 aligned nucleotide sites, of which 224 sites are potentially parsimony-informative. The length of the *trn*L intron rang from 513 bp in *Pteropyrum aucheri* to 644 bp in *Atraphaxis spinosa* and the the length of partial *trn*L-*trn*F intergenic spacer ranges from 162 bp in *A. spinosa* and *A. suaedifolia* to 288 bp in *Pteropyrum aucheri*. A 50% majority-rule consensus tree from Bayesian analysis is presented in Figure 2. This tree is, topologically, the same as the strict consensus of three most parsimonious trees (data not shown). In this tree, *Calligonum* and *Pteropyrum* are closely related sister taxa and, in turn, are well allied with the *Rumex*/*Polygonum*/*Atraphaxis*/ *Fallopia* clade.

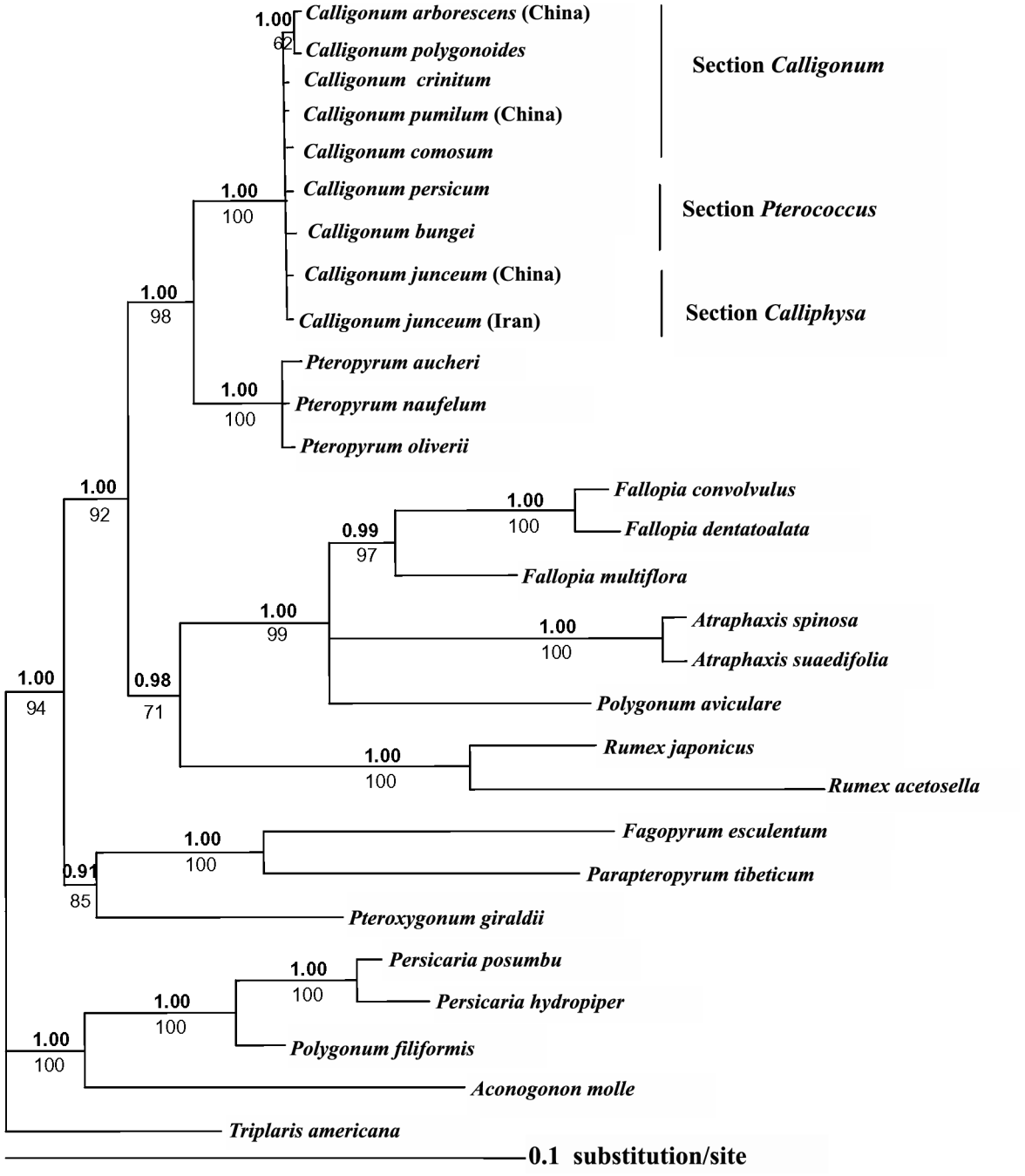

Figure 2. Fifty percent majority rule consensus tree resulting from Bayesian analysis of the cpDNA *trn*L-F data set. Numbers above branches are posterior probabilities and the numbers below them indicate MP bootstrap values. Values < 50% were not shown.

The combined sequence data: The partition homogeneity test suggested that the nrDNA ITS and *trn*L-F datasets were not congruent (P=0.012). The main topological differences between the two gene phylogenies are the positions of the *Calligonum*/*Pteropyrum* clade and *Pteroxygonum*. In spite of these conflicts and following the suggestions of several other researches (Yoder *et al*., 2001; Reeves *et al*., 2001; Wiens, 1998; Seelanan *et al*., 1997) that the ILD test may be unreliable, we combined these data sets directly. The combined dataset was 1952 nucleotide sites long, of which 507 were parsimony informative. A 50% majority-rule consensus tree from Bayesian analysis of the combined dataset is presented in Figure 3. This tree is topologically similar to the *trn*L-F tree. This Bayesian tree is, however, topologically and statistically well

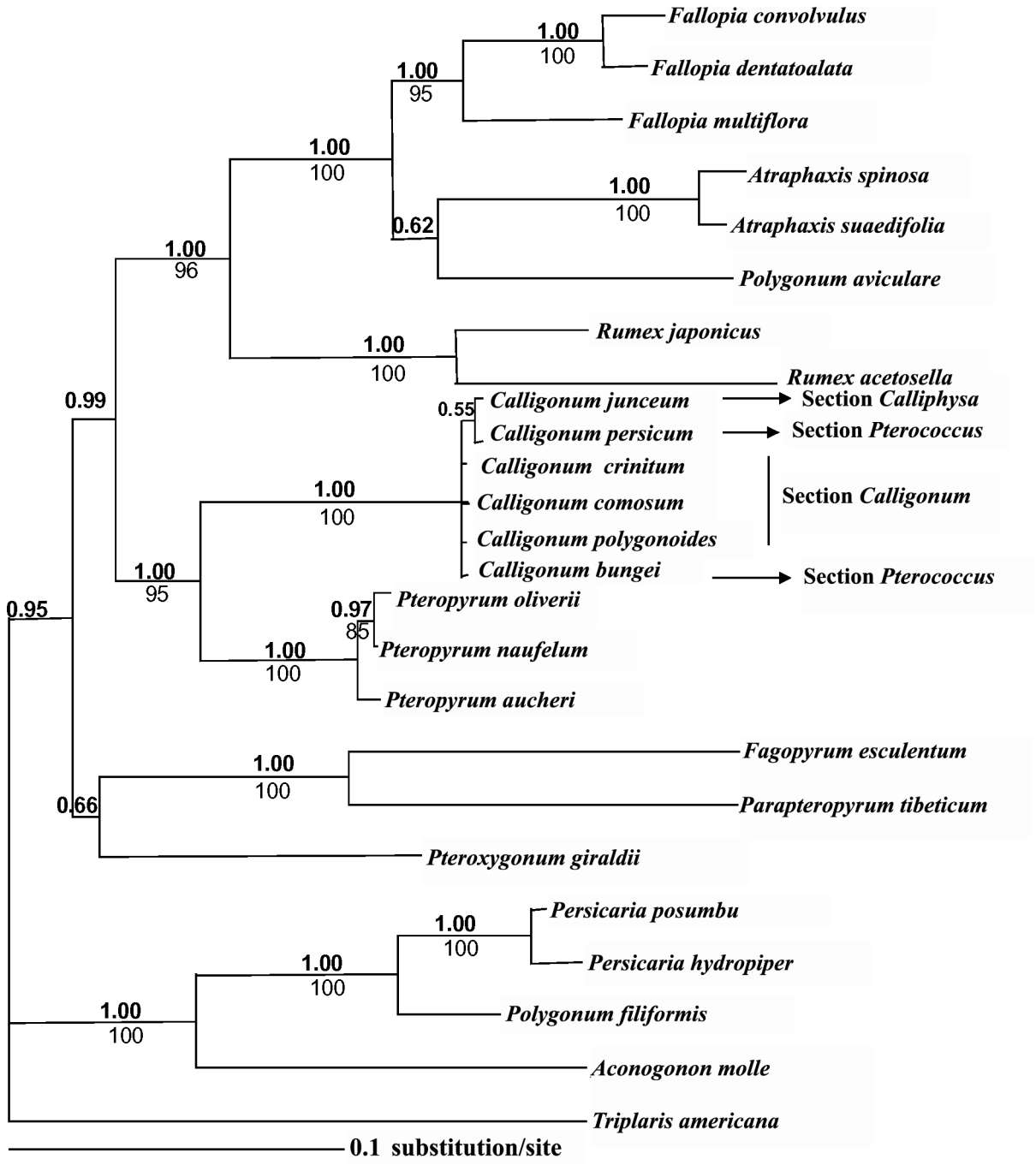

Figure 3. Fifty percent majority rule consensus tree resulting from Bayesian analysis of the combined nrDNA ITS and cpDNA *trn*L-F data set. Posterior probabilities are above branches and the numbers below them indicate MP bootstrap. Values Values < 50% were not shown.

resolved and supported than the strict consensus tree of three most parsimonious trees does (not shown). In Bayesian tree, *Pteroxygonum* is weakly allied with a clade of *Fagopyrum* and *Parapteropyrum*. *Calligonum* and *Pteropyrum* are again sister taxa and formed a sister group relationship with a clade of *Rumex*/*Polygonum*/*Atraphaxis*/ *Fallopia*.

DISCUSSION

Monophyly and relationships of *Calligonum* and *Pteropyrum*: Phylgenetic analyses of nrDNA ITS, *trn*L-F and combined nrDNA ITS-*trn*L-F datasets revealed that *Calligonum* and *Pteropyrum* are each monophyletic and closely related taxa. In a recent

cladistic analysis of morphological data, these closely related taxa also appeared to be monophyletic (Tavakkoli *et al*., 2008). The previous studies using non-molecular evidence suggested their closest relationships. such evidences include vegetative morphology: polyachanthic life-form; stem anatomy: lignified secondary walls in the collenchyma, (Haraldson, 1978); floral characters: formation of commissural veins, non-fused petaloid tepals, irregular tepal epidermal cells and presence of papillae at the base of the stamen filament (Hong *et al*., 1998; Ronse-Decraene and Akeroyd, 1988); fruit morphology: armed achenes with wings/bristles (Brandbyge, 1993; Rechinger and Schiman-Czeika, 1968); and pollen morphology: microreticulate/perforate exine sculpturing (Hong, 1995; Nowicke and Skvarla, 1979).

Sanchez and Kron (2008) and Sanchez *et al*. (2009), based on combined *rbc*L-*mat*K-*ndh*F sequences, have reported that *Calligonum* is not monophyletic with both *Pteropyrum* (*P. aucheri* and *P. olivierii*) and the monotypic *Pteroxygonum* (*Pt. giraldii*) nested within it. But, on the nrDNA ITS and the combined cpDNA-nrDNA ITS trees of Sanchez *et al*. (2009), *Calligonum* formed a well supported clade and weakly allied with *Pteropyrum* solely and *Pteropyrum-Pteroxygonum,* respectively. On the chloroplast tree resulting from their analysis *Pt. giraldii* is of a long branch (due to *ndh*F sequences, see below).

It is worthy to note that our alignment of both *mat*K and *rbc*L sequences retrieved from GenBank (determined by Sanchez *et al*., 2009), shows that *Pt. giraldii* has almost the same sequences as that of four out of five *Calligonum* species examined*,* while *Calligonum microcarpum* has the same ones as that of *Petropyrum*. But, *ndh*F sequences of *Pt. giraldii i*s completely different from that of *Calligonum* species. This indicates that Sanchez *et al*. (2009) might, most probably, determine the sequences of these taxa mistakenly. Meanwhile, the nrDNA ITS sequences of both *Calligonum* and *Pteropyrum* determined by Sanchez *et al*., are full of base-calling errors**.** All available evidence including growth habit (climbing stem with petiolate leaves), lower stamen number (usually 8), achene morphology (with three sharp horns at the base), chromosome base number (x=20), tepal venation (trifid) and perhaps the C3 photosynthetic pathway indicate that *Pteroxygonum* has no affinity with *Calligonum* (Sun *et al*., 2008; Li and Grabovskaya-Borodina, 2003; Ronse-Decraene and Akeroyd, 1988; Haraldson, 1978). This is consistent with our molecular analysis, and the nrDNA ITS tree of Sun *et al*. (2008), *Pteroxygonum* is a distinct lineage, sister to *Polygonum* (=*Persicaria*).

On the basis of growth anatomy (Haraldson, 1978) and floral characters (Hong *et al*., 1998; Ronse-Decraene and Akeroyd, 1988), it has been hypothesized that *Calligonum* and in particular *Pteropyrum* is related to *Atraphaxis* L. However, neither pollen morphological data (Hong, 1995; Nowicke and Skvarla, 1979) nor our molecular phylogenetic analyses support these hypotheses. *rbc*L (Lamb Frye and Kron, 2003), *mat*K (Kim and Park, 2005), the cpDNA (*rbc*L-*mat*K-*ndh*F) and nrDNA ITS (Sanchez *et al*., 2009) and the present nrDNA ITS and *trn*L-F phylogenies show that *Atraphaxis* related to *Polygonum* (and *Polygonella*) as well as *Fallopia*. On the other hand, Hong (1995) and Hong *et al*. (1998) based on pollen morphology and tepal surface morphology suggested a close relationship between *Pteropyrum* and the monotypic genus *Parapteropyrum (P. tibeticum)*, endemic to the Xizang Plateau (Southeast Tibet) of China. Whereas, in our phylogenetic analyses and Sanchez *et al*.'s (2009) nrDNA tree, such relationship was not appeared. *Parapteropyrum tibeticum* is instead well allied with *Fagopyrum* (PP=100, BS=100).

In short, *Calligonum* and *Pteropyrum* have no a single relative genus, but as appeared in our *trn*L-F and the combined phylogenies as well as in Sanchez *et al*.'s (2009) ones, they are related with a clade of *Rumex*/*Polygonum*/*Atraphaxis*/ *Fallopia*.

Infrageneric relationships in *Calligonum* and *Pteropyrum*: As mentioned in the introduction, based on fruit morphology, *Calligonum* has been divided into three sections: *Calligonum* (with bristled fruit), *Pterococcus* (with winged fruit) and *Calliphysa* (with membranous saccate fruit) (Rechinger and Schiman-Czaika, 1968). Our molecular analyses of both nrDNA ITS and *trn*L-F did not resolve relationships among proposed sections of *Calligonum*. This is almost consistent with the combined *rbc*L and *mat*K phylogeny of Sanchez and Kron (2008, and http://users.wfu.edu/sanca5/Adriana_Sanchez/Adriana_Sanchez/*Calligonum*.html; but see Sanchez *et al*. 2009). Lacking the resolution among *Calligonum* taxa is due to very low nucleotide substitution in both nrDNA ITS and *trn*L-F sequences However, in the Bayesian tree of combined nrDNA ITS-*trnL-F* sequences, *Calligonum junceum* (sect. *Calliphysa*) and *C. persicum* (sect. *Pterococcus*) formed a weakly supported subclade (posterior probabilities (PP) = 0.55, see Fig. 3). Ren and Tao (2004), using randomly amplified polymorphic DNA (RAPD) analyses of 14

Chinese *Calligonum* species, showed that *C. junceum* is positioned far from the remaining species studied and species having bristled fruit (sect. *Calligonum*) were not grouped in a single cluster as were the winged fruit species (sect. *Pterococcus*). In contrast to *Pteropyrum*, *Calligonum*, as one of the big genera in Polygonaceae with approximately 80 species (Mabberely, 1990), represent a rapid diversification for a short time in hot and arid deserts of Western Central Asia. Its diversification may caused by several factors including C4 photosynthetic pathway, bristled/winged fruits a dispersal units and hybridization/introgression followed by tetraploidy (Sage, 2004; Pyankov *et al*., 2000; Aparicio, 1989; Mao *et al*., 1983; Pavlov 1970; Rechinger and Schiman-Czaika, 1968).

Pteropyrum aucheri and *P. olivierii* are morphologically very similar to each other except that in the former, the shape of the leaf is linear and in the latter spatulate (Rechinger and Schiman-Czeika, 1968). Both species are distributed in arid regions, but *P. aucheri* has penetrated more to desert areas (Mozaffarian, 2004, and personal observations). However, the present molecular phylogenies and morphological cladistic analyses (Tavakkoli *et al*., 2008) did not put them close to each other. Instead, *P. olivierii* is allied with *P. naufelum*, a newly described species distributed in Iraq (Al-Khayat, 1990) and Southwest Iran (Akhani, 2004).

CONCLUSION

The present molecular data provide strong support for the monophyly of *Calligonum* and *Pteropyrum* and their closest relationship. They have no affinity with *Atraphaxis*, instead allied with a clade in which, the genus is nested. However, infrageneric relationships in *Calligonum*, due to the paucity of informative nucleotide sites in both nrDNA ITS and *trn*L-F sequences and in other cpDNA genes such as *rbc*L and *mat*K (Sanchez and Kron, 2008), is not resolved. Fast evolving genic regions including non-coding cpDNA fragments and single copy nuclear DNAs are clearly required to resolve phylogenetic relationship among *Calligonum* species.

Acknowledgements

This work was supported by a research grant from the Tarbiat Modares University.

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