

Original article:

**COMPARISON OF FIVE *CALLIGONUM* SPECIES IN TARIM BASIN
BASED ON MORPHOLOGICAL AND MOLECULAR DATA**

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ABSTRACT

Five *Calligonum* species endemic to the Tarim Basin – *C. roborowskii*, *C. kuerlese*, *C. juoqiangense*, *C. yengisaricum*, and *C. taklimakanense* – were compared using 14 morphological characteristics and nrDNA ITS and cpDNA *trnL-F* molecular data. Intraspecific and interspecific morphological variation was uncovered, with variation between species significant for most characteristics. Based on Euclidean distances, *C. roborowskii* and *C. kuerlese* were the most similar, and *C. yengisaricum* and *C. kuerlese* were the most divergent. There was very little variation in the molecular sequence data: although several variable sites were present in ITS and *trnL-F* regions, none of them were parsimony informative. Results of morphological and molecular analyses suggest that the various morphological characteristics of the studied *Calligonum* species are strongly shaped by environmental factors, with the observed intraspecific and interspecific morphological variation a product of the spatial isolation and extreme drought of the Tarim Basin.

Keywords: *Calligonum*, fruit, morphology, *trnL-F*, ITS

INTRODUCTION

The genus *Calligonum* L., a member of the family Polygonaceae, is distributed throughout North Africa, southern Europe, and Asia. In China, *Calligonum* species occur mainly in Inner Mongolia, Gansu, Ningxia, and Xinjiang. *Calligonum* is one of the dominant genera in the Xinjiang desert flora (Mao 1998), with 22 species present in the region. Most species are sand-fixation plants used for animal feed and firewood.

Species identification in *Calligonum* is based primarily on fruit morphology and

anatomy (Mao and Pan, 1986), but these diagnostic characteristics are highly variable. As a consequence of variable fruit morphology and interspecific hybridization, polymorphism is common, and wildly-differing species concepts have been proposed (Mao, 1998).

The Tarim Basin is located in southern Xinjiang, northwest China, and encompasses an area of approximately 560,000 km² (Huang and Pang, 2010). Five *Calligonum* species, all belonging to sect. *Medusa*, are endemic to the region: *C. roborowskii* A. Los., *C. kuerlese* Z. M. Mao, *C.*

juoqiangense Liou f., *C. yengisaricum* Z. M. Mao, and *C. taklimakanense* B.R Pan et G.M.Shen (Losinkaja, 1927; Liu, 1985; Gulnur et al., 2010a). These species play an important protective role in the basin ecosystem. *Calligonum kuerlese*, *C. juoqiangense*, *C. yengisaricum*, and *C. taklimakanense* are restricted to limited portions of the Tarim Basin, while *C. roborowskii* is distributed throughout the entire basin and encompasses the distribution range of the other four species. The five *Calligonum* species are morphologically similar, differing from one another only in fruit size and bristle features on each fruit rib. Because of the morphological complexity of these five endemic *Calligonum* species and their overlapping or close distributional ranges, a comparative study of their morphological characteristics and molecular structure is needed. The ability to distinguish these species would aid elucidation of *Calligonum* taxonomy and contribute to our knowledge of species diversity in the Tarim Basin ecosystem.

The main objective of this study was the analysis of morphological and molecular differences between the five *Calligonum* species endemic to the Tarim Basin. This was accomplished by examining various morphological characteristics, both vegetative and fruit, and by using nrDNA ITS and cpDNA *trnL-F* molecular markers to analyze sequence variation between these species.

MATERIALS AND METHODS

Plant materials

To obtain a broadly representative sampling of the five endemic species, we col-

lected material such that sampled populations were separated by at least 20 km, and sampled plants were separated by at least 40 m. Based on these constraints and the limited distribution area of *C. juoqiangense*, *C. yengisaricum*, *C. kuerlese*, and *C. taklimakanense*, three populations from each species and three plants from each population of *C. roborowskii*, *C. juoqiangense*, *C. yengisaricum*, *C. kuerlese*, and *C. taklimakanense* were selected for morphological study. One plant was also sampled from each species for molecular study, with *C. junceum* (Fisch. et Mey.) Litv. added as an outgroup (Table 1). Voucher specimens were deposited in XJBI, and experiments were carried out at the Key Laboratory of Biogeography and Bioresource in Arid Land, Chinese Academy of Sciences.

For the morphological portion of this study, plant height, crown length, and crown width of each plant was measured with a steel tape measure in the field. Twenty fruits were randomly selected from each plant, and their features were measured in the laboratory with electronic digital calipers. Morphological characteristics used in the analysis were plant height, crown length, crown width, fruit length, fruit diameter, bristle length, distance between bristles, distance between ribs, fruit shape, fruit size, achene length, achene width, achene shape, and achene size. Fruit and achene size were calculated as length/width, while fruit and achene shape were obtained by multiplying length by width. Variability was calculated as $\text{Stdev}/\text{Mean} \times 100$.

Table 1: Plant materials for molecular study

Species	Collected site	GenBank accession number	
		trnL_F	ITS
<i>C. roborowskii</i>	Tarim basin, Xinjiang	JX259361	JX259387
<i>C. juoqiangense</i>	Qakilik County, Xinjiang	JX259363	JX259388
<i>C. yingisaricum</i>	Yingisar County, Xinjiang	JX259367	JX259391
<i>C. kuerlese</i>	Korla City, Xinjiang	JX259356	JX259383
<i>C. taklimakanense</i>	Taklimakan desert, Xinjiang	JX259365	JX259390
<i>C. Junceum</i>	Jiangjun gebi, Xinjiang	JX987215	JX987230
<i>A. spinosa</i>	—	AB542783	JN187102

Note: Sequences from GenBank

Methods

For molecular analyses, branchlets of the five endemic *Calligonum* species were collected in the field. Total genomic DNA was extracted using the modified CTAB method of Doyle and Doyle (1987). PCR amplification of the chloroplast *trnL-F* intergenic spacer region was performed using the primer pairs *trnL-FF* (5'-CGAAATCGGTAGACGCTACG-3') and *trnL-FR* (5'-GGGGATAGAGGGACTTGAAC-3') under the following reaction conditions: an initial hold at 95 °C for 2 min 30 s, followed by 38 cycles of 95 °C denaturation for 1 min, 53 °C annealing for 45 s, and 72 °C extension for 2 min, and a final extension step at 72 °C for 7 min. Amplification of the ITS region was carried out using the primers *ITS1F* (5'-AGAAGTCGTAACAAGGTTTCCGTAGC-3') and *ITS4R* (5'-TCCCTCCGCTTATTGATATGC-3') with reaction conditions as follows: an initial hold at 94 °C for 2 min, followed by 34 cycles of 94 °C denaturation for 30 s, 58 °C annealing for 45 s, and 72 °C extension for 1 min 30 s, and a final extension at 72 °C for 10 min. DNA sequencing reactions were carried out using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) in GenoLab, Beijing. The resulting products were purified and sequenced on an Applied Biosystems 3730XL DNA Analyzer. Both forward and reverse strands were sequenced.

Data processing

Morphological data were analyzed using Excel 2007 (Mean value, variability) and SPSS 19.0 (ANOVA, Cluster Analysis) software. DNA sequence data were edited, aligned, and analyzed using Chromas 1.5 and MEGA5 software. All sequences were submitted to GenBank and Sequence data for *Atraphaxis spinosa* was downloaded from GenBank (Table 1) and added for use as an outgroup, along with *C. junceum*, during phylogenetic tree reconstruction. All gaps were treated as missing data.

RESULTS

Morphological data analysis

The most valuable fruit characteristics for addressing the infrageneric taxonomy of *Calligonum* sect. *Medusa* are fruit size (a function of fruit length and width) and bristle features. Several other plant morphological characteristics and fruit features assessed here were also found to be useful. The mean value and intraspecific and interspecific variability of 14 morphological characteristics examined in the five *Calligonum* species are shown in Table 2. As indicated by our data, different *Calligonum* species had different mean values and intraspecific variability of morphological characteristics. Interspecific variability of the 14 morphological characteristics ranged from 15.26 to 58.17, with large interspecific variability for plant height, crown length, crown width, fruit size, and bristle length. Based on one-way ANOVA, differences between species were significant for all morphological characteristics except crown length, crown width, fruit shape, and achene size.

As shown in Table 3, Euclidean distances between *C. roborowskii* and *C. yengisaricum* (211.620), *C. roborowskii* and *C. taklimakanense* (212.782), *C. yengisaricum* and *C. kuerlese* (219.325), and *C. kuerlese* and *C. taklimakanense* (213.593) were very large, whereas the distance between *C. roborowskii* and *C. kuerlese* (12.129) was very small. This can also be seen in a dendrogram generated from cluster analysis (Figure 1).

Table 2: The mean value/intraspecific, interspecific variability and ANOVA of 14 morphological characters of five *Calligonum* species

Variability	Mean value/intraspecific variability					Inter-specific variability	F value
	<i>C. roborovskii</i>	<i>C. juoqiangense</i>	<i>C. yingisaricum</i>	<i>C. kuerlese</i>	<i>C. taklimakanense</i>		
plant height (cm)	109/35.92	69/21.26	58/19.79	120/36.32	188/12.67	49.20	9.053**
crown length (cm)	296/55.94	233/34.04	137/25.70	300/62.27	164/40.15	54.84	1.124
crown width (cm)	247/45.10	133/12.33	117/27.55	250/59.19	100/17.04	58.17	2.235
fruit length (cm)	1.45/22.05	1.25/13.08	1.29/8.85	1.61/12.26	1.85/15.51	21.19	34.417**
fruit diameter (cm)	1.33/27.12	1.07/18.16	1.14/8.74	1.44/20.04	1.77/19.47	27.32	30.078**
fruit shape	1.11/10.99	1.18/8.67	1.14/7.91	1.19/39.11	1.06/11.65	20.47	1.667
fruit size (cm ²)	2.02/46.94	1.37/32.70	1.47/15.61	2.35/29.92	3.34/36.18	50.06	30.620**
bristle length (cm)	0.51/37.71	0.34/27.27	0.41/15.67	0.59/16.35	0.81/22.55	39.69	53.723**
distance between bristles (cm)	0.14/17.07	0.13/22.54	0.17/15.64	0.16/19.52	0.14/17.96	20.88	11.370**
distances between ribs (cm)	0.17/18.01	0.17/20.45	0.20/15.69	0.21/14.94	0.18/26.02	20.59	6.804**
achene length (cm)	0.90/18.67	0.80/13.95	0.86/10.79	0.91/16.48	0.88/12.01	15.26	3.317*
achene diameter (cm)	0.39/13.82	0.39/23.41	0.38/10.61	0.33/25.83	0.35/16.68	19.39	4.063**
achene shape	2.34/23.17	2.14/14.18	2.31/13.46	2.84/16.84	2.57/21.98	20.90	10.736**
achene size (cm ²)	0.35/23.89	0.32/37.03	0.33/16.17	0.31/39.33	0.31/21.04	28.70	1.154

* Significant level $P < 0.05$, ** significant level $P < 0.01$ (2-tailed)

Table 3: Euclidean distance matrix for five *Calligonum* species inferred from 14 morphological characteristics

Species	<i>C. roborovskii</i>	<i>C. juoqiangense</i>	<i>C. yingisaricum</i>	<i>C. kuerlese</i>	<i>C. taklimakanense</i>
<i>C. roborovskii</i>	0.000				
<i>C. juoqiangense</i>	136.258	0.000			
<i>C. yingisaricum</i>	211.620	97.944	0.000		
<i>C. kuerlese</i>	12.129	144.155	219.325	0.000	
<i>C. taklimakanense</i>	212.782	141.481	133.878	213.593	0.000

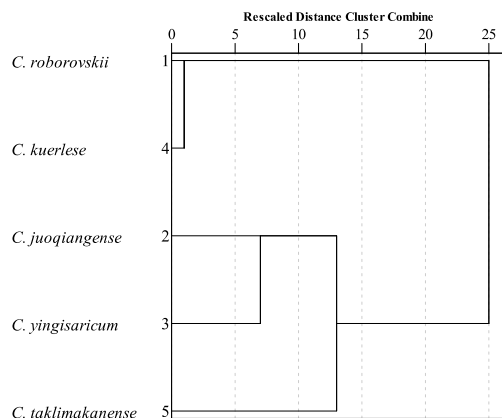


Figure 1: Dendrogram from cluster analysis of five *Calligonum* species based on 14 morphological characteristics

Molecular data analysis

DNA was extracted from six samples, one for each species, resulting in good yield (35–50 ng/ μ L) of high quality DNA. Most of the samples showed good PCR amplification. ITS amplification failed for a few samples, requiring the reactions to be repeated. All PCR products corresponding to the two DNA regions were successfully sequenced, resulting in the generation of high quality bidirectional sequences.

The resulting *trnL-F* and ITS datasets comprised 537 and 627 bp, respectively, and showed very little sequence variation. Although there were a few variable sites – nine in the *trnL-F* and one in the ITS region – none were parsimony informative (Table 4). Pairwise distances among the five *Calligonum* species based on *trnL-F* and ITS sequence data were all less than 0.015.

A phylogenetic tree generated using maximum likelihood (ML) and based on the *trnL-F* dataset (Figure 2) indicates that *C. taklimakanense* is sister to an unresolved clade consisting of *C. junceum*, *C. kuerlese*, *C. juoqiangense*, *C. roborovskii*, and *C. yingisaricum*, the bootstrap support for that clade is 85 %. In contrast, the ITS tree (Figure 3) strongly supports (92 % bootstrap value) the five Tarim Basin *Calligonum* species as a distinct clade sister to *C. junceum*. ML analysis of a combined *trnL-F*-ITS dataset recovered the topology shown in Figure 4, in which the Tarim Basin clade is still well-supported (86 % bootstrap value) as a sister clade to *C. junceum*. Within the Tarim Basin clade, *C. roborovskii* and *C. taklimakanense* are in a sister relationship.

Table 4: *trnL-F* and ITS sequence variation among five *Calligonum* species

Species	<i>trnL-F</i> sequences variable site									ITS sequences variable site
	6	7	8	9	10	11	42	208	454	
<i>C. roborovskii</i>	A	G	T	G	A	G	A	T	T	A
<i>C. juoqiangense</i>	A	G	T	G	A	G	A	T	C	A
<i>C. yingisaricum</i>	A	G	T	G	A	G	C	T	C	A
<i>C. kuerlese</i>	A	G	T	G	A	G	A	T	C	A
<i>C. taklimakanense</i>	G	T	G	A	G	A	A	G	C	G

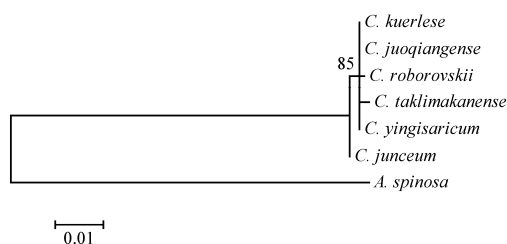


Figure 2: ML tree inferred from *trnL-F* sequence data (using the Kimura two-parameter [K2P] model of evolution and 1000 bootstrap replicates)

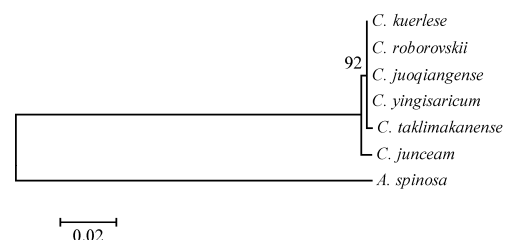


Figure 3: ML tree inferred from ITS sequence data (K2P model; 1000 bootstrap replicates)

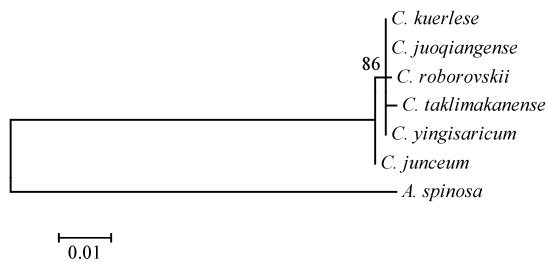


Figure 4: ML tree inferred from combined *trnL-F* and ITS sequence data (K2P model; 1000 bootstrap replicates)

DISCUSSION

Morphological characteristics are the outward manifestation of an organism. Morphological variation between species is often used to resolve taxonomic problems. *Calligonum* fruit morphology has been an important subject of taxonomic studies in the genus. In a previous study, intraspecific and interspecific morphological variation in fruit characteristics of some *Calligonum* species was studied, and the variation was attributed to genetic variability and microscale adaptation to different habitat factors, including geographical distribution, soil characteristics, and climate (Shi et al., 2009, 2011; Gulnur and Pan, 2008; Gulnur et al., 2010b; Tao and Ren, 2004; Tao et al., 2001). The morphological analysis presented here indicates that intraspecific and interspecific morphological variation exists in Tarim Basin *Calligonum* species, and that most examined morphological characteristics differ significantly between species. The morphological fruit plasticity of *Calligonum* may be an adaptive response that favors a plant's fitness over time.

Although morphological characteristics in plant species are affected by both genetic and environmental factors, sometimes genetic factors are not able to play a significant role. For example, Li et al. (2004) examined the relationship between morphological and genetic variation in *Dipteronia dyeriana* and discovered that while morphological variation in this species was intensely influenced by environmental fac-

tors, there was no significant correlation with population genetic background.

The ITS and *trnL-F* molecular data in this study showed very little sequence variation, and there were not enough parsimony informative sites to resolve relationships among the Tarim Basin *Calligonum* species; this is generally consistent with the results of Tavakkoli et al. (2010) and Sanchez et al. (2009). We also screened other DNA regions, such as *rbcL*, *matK*, *accD*, *atpB-rbcL*, *atpF-H*, *psbA-trnH*, *psbK-I*, *rpoB*, *rpoC1*, and *psaA*, for possible inclusion in our study of *Calligonum* taxonomy; there was insufficient variation in these regions and the data is not presented here.

Plant species can adjust to environment changes by exploiting phenotypic plasticity, adapting through natural selection, or migrating to follow conditions to which they are adapted (Nicotra et al., 2010). Environmental factors in a given habitat can play an important role in determining morphological characteristics in plant species. For example, *Echium plantagineum* showed significant differences in plant height, seed size, and seed weight in different habitats and under different soil conditions; in addition, through competition and other indirect effects, the presence of other species in natural habitats appeared to trigger a plastic response in *E. plantagineum* by influencing its growth performance (Sharma and Esler, 2008). Furthermore, spatial isolation of populations or species can lead to intraspecific or interspecific morphological variation.

The morphological and molecular results of this study suggest that morphological characteristics of *Calligonum* in the Tarim Basin may be primarily determined by environmental factors such as the harsh climate. Further phylogeographic research and complete genomic studies, however, are needed to confirm this hypothesis.

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