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A phylogeny of *Calligonum* L. (Polygonaceae) yields challenges to current taxonomic classifications

Pei-Liang Liu[1], Wei Shi[2, 3, 4*], Jun Wen[4], Shomurodov Khabibullo Fayzullaevich[5] and Borong Pan[2, 3]

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ABSTRACT

Calligonum is the only C_4 genus within Polygonaceae. We applied DNA sequences from the nuclear ribosomal internal transcribed spacer (nrITS) and five plastid genome regions (*psbA-trnH*, *ycf6-psbM*, *trnL-F*, *rpl32-trnL* and *rbcL*) to reconstruct the phylogeny of *Calligonum*. The nrITS and the combined plastid DNA regions were analysed separately. The phylogeny of the five plastid genome regions supports the treatment of the *Calligonum mongolicum* complex as a single species with intra-specific geographic structure, and suggests independent hybrid origins for the polyploid species *C. caput-medusae* and *C. arborescens* through comparisons with the nrITS tree. We detected phylogenetic incongruence between the nrITS and plastid DNA trees and hypothesized reticulate evolution or hybrid speciation in the genus. Divergence time dating based on nrITS determined that the most recent common ancestor of *Calligonum* species began diversification 3.46 million years ago [mya; 95 % high probability density (HPD): 1.87-5.71 mya], and diversification began in the Central Asia and China clade ca. 2.68 mya (95 % HPD: 1.28-4.59 mya). We expect that future studies employing next generation sequencing methods, such as RAD-seq, coupled with denser inter- and intra- specific taxonomic sampling, may prove to be cost-effective methods for further investigation of the evolutionary history of this genus.

Keywords: desert plant, ITS, plastid sequence, Central Asia, North Africa

Introduction

The genus *Calligonum* (Polygonaceae) has long been of interest to botanists due to the unique fruit morphology characterizing each of its four sections (Losinskaja 1927; Komarov 1970; Soskov 1975a; Tao & Ren 2004; Gulinuer 2008; Kang *et al.* 2008; Shi *et al.* 2009; 2011; Kong *et al.* 2016) and problematic delimitation of its species (Li *et al.* 2014; Gouja *et al.* 2015) that may result from hybrid speciation and reticulate evolution (Burke *et al.* 2010; Dhief *et al.* 2011; Soskov 2011; Gouja *et al.* 2014; Li *et al.* 2014). There are at least 161 accepted species names ascribed to *Calligonum*, but, of these, only ca. 40 to 85 may represent entities meriting species status (Sanchez *et al.* 2011; Soskov 2011). In several prior studies, plastid and nuclear DNA sequences have been used for phylogenetic reconstructions to aid in species delimitation in this genus (Shi *et al.* 2009; 2013; 2016; 2017; 2019) as well as to infer its position within Polygonaceae (Zhou *et al.* 2003; Sanchez *et al.* 2009; 2011; Soskov 2011; Sun & Zhang 2012; Schuster *et al.* 2013;) and reconstruct the biogeographic history of its taxonomic sections (Wen *et al.* 2015; 2016a; b). Nevertheless, there remains a lack of DNA data for elucidating the mechanisms

1 College of Life Sciences, Northwest University, 710069, Xi'an, Shaanxi,China
2 State Key Laboratory of Desert and Oasis Ecology, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, 830011, Urumqi, Xinjiang, China
3 Turpan Eremophytes Botanic Garden, Chinese Academy of Sciences, 838008, Turpan, China
4 Department of Botany, National Museum of Natural History, Smithsonian Institution,20013-7012, Washington, DC, USA
5 Laboratory of Geobotany, Institute of Botany, Academy of Sciences of the Republic of Uzbekistan, 100125, Tashkent, Uzbekistan

* Corresponding author: water5116@163.com

that have contributed to taxonomic complexities in *Calligonum*, especially to determine the possible roles of reticulate evolution and hybrid speciation in its evolutionary history.

The complex taxonomical and evolutionary history of *Calligonum* is reflected in its fruit morphology (Bao & Grabovskaya-Borodina 2003; Shi *et al.* 2009; Feng *et al.* 2010; Soskov 2011; Shi *et al.* 2016). Fruit morphology represents the primary basis for delimiting the four sections of *Calligonum* (Bao & Grabovskaya-Borodina 2003): Sect. *Calliphysa*, which has membranous-saccate fruits, Sect. *Pterococcus*, which possesses winged fruits, Sect. *Calligonum*, which has non-membranous fruits with both wings and seta, and Sect. *Medusa*, which exhibits seta, but is neither winged nor membranous.

Calligonum rubicundum (a member in Sect. *Pterococcus*) has a complex fruit morphology and can be tetraploid or hexaploid within a narrow distribution (Kong *et al.* 2016), which also caused their taxomonical challenges in the past (Soskov 1975a; Bao & Grabovskaya-Borodina 2003; Soskov 2011). Thus, the karyotypes also gave the evidences in its complex biosystematics (Soskov 1975b; Wang & Yang 1985; Wang & Guan 1986; Shi *et al.* 2009; Shi & Pan 2015). Polyploidy in *Calligonum* is also likely to have arisen independently multiple times, such as in *C. caput-medusae* ($2n = 6x = 54$) and *C. arborescens* ($2n = 4x = 36$) of Sect. *Medusa* (Wang & Yang 1985; Wang & Guan 1986; Sabirhazi & Pan 2009; Shi & Pan 2015). Moreover, several species exhibit intraspecific karyotypic variation, such as in *C. mongolicum* of Sect. *Medusa*. This species possesses two karyotypes with chromosome numbers $2n = 2x = 18$ and $2n = 3x = 27$ that can occur simultaneously within populations (Shi & Pan 2015). *C. mongolicum* also has heterogeneous phenotypes that have led to erecting several additional species or subspecific ranks to try to accommodate its diversity (Shi *et al.* 2016; 2017) yielding a *C. mongolicum* complex (CM complex, hereafter). The CM complex consists of *C. mongolicum* and six additional putative species: *C. pumilum*, *C. gobicum*, *C. chinense*, *C. alashanicum*, *C. zaidamense* and *C. roborowskii*. Throughout *Calligonum*, the complexity of karyotypes within and among species and frequency of polyploidy shows strong support for reticulate or hybrid evolutionary processes (Wang & Yang 1985; Wang & Guan 1986; Shi & Pan 2015). Within Sections various chromosome numbers have been reported, every section including diploid, triploid, tetraploid, and hexaploid species meanwhile. All of the above biosystematics factors in *Calligonum* lead its complex and challenges in its current taxonomic classifications.

The occurrence of natural hybridization in *Calligonum* has been proposed based on artificial hybridization experiments (Tavakkoli *et al.* 2008; Soskov 2011; Shi *et al.* 2017) and seems likely according to observations of morphology and the frequency of polyploid species. However, hybrid speciation and reticulate evolution have not yet been effectively demonstrated within a phylogenetic framework using DNA data, such as based on incongruence between plastid and nuclear datasets (Mallet 2007; Soltis & Soltis 2009; Bartha *et al.* 2013; Gambette *et al.* 2016). Hybrid speciation and polyploidy as well as ancient and ongoing reticulation may have facilitated adaptation of species of *Calligonum* to heterogeneous environmental patches over large geographic ranges (Pyankov *et al.* 2000; Su & Yan 2006) and simultaneously resulted in high rates of morphological heterogeneity, which can confound traditional taxonomic approaches. Therefore, using molecular phylogeny to elucidate cases of hybrid speciation and reticulate evolution may help to delimit species of *Calligonum* as well as provide new insights into the taxonomical relationships and biosystematics among them.

In this study, we reconstructed phylogenies of *Calligonum* independently from sequences of nuclear nrITS and five combined plastid regions (*psbA-trnH*, *ycf6-psbM*, *trnL-F*, *rpl32-trnL* and *rbcL*). Our primary objectives were to (1) determine relationships among species and (2) infer species boundaries using the phylogenies. Additionally, we sought to (3) detect cases of hybrid speciation and reticulate evolution in *Calligonum* based on incongruence between the nrITS and plastid phylogenies. We also estimated divergence times in *Calligonum* providing a time scale for the evolutionary history of *Calligonum*. We believe that our study sheds new light on the evolutionary history *Calligonum* as well as supports future taxonomic revision in the genus.

Materials and methods

Species identification and sampling

We collected samples from the shoots of individuals in *Calligonum* mostly in the field from the northwest China including five provinces (Xinjiang, Qinghai, Inter Mongolia, Gansu, and Ningxia) during summers from 2006 to 2015 (Fig. 1). We obtained several additional samples from germplasm resources maintained in the Turpan Eremophytes Botanic Garden, Chinese Academy of Sciences and from herbarium specimens. Information of all the samplings for this study was shown and cited in Table 1. The data generated in our previous study (Shi *et al.* 2019) were also incorporated in the present analyses and the information of samplings can be found therein. We also expanded our sampling by downloading available DNA sequences from GenBank, in which the samples in North Africa have been labeled in the Fig. 1, and the accession numbers of the sequences used in this study also can be found in Table 1. We included representative species of *Pteroxygonum* Dammer & Diels and *Pteropyrum* Jaub. & Spach in our sampling as outgroups based on prior molecular phylogenetic studies (Sun *et al.* 2008; Schuster *et al.* 2011; Schuster *et al.* 2013).

Species of *Calligonum* can be readily assigned to one of four sections according to Mao (1992) and Bao & Grabovskaya-Borodina (2003) based on fruit characteristics, namely: length of fruits, width of fruits, the length of setae or wings, the space between setae or wings, the space between ribs, the length of achenes, the width of achenes, and the number of rows of bristles on each rib of achenes. We used these characteristics as well as geography to identify species. However, species within species complexes are challenging to be identified non-subjectively using morphology. Therefore, we treated the CM complex as well as a complex of *C. rubicundum* (CR complex, hereafter) each as single species, which we have labeled throughout the study according to the geographic origins of individual samples (see Tab. 1 and Shi *et al.* 2019).

Molecular protocols

We extracted total genomic DNA of all samples from fresh or silica gel dried leaves following the protocol of the protocols of Doyle & Doyle (1990) and Doyle *et al.* (2004). We amplified nrITS regions using "ITS5a" and "ITS4" primers (Stanford *et al.* 2000; Alvarez & Wendel 2003), and we amplified *psbA- trnH*, *ycf6-psbM*, *rpl32-trnL*, *trn*L-F and *rbcL* using primers based on several prior studies (Demesure *et al.* 1995; Small *et al.* 1998; Shaw *et al.* 2005; 2007; Falchi *et al.* 2009). We selected the plastid DNA regions *ycf*6-*psbM*, *rpl*32-*trnL*, and *rbcL* because they are known to be variable within *Calligonum* (Gouja *et al.* 2014; 2015), but this study represents the first time that all five markers have been combined to reconstruct phylogenetic relationships in the genus. Amplification of all DNA markers was via standard PCR using 10 ng of genomic DNA, 200 μM of each dNTP, 4 pmol of each primer, 0.5 U Taq polymerase (Bioline, Randolph, MA, USA), and 2.5 mM $MgCl_2$ in a volume of 25 μL. We performed PCR using a PTC-225 Peltier thermal cycler with cycling parameters as follows: a 95 °C enzyme activation for 5 min, 32 cycles of 94 °C for 30 s, primer specific annealing temperatures and durations (ITS: 55°C for 60s, five plastid primers: 53 °C for 40 s), and 72 °C for 60 s with a final extension of 72 °C for 10 min. We purified the PCR products with EXO-SapIT (US Biological, Swampscott, MA, USA) or a PCR Product Purification kit (Shanghai SBS, Biotech Ltd., China). We carried out cycle sequencing using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) with 5 ng of primer, 1.5 μL of sequencing dilution buffer and 1 μL of cycle sequencing mix in a 10 μL reaction volume. Cycle sequencing conditions were as follows: 30 cycles of 30s denaturation (96 °C), 30s annealing (50 °C) and 4min elongation (60 °C). 10 μL of the sequencing products were separated on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). Alternatively, for some samples, we used a DYEnamic ET Terminator Kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K.) for sequencing on an ABIPRISM 3730 automatic DNA sequencer (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China). In all cases, we sequenced forward and reverse DNA strands to help ensure the reliability of base calls.

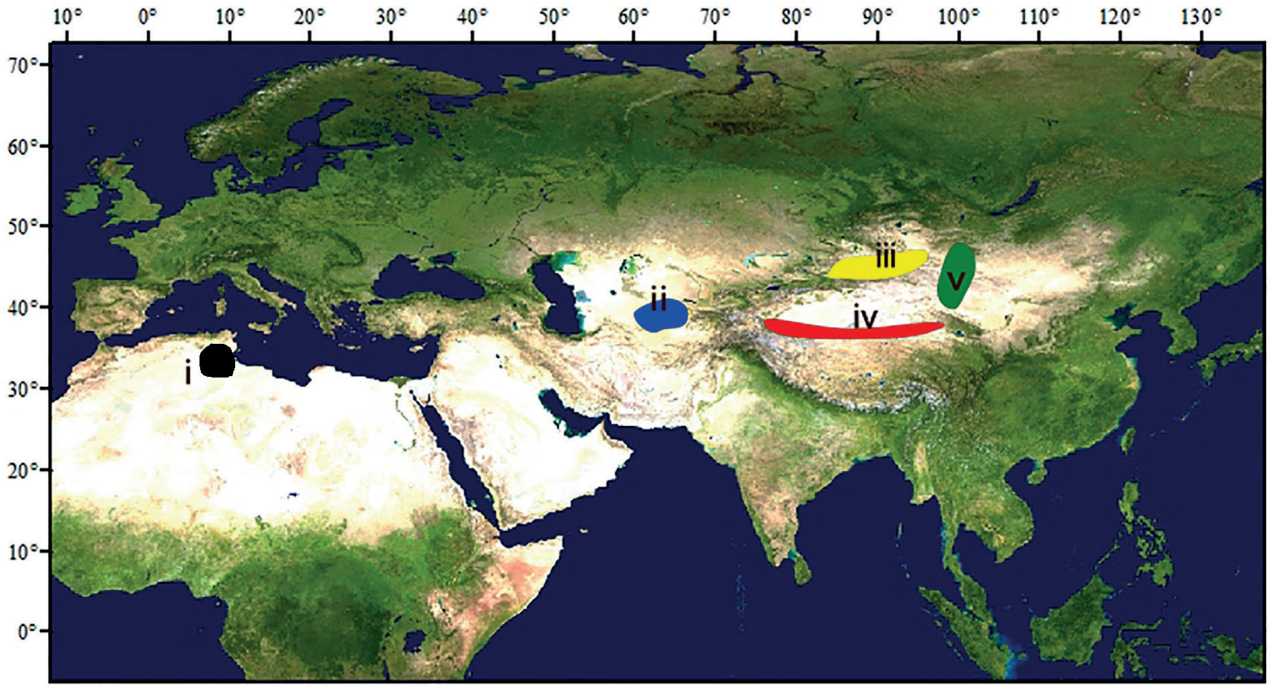

Figure 1. Map of the distribution of the *Calligonum* samples: (**i**) the samples in the Sahara desert; (**ii**) the samples in Kyzylkum Desert; (**iii**) the samples in Gurbantunggut desert; (**iv**) the samples in the other deserts of China; (**v**) the samples in the Taklimakan desert.

Table 1. Collection information and GenBank accession numbers of the samplings used in this study. Asterisks (*) indicate new sequences generated in this study. Dashes (-) indicate missing data.

Calligonum Sect. / Outgroups	Species	Location	Latitude	Longitude	Elevation	GenBank accession number						Voucher Num.
						ITS	*psbA-trnH*	*trnL–F*	*ycf6-psbM*	*rpl32-trnL*	*rbcL*	
Sect. *Medusa*	*C. mongolicum*	Erjinaqi, Inter Mongolia	N41°27'	E100°26'	1002m	KU050846 KU050848	MN449309 MN449310 MN449311 MN449312	MN449258 MN449259 MN449260 MN449261	MN449070 MN449071 MN449072 MN449073	MN449121 MN449122 MN449123 MN449124	MN449172 MN449173 MN449174 MN449175	C1101-C1110
		Hulishan, Inter Mongolia	N41°58'	E100°35'	899m	MN449220 MN449221	MN449313 MN449314	MN449262 MN449263	MN449074 MN449075	MN449125 MN449126	MN449176 MN449177	C1111-C1120
		Qingtongxia, Ninxia	N38°01'	E105°55'	1134m	KU050847 KU050853	MN449315 MN449316	MN449264 MN449265	MN449076 MN449077	MN449127 MN449128	MN449178 MN449179	C1121-C1130
		Mazongshan, Gansu	N41°48'	E098°42'	1364m	MN449222 MN449223 -	MN449317 MN449318 MN449319	MN449266 MN449267 MN449268	MN449078 MN449079 MN449080	MN449129 MN449130 MN449131	MN449180 MN449181 MN449182	C1145-C1154
		Liuyuan, Gansu	N43°20'	E091°23'	1273m	KU050844 KU050845	MN449320 MN449321	MN449269 MN449270	- MN449081	MN449132 MN449133	MN449183 MN449184	C1166-C1175
		Kelamayi, Xinjiang	N47°19'	E086°46'	574m	MN449224 MN449225	MN449322 MN449323	MN449271 MN449272	MN449082 MN449083	MN449134 MN449135	MN449185 MN449186	C2101-C2110
		Wuerhe, Xinjiang	N46°08'	E086°12'	415m	KU050849 KU050850	MN449324 MN449325	MN449273 MN449274	MN449084 MN449085	MN449136 MN449137	MN449187 MN449188	C2133-C2142
		Xinxinxia, Xinjiang	N42°45'	E095°28'	1744m	MN449226 MN449227 MN449228 MN449229	MN449326 MN449327 MN449328 MN449329	MN449275 MN449276 MN449277 MN449278	MN449086 MN449087 MN449088 MN449089	MN449138 MN449139 MN449140 MN449141	MN449189 MN449190 MN449191 MN449192	C2165-C2174
		Qijiaojing, Xinjiang	N43°35'	E091°25'	1142m	KU050852 KU050841	MN449330 MN449331	MN449279 MN449280	MN449090 MN449091	MN449142 MN449143	MN449193 MN449194	C2175-C2184
		Hami1, Xinjiang	N43°23'	E091°32	1038m	- KU050843 -	MN449290 MN449291 MN449292	MN449239 MN449240 MN449241	MN449051 MN449052 MN449053	MN449102 MN449103 MN449104	MN449153 MN449154 MN449155	C2011-C2020
		Hami2, Xinjiang	N42°44'	E093°55'	812m	MN449205 MN449206	MN449293 MN449294	MN449242 MN449243	MN449054 MN449055	MN449105 MN449106	MN449156 MN449157	C2178-C2186
		Tashan, Xinjiang	N45°01'	E090°03'	1018m	MN449207 MN449208 MN449209	MN449295 MN449296 MN449297	MN449244 MN449245 MN449246	MN449056 MN449057 MN449058	MN449107 MN449108 MN449109	MN449158 MN449159 MN449160	C2274-C2283
		Chaidamu, Qinhai	N39°09'	E089°47'	1680m	MN449210 -	MN449298 MN449299	MN449247 MN449248	MN449059 MN449060	MN449110 MN449111	MN449161 MN449162	C0121-C0130
		Kumishi, Xinjiang	N42°14'	E088°13'	919m	MN449211 MN449212 MN449213	MN449300 MN449301 MN449302	MN449249 MN449250 MN449251	MN449061 MN449062 MN449063	MN449112 MN449113 MN449114	MN449163 MN449164 MN449165	C0152-C0161
		Heshuo, Xinjiang	N42°16'	E082°59'	1105m	MN449214	MN449303	MN449252	MN449064	MN449115	MN449166	C0122-C0131
		Mingfeng, Xinjiang	N36°45'	E082°59'	1600m	MN449215 MN449216 MN449217	MN449304 MN449305 MN449306	MN449253 MN449254 MN449255	MN449065 MN449066 MN449067	MN449116 MN449117 MN449118	MN449167 MN449168 MN449169	C0174-C0184
		Yutian, Xinjiang	N36°45'	E082°02'	1648m	MN449218 MN449219	MN449307 MN449308	MN449256 MN449257	MN449068 MN449069	MN449119 MN449120	MN449170 MN449171	C0147-C0158
	C. roborowskii	Kumishi, Xinjiang	N43°14›	E087°53'	1001m	MZ303080* MZ303081*	MZ303124* MZ303125*	MZ303240* MZ303241*	MZ303281* MZ303282*	MZ303199* MZ303200*	-	C0012
		Luntai, Xinjiang	N41°49'	E083°55'	1019 m	MZ303082* MZ303083* MZ303084*	MZ303126* MZ303127* MZ303128*	MZ303242* MZ303243* MZ303244*	MZ303283* MZ303284* MZ303285*	MZ303201* MZ303202* MZ303203*	-	C0021-C0022
		Rouqiang, Xinjiang	N39°04'	E088°21'	866 m	MZ303085*	MZ303129*	MZ303245*	MZ303286*	MZ303204*	-	C0035, C0037
		Rouqiang, Xinjiang	N39°00'	E088°21'	869 m	MZ303086*	MZ303130*	MZ303246*	MZ303287*	MZ303205*	-	C0048-C0049

Table 1. Cont.

Calligonum Sect. / Outgroups	Species	Location	Latitude	Longitude	Elevation	GenBank accession number						Voucher Num.
						ITS	*psbA-trnH*	*trnL–F*	*ycf6-psbM*	*rpl32-trnL*	*rbcL*	
Sect. *Medusa*	*C. ebinuricum*	Jinhe, Xinjiang	N44°38'	E083°11'	370m	MN449236 MN449237 MN449238	MN449336 MN449337 -	- MN449285 MN449286	MN449096 MN449097 MN449098	MN449148 MN449149 -	MN449199 MN449200 MN449201	C1158-C1167
	C. caput-medusae	Tulufan, Xinjiang	N42°51'	E089°55'	23m	MZ303071*	MZ303115*	MZ303231*	MZ303272*	MZ303190*	MZ303156*	C0044
		-	-	-	-	JN187106	-	-	-	-	-	-
	C. arborecens	Huocheng, Xinjiang	N44°05'	E080°29'	639m	MN449230 MN449231	MN449332 MN449333	MN449281 MN449282	MN449092 MN449093	MN449144 MN449145	MN449195 MN449196	C2322-C2323
	C. polygonoides	Hoshat, Egypt	-	-	-	MZ303079*	-	-	-	-	-	MO4608487
		-	-	-	-	AB542779	-	AB542790	-	-	MK097159	-
	C. arich	ElBorma, Tunisia	N31°39'	E09°28'	-	KC585438 KC585439 KC585440	-	KC585502 KC585503 KC585504	-	-	KC585470 KC585471 KC585472	-
		Jbil, Tunisia	N32°59'	E09°00'	-	KC585441 KC585442 KC585443	-	KC585505 KC585506 KC585507	-	-	KC585473 KC585474 KC585475	-
		Kamour, Tunisia	N32°34'	E09°28'	-	KC585433 KC585434	-	KC585497 KC585498	-	-		-
	C. azel	ElBorma, Tunisia	N31°39'	E09°28'	-		-		-	-		-
		Tiert, Tunisia	N30°47'	E10°17'	-		-		-	-		-
	C. comosum	Iran	-	-	-	AB542778	-	-	-	-	-	-
		Douz,Tunisia	N33°14'	E09°20'	-	KC585419	-	KC585483	-	-	KC585451	-
		ElBorma, Tunisia	N31°39'	E09°28'	-	KC585428 KC585429 KC585430	-		-	-		-
		ElOuaaraa Tunisia	N32°40'	E10°36'	-		-		-	-		-
		Jbil, Tunisia	N32°59'	E09°00'	-		-		-	-		-
		Kamour, Tunisia	N32°34'	E09°28'	-		-		-	-		-
		Tiert, Tunisia	N30°47'	E10°17'	-		-		-	-		-
	C. korlaense	-	-	-	-		-		-	-		-
	C. juochiangense	-	-	-	-	JX259388 JX259389	-	JX259362 JX259363	-	-	JX259333 JX259334	-
	C. microcarpum	-	-	-	-	GQ206244	-	-	-	-	-	-
	C. crinitum	-	-	-	-	AB542776	-	AB542787	-	-	KX015751	-
	C. eriopodum	-	-	-	-	GQ206242	-	-	-	-	GQ206216	-
	C. molle	-	-	-	-	GQ206245	-	-	-	-	GQ206219	-
	C. taklimakanense	-	-	-	-	JX259390	-	JX259365	KP985630	KP985587	JX259336	-
	C. trifarium	-	-	-	-	JQ731666 JQ731667	-	JX987219	KP985631	KP985588	JQ731661	-
	C. yengisaricum	-	-	-	-	JX259391	-	JX259366	KP985633	KP985590	JX259338	-
Sect. *Calligonum*	*C. colubrinum*	Qitai, Xinjiang	N44°34'	E089°59'	521m	-	MZ303116*	MZ303232*	MZ303273*	MZ303191*	MZ303157*	C2002
	C. densum	-	-	-	-	-	JQ009235	JQ009291	-	-	JQ009273	-

Table 1. Cont.

Calligonum Sect. / Outgroups	Species	Location	Latitude	Longitude	Elevation	GenBank accession number						Voucher Num.
						ITS	*psbA-trnH*	*trnL–F*	*ycf6-psbM*	*rpl32-trnL*	*rbcL*	
Sect. *Calligonum*	*C. klementzii*	Qitai, Xinjiang	N44°14'	E090°9'	751m	MZ303072* MZ303073* MZ303074* MZ303075* MZ303076* MZ303077* MZ303078*	MZ303117*	MZ303233*	MZ303274*	MZ303192*	MZ303158*	C2224
	C. squarrosum	Qitai, Xinjiang	N44°18'	E090°6'	736m	MZ303093* MZ303094* MZ303095* MZ303096* MZ303097* MZ303098* MZ303099* MZ303100* MZ303101*	MZ303139* MZ303140* MZ303141* MZ303142*	MZ303255* MZ303256* MZ303257* MZ303258*	MZ303296* MZ303297* MZ303298* MZ303299*	MZ303214* MZ303215* MZ303216* MZ303217*	MZ303173* MZ303174* MZ303175* MZ303176*	C2212-C2215
Sect. *Pterococcus*	*C. aphyllum*	-	-	-	-	-	JQ009234	JQ009290	KP636666	KP636655	JQ009272	-
	C. bungei	-	-	-	-	AB542775	-	AB542786	-	-	-	-
	C. persicum	-	-	-	-	AB542777	-	AB542788	-	-	-	-
	C. rubicundum	Habahe, Xinjiang	N47°44'	E086°02°	607m	MZ303089* MZ303090* MZ303091* MZ303092*	MZ303135* MZ303136* MZ303137* MZ303138*	MZ303251* MZ303252* MZ303253* MZ303254*	MZ303292* MZ303293* MZ303294* MZ303295*	MZ303210* MZ303211* MZ303212* MZ303213*	MZ303169* MZ303170* MZ303171* MZ303172*	C2275-C2278
		Buerjin, Xinjiang	N47°45'	E086°48'	516 m	MZ303088*	MZ303133* MZ303134*	MZ303249* MZ303250*	MZ303290* MZ303291*	MZ303208* MZ303209*	MZ303167* MZ303168*	C2105-C2107
		Altay, Xinjiang	N47°34'	E087°56'	567 m	MZ303087*	MZ303131* MZ303132*	MZ303247* MZ303248*	MZ303288* MZ303289*	MZ303206* MZ303207*	MZ303165* MZ303166*	C2116-C2118
	C. leucocladum	Xiaoguai, Xinjiang	N45°17'	E085°02'	270	-	MZ303120* MZ303121* MZ303122* MZ303123*	MZ303236* MZ303237* MZ303238* MZ303239*	MZ303277* MZ303278* MZ303279* MZ303280*	MZ303195* MZ303196* MZ303197* MZ303198*	MZ303161* MZ303162* MZ303163* MZ303164*	C2160-C2163
		Jinhe, Xinjiang	N44°33'	E082°39'	295m	-	MZ303118* MZ303119*	MZ303234* MZ303235*	MZ303275* MZ303276*	MZ303193* MZ303194*	MZ303159* MZ303160*	C2152-C2154
Sect. *Calliphysa*	*C. calliphysa*	Mulei, Xinjiang	N44°36'	E090°40'	574m	MZ303067* MZ303068* MZ303069*	MZ303106* MZ303107* MZ303108* MZ303109*	MZ303222* MZ303223* MZ303224* MZ303225*	MZ303263* MZ303264* MZ303265* MZ303266*	MZ303181* MZ303182* MZ303183* MZ303184*	MZ303147* MZ303148* MZ303149* MZ303150*	C0112-C0121
		Qitai, Xinjiang	N44°59'	E089°58'	540m	MZ303070*	MZ303110* MZ303111*	MZ303226* MZ303227*	MZ303267* MZ303268*	MZ303185* MZ303186*	MZ303151* MZ303152*	C2301-C2304
		Beitashan, Xinjiang	N45°02'	E090°04'	1075m	MZ303064* MZ303065* MZ303066*	MZ303102* MZ303103* MZ303104*	MZ303218* MZ303219* MZ303220*	MZ303259* MZ303260* MZ303261*	MZ303177* MZ303178* MZ303179*	MZ303143* MZ303144* MZ303145*	C2184-C2186
		Wusu, Xinjiang	N44°24'	E084°38'	755m	-	MZ303112* MZ303113* MZ303114*	MZ303228* MZ303229* MZ303230*	MZ303269* MZ303270* MZ303271*	MZ303187* MZ303188* MZ303189*	MZ303153* MZ303154* MZ303155*	C2189-C2190
		Jinhe, Xinjiang	N44°41'	E082°54.8'	514m	-	MZ303105*	MZ303221*	MZ303262*	MZ303180*	MZ303146*	C2198-C2199
Outgroups	*Pteropyrum aucheri*	-	-	-	-	AB542780	JQ009241	AB542791	-	-	GQ206227	-
	Pteropyrum naufelum	-	-	-	-	AB542781	-	AB542792	-	-	-	-
	Pteropyrum olivierii	-	-	-	-	AB542782	-	AB542793	-	-	GQ206228	-
	Pteroxygonum giraldii	Ningshan, Shaanxi	N 33°49'	E108°40'	1501m	MN449235	MN449340	MN449289	MN449101	MN449152	MN449204	P. L. Liu 431

We assembled and curated the raw DNA sequencing results in Sequencher 4.5 (GeneCodes, Ann Arbor, MI, USA) and submitted all new sequences to GenBank (Tab. 1). We conducted multiple sequence alignments for the nrITS and combined plastid datasets using MUSCLE (Edgar 2004) implemented in Geneious v.10.0.6 (Kearse *et al.* 2012) with default settings followed by manual adjustments, and we coded indels in the DNA alignments as binary characters using the simple coding method (Simmons & Ochoterena 2000) in SeqState (Muller 2005).

Phylogenetic analyses

We conducted phylogenetic analyses independently for the nrITS and plastid alignments. Prior to phylogenetic analysis, we determined the best-fit substitution models for the nrITS and the combined plastid sequences using jModelTest v.2.1.7 and the Bayesian information criterion (Darriba *et al.* 2012). The best models were HKY+G and HKY+I+G for nrITS and the combined plastid data, respectively. The model applied to the coded binary character partitions was a default Standard Discrete Model in MrBayes (Ronquist *et al.* 2011). Our phylogenetic analyses consisted of Bayesian inference (BI) in MrBayes v.3.2.5 (Ronquist & Huelsenbeck 2003) and maximum likelihood (ML) in RAxML v.8.2 (Stamatakis 2014). For BI, we conducted two independent analyses with one cold and three incrementally heated chains, which we ran for 10,000,000 generations with sampling of the cold chain every 1,000 generations.

The BI analyses yielded final split frequencies of less than 0.01, showing convergence between the paired runs. We discarded the first 2,500 trees from each run as burn-in phase and used the remaining trees from both runs to construct a 50 % majority-rule consensus for obtaining posterior probabilities (PP). For ML, we performed a rapid bootstrap analysis (MLBS) with 1,000 replicates from a random starting tree. Within RAxML we optimized the GTR+G model under the GTRGAMMA command. We visualized all trees in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). The accessions or clades exhibiting hard incongruence (HI) were identified by visual inspection of the nrITS and combined plastid phylogenetic trees for well supported conflicting placements (Mason-Gamer & Kellogg 1996), using a threshold of ≥0.90 Bayesian posterior probability (PP) in both topologies.

Estimation of divergence times

We estimated divergence times in BEAST v.2.4.3 (Bouckaert *et al.* 2014) according to the nrITS dataset, which included more taxa than the plastid dataset. Within BEAST, we applied the HKY+G substitution model based on the outcome from jModelTest, a log-normal relaxed clock model, and a Yule model of tree branching processes. We calibrated the stem age of *Calligonum* based on fossil pollen from the Pliocene (2.6 – 5.3 million years, mya) of the Sahara (Muller 1981) using a log-normal prior on the distribution of ages with an offset of 2.6 Ma, a mean of 1.0 Ma and a standard deviation of 1.0 Ma. We ran two independent analyses in BEAST for 200,000,000 generations with sampling every 1,000 generations. We confirmed the convergence between the two runs using Tracer v.1.6 (http://beast.bio.ed.ac.uk/Tracer). After removing a 10 % burn-in from each run, we combined the results in LogCombiner of the BEAST package (Bouckaert *et al.* 2014). Effective sample sizes (ESSs) of all parameters exceeded 200 in the combined results. We determined the maximum clade credibility (MCC) tree using TreeAnnotator (Bouckaert *et al.* 2014), annotating only those branches with posterior probability greater than 0.5. We visualized the result in FigTree v.1.4.3.

Results

Characteristics of the nrITS and plastid sequences

The nrITS sequence alignment used for the phylogenetic tree reconstruction included 140 sequences: 136 accessions comprising the ingroup and four accessions representing the outgroups. The total length of the aligned nrITS sequences was 570 bp including 360 variable sites and binary characters representing 30 indels. The combined plastid DNA matrix included 148 sequences: 144 representing the ingroup and four accessions for the outgroup. The total length of the combined plastid DNA matrix was 3528 bp including 1005 variable sites with binary characters representing 118 indels. The best ML trees (Figs. S1, S2 in supplementary material) contradicted the Bayesian consensus trees (Fig. 2) at only a few nodes with bootstrap support percentage (MLBS) ≤ 50 % (*i.e.*, soft incongruence).

Phylogenetic results from nrITS

Based on the nrITS dataset, we recovered 15 major clades (Fig. 2), A-O. Clade D (PP = 1, MLBS = 60 %) contains all species in central Asia and China. Clade B (PP = 0.53, MLBS = 80 %) included species distributed within China. Within Clade B, relationships among the four sections were unresolved, and accessions of the CM complex from the Gurbantunggut Desert were placed variously among three sections (clade A, PP = 0.73, MLBS = 30 %). All other accessions of the CM complex, which are distributed in the Taklimakan desert and nearby in the south of the Xinjiang autonomous region, were clustered with sympatric species (clade B) but did not form a monophyletic group. Clade C consisted only of *C. ebinuricum* (PP = 1, MLBS = 81 %), while *C. jeminaicum* formed an independent clade (PP = 1, MLBS = 97 %) showing a high level of divergence from other species in clade A.

Calligonum calliphysa (in clades E and G) was the sole species of Sect. *Calliphysa* but was nested within the Central Asia and China clade D, but did not form a monophyletic group. Several polyploids, *C. crinitum, C. comosum* and

C. polygonoides, formed a clade F (PP = 0.99, MLBS = 62 %), while the polyploid *C. arborescens* was resolved as separated from them (clade I, PP = 0.98, MLBS = 69 %). Additionally, *C. bungei* and *C. persicum* were the sole species clustered in clade H, which, in turn, clustered within clade D, as well as several other resolved clades (A, B, C, E, F, G and I) and several unresolved species, such as *C. eripodum* and *C. microcarpum*.

The polyploid species *C. comosum* is widespread with populations in northern Africa, Europe, and Central Asia. However, all populations were resolved with Central Asia species (PP = 1, MLBS = 60 %, clade D), except for one sample, which was clustered with other two species (*C. crinitum* and *C. polygonoides*) in clade F (PP = 0.99, MLBS = 62 %). *Calligonum azel*, which is limited to the Sahara Desert was resolved with Branch M (PP = 1, MLBS = 70 %), but *C. arichi*, which is also restricted to the Sahara, occurred separately on clade N (PP = 1, MLBS = 79 %). Populations of both *C. azel* and *C. arichi* formed mutually monophyletic groups.

According to both the BI and ML trees of nrITS, species from northern Africa, such as *C. comosum, C. azel* and *C. arichi*, appear to have diversified earlier than Central Asia species and represent lineages that have fewer species. In contrast, *Calligonum* of Central Asia appears to have undergone relatively recent diversification and exhibits greater species richness. For example, endemic species in China, such as *C. taklimakanense*, *C. ebinuricum* and *C. roborowskii* may represent radiations into the Taklimakan or Gurbantunggut Deserts and have close relationships with the CM complex.

Some polyploid in *Calligonum*, such as *C. caput-medusas*, *C. rubicundum* and *C. roborowskii* had independent origins according to the tree topologies. For example, *C. caput-medusas* ($2n = 4x = 36$) was relatively distant from other species in China. However, *C. rubicundum* ($2n = 4x = 36$) seemed to have a close relationship with other polyploid species from the Gurbantunggut Desert, as did *C. roborowskii* with polyploids from the Taklimakan.

Phylogenetic results from the plastid DNA data

Based on the plastid DNA phylogeny (Fig. 3), the four sections of *Calligonum* could not be completely resolved. Nevertheless, all populations of the CM complex were clustered into a clade with high support (0.93 PP, 72 % MLBS, clade *a*), and within this clade, subclades were resolved according to the geographic origins of samples (clades *a*1-*a*5). Similarly, samples of the polyploid species, *C. roborowskii*, comprised a clade (0.62 PP, 64 % MLBS, clade *b*). However, accessions of *C. calliphysa* did not form a monophyletic group except for two populations in Betashan and Qitai (0.58 PP, 82 % MLBS, clade *c*), which are closer geographically to one another than two other populations of the species in two counties named Mulei and Wusu in Xinjiang. *C. leucocladum* and *C. rubicundum* were also not monophyletic based on the plastid DNA tree.

Divergence time dating

According to our divergence time dating based on nrITS, an ancestor of *Calligonum* diversified beginning 3.46 Ma (95 % HPD 1.87-5.71 Ma; Figs. 2, S3 in supplementary material). The Central Asia and China clade (clade D) underwent diversification ca. 2.68 Ma (95 % HPD: 1.28-4.59 Ma), and *Calligonum* in China (Clade B) diversified beginning 2.31 Ma (95 % HPD: 1.05-4.00 Ma).

Discussion

Relationships within Calligonum based on nrITS

We found that the nrITS phylogeny supports separation of the Central Asia and China species from the northern African desert species, *C. comosum*, *C. azel* and *C. arich*. These three species have overlapping distributions in Tunisia and are morphologically distinct from one another and from other species (Gouja *et al.* 2014; 2015). Within these species, the typical karyotypes are $2n = 2x = 18$ for *C. azel* and *C. arich* and $2n = 2x = 18$ or a tetraploid type, $2n = 4x = 36$, for *C. comosum*. The tetraploid cytotypes in *C. comosum* and behaviors of chromosomes in *C. azel* during early prophase suggest that *C. azel* and *C. arich* may be progenitors of *C. comosum* (Dhief *et al.* 2011). However, a recent molecular phylogenetic study based on nrITS showed that these species were distinct (see Fig. 3 in Gouja *et al.* 2014). Our results, combined with the former conclusion, can be treated as the taxonomic evidences for the three species in northern Africa.

Polyploidy, namely allopolyploidy, sometimes results in paraphyletic intraspecific relationships resolved by nuclear genes, such as nrITS (Soltis & Soltis 1999; Ruiz-Garcia *et al.* 2005), because allopolyploid species may possess two or more copies of the gene (or types, in the case of nrITS) from divergent progenitors (Schupp & Feener, 1991; Feliner & Rossello, 2007; Folk *et al.* 2018). This may explain the intraspecific paraphyly of *C. comosum*, *C. rubicundum*, *C. klementzii*, *C. roborowskii*, *C. caput-medusae* and *C. arborescens*, all of which are polyploid, in the nrITS phylogeny. In cases such as these, chromosomal data may complement molecular phylogeny for determining species relationships (Stebbins 1971). However, karyological studies have been performed for only 16 species of *Calligonum*, in part because their small chromosome sizes make karyotyping difficult (Mao 1984; Wang & Yang 1985; Wang & Guan 1986; Mao 1992; Ferchichi 1997; Shi & Pan 2015). The taxonomical relationships and biosystematics among the allopolyploid species were challenges and should be elucidated by the other multi-evidences, such as morphology, karyotypes and high-throughput sequencing database.

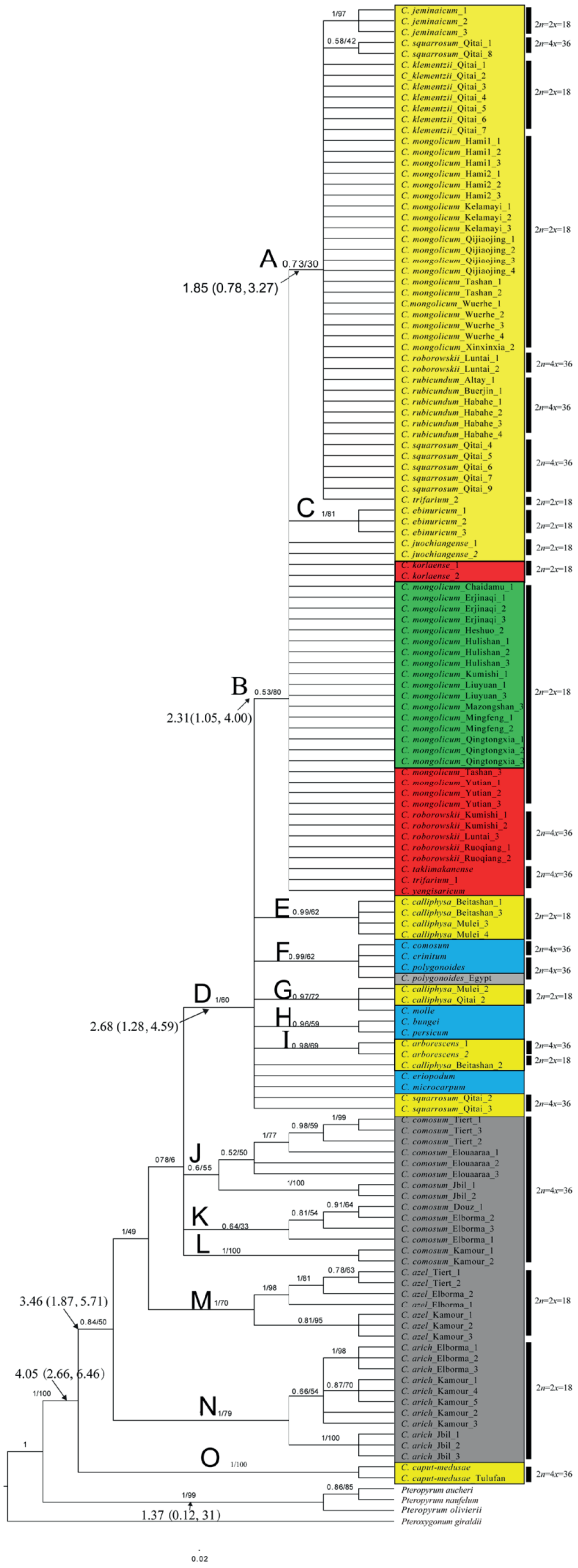

Figure 2. Majority rule tree resulting from Bayesian Inference of the nrITS DNA sequences of *Calligonum*. Bayesian posterior probabilities and maximum likelihood bootstrap support values are given above the branches. Divergence time of an interested node is given with a mean age and its 95 % high probability density (HPD). The colors of the samples are in agreement with the geographical distribution labeled in Figure 1.

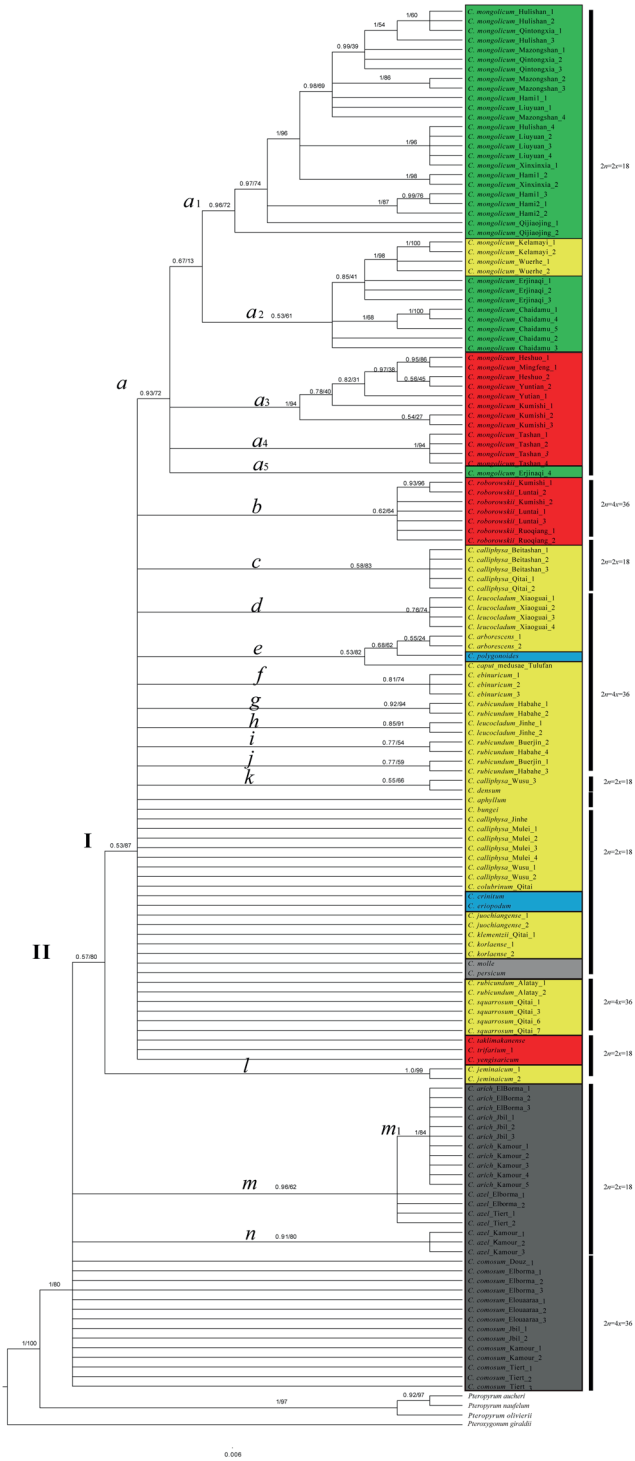

Figure 3. Majority rule tree resulting from Bayesian inference of the combined plastid DNA sequences (*psbA-trnH*, *ycf6-psbM*, *rpl32-trnL*, *rbcL* and *trnL-F*) of *Calligonum*. Bayesian posterior probabilities and maximum likelihood bootstrap support values are given above the branches. The colors of the samples are in agreement with the geographical distribution labeled in Figure 1.

Relationships within Calligonum based on the plastid DNA data and conflicts with the nrITS data

Overall, plastid regions show great promise as DNA barcodes for species delimitation in angiosperms because maternal inheritance of the plastid DNA genome combined with limited seed dispersal in many species may operate together to facilitate clear lineage sorting and limit organellar introgression (Stenz *et al.* 2015; Gambette *et al.* 2016; Morrison 2016). However, hybrid speciation or evolutionary reticulation may yield incongruence between nuclear and plastid DNA phylogenies. Incongruence can also arise due to incomplete lineage sorting or intragenomic recombination (Rieseberg & Brunsfeld 1992; Xu *et al.* 2012). Incomplete lineage sorting may be a more probable explanation for incongruence when divergence occurred recently (Sang *et al.* 1995). Both reticulate evolution or hybrid speciation and incomplete lineage sorting have probably occurred in *Calligonum*, but based on comparisons between the nrITS and plastid DNA trees, these confounding evolutionary processes appear to have occurred more often (or exclusively) among Central Asia lineages compared to northern African ones. The lack of hybridization or reticulation observed for northern African species may reflect mechanisms of isolation and speciation unique to the Sahara Desert (Gouja *et al.* 2014) that can be investigated in future studies using *Calligonum* as a model.

In the nrITS tree, accessions of the CM complex did not form a monophyletic clade. However, in the plastid phylogeny, the CM complex was resolved as a monophyletic group. Several explanations may exist for this unanticipated result, including nuclear gene flow between the CM complex and several other species or incomplete lineage sorting of nrITS among the CM complex and other descendants of a common ancestor. We also observed conflicts in the placements of some polyploids between trees, such as of *C. comosum*, *C. rubicundum*, *C. klementzii*, *C. roborowskii*, *C. caput-medusae* and *C. arborescens* (Figs. 2, 3) and their status as polyploids makes hybridization seem like the most probable explanation.

Species delimitation in the CM complex

C. mongolicum is widely distributed from Xilinhot, Inner Mongolia in the east to the Kyzyl Kum Desert of Uzbekistan in the west and from Milan, Xinjiang in the south to Baitashan, Qitai and Karamay, Xinjiang in the north with a longitudinal range of ca. 30° (Pavlov 1936; Drobov 1953; Baitenov & Pavlov 1960; Sergievskaya 1961; Kovalevskaja 1971; Shi *et al.* 2011). All other putative species within the CM complex have more limited geographic distributions within the range of *C. mongolicum* (Losinskaja 1927; Bao & Grabovskaya-Borodina 2003).

In some treatments, *C. mongolicum* is circumscribed to include all or most of the other controversial species (*e.g.*, Soskov 1975a; b), but in other treatments, some of the controversial species are given species status, especially on the basis of fruit morphology (Mao 1984; Bao & Grabovskaya-Borodina 2003). Nevertheless, fruits within the CM complex are, overall, quite similar (Mao & Pan 1986; Shi *et al.* 2011; Soskov 2011). Therefore, several recent studies have sought to use multiple lines of evidence from molecular phylogeny, morphology, reproductive processes and karyotypes to resolve the complicated taxonomy of the CM complex (Shi *et al.* 2009; 2011; 2013; 2016). In the most current taxonomic treatments (Shi *et al.* 2013; 2016), all members of the CM complex are merged within *C. mongolicum* except for *C. roborowskii*, which is the sole polyploid species occurring in the Taklimakan Desert. Our plastid DNA results support this current taxonomic perspective in showing that *C. roborowskii* is separated from the rest of the CM complex. The taxonomical relationship in CM complex have been strengthened and confirmed in here again. However, the biosytematics and phylogeny of CM complex is still uncertain and unstable.

Evolutionary radiation in Calligonum

Polyploidy is often thought to facilitate range expansion of species and the maintenance of relatively widespread geographic ranges (Soltis & Soltis 2009). Several polyploid species in *Calligonum* (*e.g.*, *C. roborowskii*, *C. caput-medusae*, *C. arborescens* and so on) have large wide distributions, such as throughout the Sahara, Taklimakan, or other deserts of Central Asia. Notably, the CM complex, which includes polyploid cytotypes, occurs in deserts throughout Central Asia and has likely undergone rapid evolution based on its morphological heterogeneity. Similarly, in northern Africa, the tetraploid cytotypes of *C. comosum* have a large geographic distribution (Ferchichi 1997). By contrast, many diploids of *Calligonum*, such as *C. azel* and *C. arich*, are relatively narrowly endemic and, within their ranges, exhibit a low frequency of distribution (*i.e.*, one to five plants per ha). Thus, polyploidy in *Calligonum* may drive diversification of the genus within a biogeographic framework, such as by facilitating occupation of new niches or new geographic areas. This may be followed by isolation or adaptive specialization resulting in speciation.

Divergence time estimates based on nrITS show that the Asian and African groups of *Calligonum* diverged from one another ca. 3.46 Ma (95 % HPD: 1.87-5.71, Fig. 1), while the large central Asian and Chinese groups separated ca. 2.68 Ma (95 % HPD: 1.28-4.59 Ma). These times likely reflect the adaptation of *Calligonum* to increasing regional aridity and the expansion of sandy deserts in the interior of Asia during the Pleistocene (Meng *et al.* 2015).

Conclusions

In this study we used nrITS and five plastid DNA regions to reconstruct a phylogeny of *Calligonum* for further resolving relationships within the genus, evaluating the roles of hybridization and reticulation in its complicated taxonomy,

evolutionary history, and providing some frameworks for addressing open questions. We found that both nrITS and the combined plastid DNA regions showed utility for separating lineages at higher taxonomic levels (or deeper nodes), such as species representing a northern African clade from a clade of central Asian species. However, at lower taxonomic ranks, the plastid DNA regions represented a more promising tool for species delimitation evidenced by clusters of *C. calliphysa*, *C. roborowskii* and *C. rubicundum*, which are each highly morphologically distinct, into separate clades. The plastid DNA also showed that the CM complex is best regarded as a single species, *C. mongolicum*, except for *C. roborowskii*. Incongruence between the plastid DNA and ITS trees coupled with evidence from polyploid cytotypes suggests that hybridization has been an important driver of evolution within *Calligonum* of central Asia and much less so in northern African *Calligonum*. In future studies, next generation sequencing methods, such as RAD-seq (Baird *et al.* 2008; Hollingsworth *et al.* 2009; Zimmer & Wen 2012; Wang *et al.* 2013; Wu *et al.* 2014; Hollingsworth *et al.* 2016; Schumer *et al.* 2016) may be useful for further resolving species boundaries and relationships in the genus as well as testing specific hypotheses of hybrid speciation and reticulate evolution.

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