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Species-level phylogeographical history of the endemic species *Calligonum roborovskii* and its close relatives in *Calligonum* section *Medusa* (Polygonaceae) in arid north-western China

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Quaternary climatic oscillations appear to have influenced the genetic diversity and evolutionary history of arid-adapted plants. To understand the processes involved and reveal evolutionary relationships, haplotypes were examined from *Calligonum roborovskii*, an endemic species occurring in the arid zones across the desert regions of north-western China, and seven other species also from *Calligonum* section *Medusa*, including *C. gobicum*, *C. mongolicum* and the narrow endemic species *C. ebi-nuricum*, *C. pumilum*, *C. taklimakanense*, *C. trifarium* and *C. yengisaricum*. Forty-three haplotypes were identified in 422 individuals from 51 natural populations, from variation of two plastid DNA intergenic spacers (*rpl32-trnL* and *ycf6-psbM*). A high level of total genetic diversity was found across species for which more than two populations were examined, including *C. gobicum*, *C. mongolicum*, *C. pumilum* and *C. roborovskii*. A distinct isolation-by-distance pattern in each of these species was suggested by the Mantel test, indicating that restricted gene flow caused high genetic differentiation among populations. Three haplotypes were shared by two or three species each, but the other 40 haplotypes were species-specific. The 43 haplotypes split into three major clades, but not species-specific lineages; most of the *Calligonum* species were not reciprocally monophyletic, probably due to incomplete lineage sorting or introgression. The identified haplotypes were dated to 1.97 Mya (95% highest posterior density: 2.95–0.99 Mya) and diverged until the late Pleistocene, possibly linked to aridification and enlargement of deserts caused by climate changes. Variation of desert habitats during the Pleistocene might play a key role in causing the divergence. *Botanical Journal of the Linnean Society*, 2016, **180**: 542–553

ADDITIONAL KEYWORDS: aridification – climate change – habitat fragmentation – haplotype variation – speciation.

INTRODUCTION

Among the remarkable climatic changes of the Cenozoic, the climatic oscillations of the Quaternary greatly affected the genetic structure and evolutionary history of extant species, especially in temperate zones of the Northern Hemisphere (Comes & Kadereit, 1998; Taberlet *et al.*, 1998; Hewitt, 2000; Hampe *et al.*, 2003). During glacial expansion, many species had to migrate southward or retreat to separate refugia and then experienced demographic expansion and recolonization during relatively warm interglacials

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(Hewitt, 2000). These cycles resulted in fragmentation of the geographical distributions of many species, allopatric divergence among populations and the likelihood of intraspecific differentiation (Wang *et al.*, 2013).

Arid lands of the temperate zone are also well known for having experienced climate changes during the Quaternary, consisting particularly of long-term cooling and drastic aridity (Hesse, Magee & van der Kaars, 2004; Xu *et al.*, 2010b). North-western China is located in the interior of Eurasia, distant from oceans. However, the extreme aridity of this region results principally from the late Cenozoic uplift of the Qinghai–Tibetan Plateau (QTP) (Guo *et al.*, 2002; Sun *et al.*, 2010). With uplift of the QTP, westerly winds weakened and the Mongolian–Siberian high pressure intensified, resulting in less precipitation and greater cold (Fang *et al.*, 2002b; Guo *et al.*, 2002). Later, aridification intensified greatly, accompanied by cooling during the Pleistocene and continued uplift of mountain ranges (Sun, Zhu & Bowler, 2004; Sun & Zhang, 2009). Increased aridity accelerated desertification in north-western China and produced large-scale expansion of deserts, such as the Gurbantunggut Desert of the Junggar Basin and the Taklimakan Desert of the Tarim Basin (Sun, Ding & Liu, 1998; Fang *et al.*, 2002a, b; Ding *et al.*, 2005; Shi, Cui & Su, 2005). In particular, the large mobile dunes of the Taklimakan Desert were formed (Shi *et al.*, 2005), which might have acted as an effective stimulus to promote adaptation of plants to different desert habitats (Wang *et al.*, 2013).

During the Pleistocene, aridification of north-western China as a whole and expansion of deserts took place on a large scale (Fang *et al.*, 2002b; Yang *et al.*, 2004, 2011). As evidenced by phylogeographical studies in north-western China, aridification and expansion of deserts have been deemed to play key roles in determining genetic diversity, promoting diversification (Guo *et al.*, 2002, 2010; Garrick *et al.*, 2009; Ge *et al.*, 2011; Li *et al.*, 2012; Wang *et al.*, 2013), and affecting the contraction–expansion dynamics of desert species in arid lands. Climatic change cycles were probably related to the aridity of glacial stages and the movement of deserts (Meng & Zhang, 2013). Meng & Zhang (2013) suggested that the range enlargement of species of *Lagochilus* Bunge ex Benth. (Lamiaceae) during the expansion of deserts was an adaptation to dry and cold environments during the Quaternary. Su & Zhang (2014) proposed that the enlargement of the Taklimakan Desert during the mid-Pleistocene triggered the range dispersal of *Hexinia polydichotoma* (Ostenf.) H. L. Yang (Asteraceae). Quaternary climate cycles also profoundly changed the hydrology of this region (Fang *et al.*, 2002b); for example, the climate of the Junggar and Tarim basins experienced multiple transitions between dry and humid conditions, resulting in expansion and contraction cycles of river systems (Feng, Su & Jin, 1999; Yang *et al.*, 2002; Zhang *et al.*, 2003). These would not only have caused shifts in the sizes of the deserts and their oases (Zhang *et al.*, 2003), but also have resulted in submergence of many habitats around the rivers, inevitably leading to population extinction. Such a process has been revealed in a study of *Tetraena mongolica* Maxim. (Zygophyllaceae) in Inner Mongolia (Ge *et al.*, 2011). These geological processes also often affected population genetic structure and diversification (Garrick *et al.*, 2009; Ge *et al.*, 2011; Su & Zhang, 2014).

Climate cycles and associated environment changes in the Quaternary made habitat fragmentation common (Guo *et al.*, 2010; Li *et al.*, 2012; Su & Zhang, 2014) and this had a profound effect on species distributions and their demographic history (Young, Boyle & Brown, 1996; Fahrig, 2003). Some studies have shown that habitat fragmentation reduced genetic diversity between populations, mainly due to restricted gene flow, genetic drift and inbreeding in small populations (Aguilar *et al.*, 2008). In contrast, other studies showed that overall genetic diversity could be maintained or increased by habitat fragmentation because of the occurrence of allopatric speciation caused by isolation (Carson, 1990; Xu *et al.*, 2010a; Hou & Lou, 2011; Zhang, Meng & Rao, 2014). Therefore, additional study of desert species with naturally fragmented distribution patterns is needed to investigate range fluctuations and diversification of species across the desert regions potentially triggered by the past climatic and/or geological changes in arid north-western China.

Species in arid lands of China are often naturally sparsely distributed and relictual, such as *Calligonum* L. *Calligonum* is a Tethyan representative of Polygonaceae (Wang & Guan, 1986), comprising 35 desert species, of which 24 are found in China (Li *et al.*, 1998; Sabirhazi *et al.*, 2010), 16 are endemic to China and 13 are found in Xinjiang (Mao & Pan, 1986). Based on fruit characters, *Calligonum* is classified into four sections, *Calliphysa* (Fisch. et Mey.) Borszcz., *Calligonum*, *Pterococcus* (Pall.) Borszcz. and *Medusa* Sosk. et Alexandr. (Mao & Pan, 1986). Compared with other three sections, section *Medusa* is characterized by a fruit having bristles on the ribs, often not flat at base and lacking wings . They are entomophilous shrubs and pollination is primarily by insects. Seed dispersal is mainly by gravity and thus dispersal capabilities are limited.

Calligonum section *Medusa* includes 15 well-documented species distributed in north-western China,

inhabiting gobi or sand deserts, 11 of which are endemic to north-western China (Li *et al.*, 1998). Therefore, north-western China is regarded as one of the centres of diversity of section *Medusa* (Feng, Pan & Yan, 2008b). Most endemic species in section *Medusa* are narrowly distributed, occurring in only one region in Xinjiang (e.g. *C. jeminaicum* Z.M.Mao, *C. taklimakanense* B.R.Pan & K.M.Shen, *C. trifarium* Z.M.Mao, *C. yengisaricum* Z.M.Mao), two regions in Xinjiang (e.g. *C. korlaense* Z.M.Mao) or a few regions (e.g. *C. alaschanicum* Losinsk., *C. chinense* Losinsk., *C. pumilum* Losinsk., *C. zaidamense* Losinsk.). Only one endemic species, *C. roborovskii* Losinsk., is widely distributed along the edge of deserts, mainly in western Gansu and southern and eastern Xinjiang (Li *et al.*, 1998) (Fig. 1). As an exemplary naturally fragmented distributed species of north-western China, it provides the opportunity to investigate how the Quaternary history shaped the process of genetic diversity and diversification of desert plants.

As addition of individual or population representatives from congeneric species is also important when investigating the evolutionary history of recovered haplotypes for a particular species (Liu *et al.*, 2012, 2014), we added seven other species from section *Medusa*, including *C. gobicum* Bunge ex Meisn., *C. mongolicum* Turkz. and the narrow endemics *C. ebi-nuricum* Ivanova ex Y.D.Soskov, *C. pumilum*, *C. taklimakanense*, *C. trifarium* and *C. yengisaricum* (Li *et al.*, 1998; Sabirhazi *et al.*, 2010). The taxonomy of *Calligonum* spp. follows Mao & Pan (1986) and Li *et al.* (1998). A phylogeographical study of *C. roborovskii* and closely related species could help to understand better the effects of climatic and environmental changes on the evolutionary processes of arid-adapted plants with fragmented populations in arid north-western China.

In this study, we used two plastid DNA non-coding regions (*rpl32-trnL* and *ycf6-psbM*) to investigate the phylogeography of *C. roborovskii* and other species from section *Medusa*. The aims of this study were: (1) to reveal the genetic diversity and population differentiation of *C. roborovskii* with natural habitat fragmentation and compare these with those for related species; (2) to test phylogenetic relationships

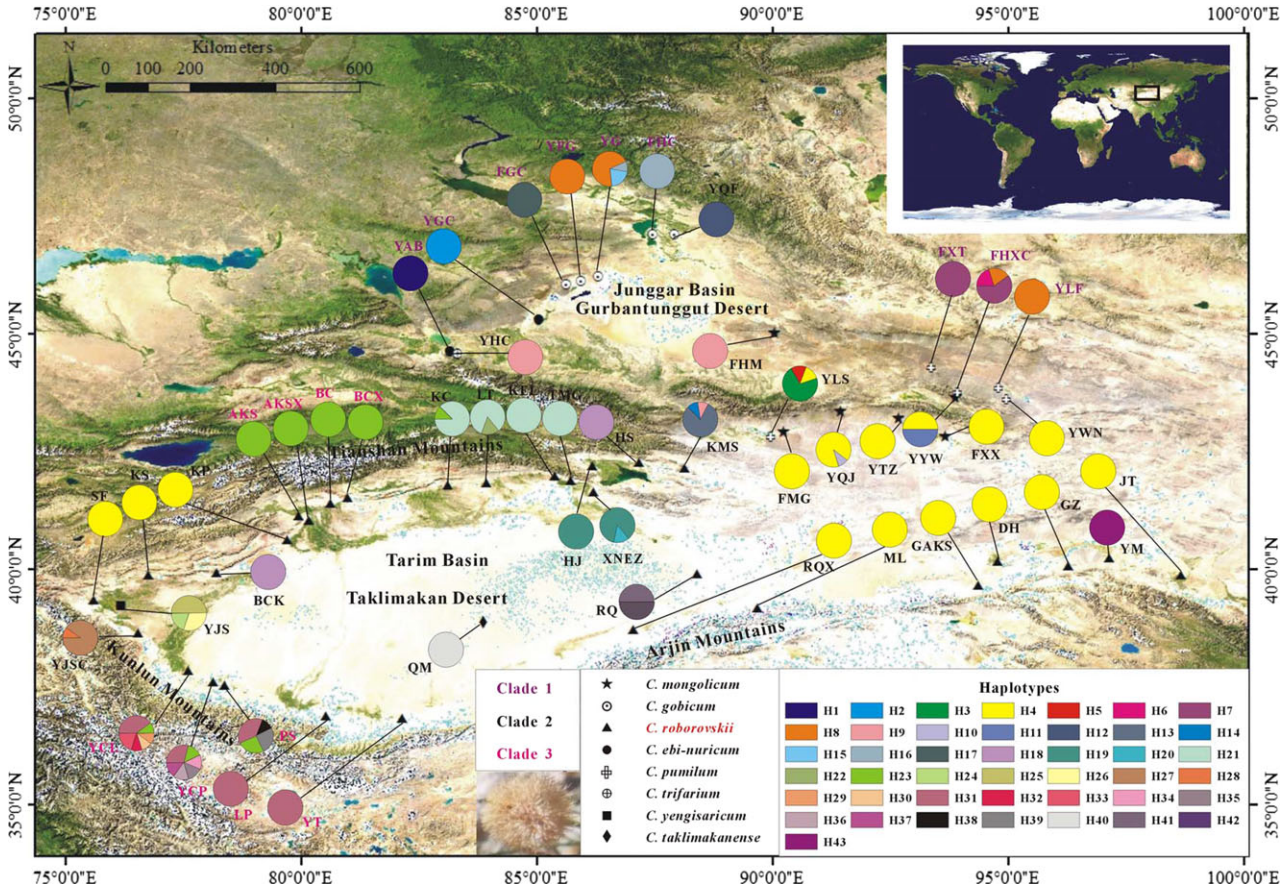

Figure 1. Geographical distribution of plastid DNA haplotypes of *Calligonum roborovskii* and its relatives in section *Medusa*. Population codes, numbers and haplotypes correspond to those in Table 1.

among the species; and (3) to infer the demographic history of *C. roborovskii* and related species during Quaternary climatic changes.

MATERIAL AND METHODS

Species study and population sampling

The endemic species *C. roborovskii* and seven related species, *C. ebi-nuricum*, *C. gobicum*, *C. mongolicum*, *C. pumilum*, *C. taklimakanense*, *C. trifarium* and *C. yengisaricum*, were examined in this study, of which *C. ebi-nuricum*, *C. pumilum*, *C. taklimakanense*, *C. trifarium* and *C. yengisaricum* are narrow endemics to Xinjiang. In total, 422 individuals were sampled at 51 localities (Supporting Information, Table S1), including 30 populations of *C. roborovskii*, six populations of *C. mongolicum*, five populations each of *C. pumilum* and *C. gobicum*, two populations of *C. ebi-nuricum*, and one population each of *C. taklimakanense*, *C. trifarium* and *C. yengisaricum*. These sampling sites covered most of the distribution areas of endemic *Callogonum* spp. Sampled individuals were separated from each other by at least 50 m to avoid the collection of clones or close relatives. Details including the latitude, longitude and elevation of each population were recorded using GPS. Young healthy leaves were dried immediately in the field using silica gel. Voucher specimens were deposited in the Herbarium of the Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences (XJBI). Identification of plant specimens was the responsibility of Y.F., who has been engaged in the taxonomy of *Calligonum* for many years.

DNA extraction, amplification and sequencing

We extracted total genomic DNA from *c.* 100 mg of silica-gel-dried leaves following a modified CTAB protocol (Doyle & Doyle, 1987). Initially, ten plastid DNA segments (*trnS-trnG*, *atpB-rbcL*, *trnQ-rps16*, *rpl32-trnL*, *psbK-psbI*, *psbA-trnH*, *rps16*, *rps12-rpl20*, *trnL-trnF* and *ycf6-psbM*) were used to screen for genetic variations, but only *rpl32-trnL* (Falchi *et al.*, 2009) and *ycf6-psbM* (Demesure, Sodzi & Petit, 1995) were found to have sufficiently high levels of variation.

The polymerase chain reaction (PCR) was carried out in total reaction volumes of 30 μL, including 2.4 μL 2.5 mM dNTP solution in an equimolar ratio, 1.5 μL 10× PCR buffer, 1.5 μL 25 mM $MgCl_2$, 1.2 μL each primer at 50 ng μL^{-1}, 0.5 μL Taq polymerase and 2 μL genomic DNA at 5 ng μL^{-1}. The PCR amplifications were performed as follows: an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. PCR products were examined with gel electrophoresis using a 0.8% agarose gel in a 0.5× TAE (pH 8.3) buffer, then stained with ethidium bromide to confirm a single product and purified using the PCR product purification kit (Qiagen). Sequencing reactions were conducted on an ABI 3730 automated sequencer by Sangon Biotech. Sequence alignment was mostly done using CLUSTAL X v.1.81 (Thompson *et al.*, 1997) and refined manually. Newly obtained haplotype sequences were deposited in the NCBI GenBank database under accession numbers KP985549–KP985591 for *rpl32-trnL* and KP985592–KP985634 for *ycf6-psbM*.

Data analysis

Population genetic structure and demographic analyses

Based on the two plastid DNA fragments, population genetic diversity was quantified using indices of polymorphic sites, nucleotide diversity (π), haplotype diversity (h) and number of haplotypes, in the program ARLEQUIN v.3.5 (Excoffier & Lischer, 2010). Average gene diversity within populations (H_s), total gene diversity (H_T) and between-population differentiation (G_{ST} and N_{ST}) were estimated using the program PERMUT (available at www.pierroton.inra.fr/genetics/labo/Software/) with 1000 permutation tests. A higher N_{ST} than G_{ST} indicates the presence of phylogeographical structure (Zhang *et al.*, 2005).

To study the genetic structure, an analysis of molecular variance (AMOVA) was employed using the software ARLEQUIN v.3.5 (Excoffier & Lischer, 2010). The relationships among plastid DNA haplotypes were examined with Network v.4.6 using the median-joining network method (Bandelt, Forster & Rohl, 1999). A Mantel test was performed in Alleles In Space v.1.0 with 1000 random permutations to assess the significance of isolation by distance between populations (Miller, 2005).

Phylogenetics and divergence time

Atraphaxis manshurica Kitag. and *A. bracteata* A.Los. were chosen as outgroups in phylogenetic analyses and molecular dating. The phylogenetic reconstruction based on the combined sequence matrix was performed using Bayesian inference (BI). Nucleotide substitution model (GTR+ G) parameters were determined for BI using the Akaike information criterion (AIC) in Modeltest v.3.7 (Posada & Crandall, 1998). BI was conducted using MrBayes v.3.2 (Ronquist *et al.*, 2012) (N_{ST} = 6, rates = gamma). The Markov chain Monte Carlo (MCMC) algorithm

was run for 50 000 000 generations, with four incrementally heated chains. Analyses included starting from a random tree and sampling every 1000 generations. The first 10% of trees were discarded as burn-in. The remaining trees were used to construct a 50% Bayesian consensus tree and the proportion of bifurcations found in this consensus tree was give as posterior clade probabilities (pP) to estimate the robustness of the BI trees.

As there were no fossils of *Calligonum* and no suitable substitution rates available, we used the range of synonymous substitution rates of plastid DNA genes to estimate divergence times, 0.0011-0.0029 substitutions per site per Myr (0.22–0.58% Myr^{-1}) (Wolfe, Li & Sharp, 1987). Bayesian analysis was carried out in BEAST v.1.8.2 (Drummond *et al.*, 2012). BEAUti was first used to set criteria for the analysis. The Bayes factor (BF) was calculated by Tracer v.1.6 to check the effective sample sizes (ESSs) (> 200) after the first 20% of generations had been discarded as burn-in. A Bayesian MCMC approach was used and the MCMC was run for 50 000 000 generations and trees were sampled every 1000 generations. In addition, the GTR substitution model with four gamma categories and the uncorrelated log-normal relaxed clock were performed. The maximum clade credibility tree was generated using Tree Annotator v.1.8.2 (Drummond *et al.*, 2012). Final trees were edited in FigTree v1.3.1 (http://tree.bio.ed.as.uk/software/figtree/).

RESULTS

Haplotype distributions and their evolutionary relationships

The aligned fragment lengths were 823 bp for *rpl32-trnL* and 890 bp for *ycf6-psbM*. Average nucleotide frequency for the combined sequences was A (35.99%), T (34.11%), C (14.99%) and G (14.91%); they were A/T rich, with an average content of 70.10%. Thirty-six nucleotide substitutions and 11 indels were found in the concatenated sequences (Supporting Information, Table S2). Based on all variable sites, 43 haplotypes were identified (Table 1, Fig. 1). *Calligonum roborovskii* had the largest number of haplotypes (26) and sample size (252). The number of haplotypes obtained was six in *C. pumilum*, five in *C. gobicum*, four in *C. mongolicum*, three in *C. yengisaricum*, two in *C. ebi-nuricum*, and one each in *C. taklimakanense* and *C. trifarium* (Table 1, Fig. 1). Haplotype H4 was the most widely distributed, being shared by three species (*C. mongolicum*, *C. pumilum* and *C. roborovskii*). Haplotypes H8 and H9 were shared by two (*C. gobicum* and *C. pumilum*) and three species (*C. mongolicum*, *C. roborovskii* and *C. trifarium*,) respectively. The remaining 40 haplotypes were species-specific (Table 1, Fig. 1).

Three main clades were resolved using the network from the plastid DNA haplotypes (Fig. 2). In Clade 1, there were no network relationships or haplotypes shared between *C. roborovskii* and its related species. The clade included eight haplotypes, belonging to *C. ebi-nuricum*, *C. gobicum* and *C. pumilum*. Clade 2 showed network relationships between *C. roborovskii* and the other species. It was divided into two parts, Clade 2-1 and Clade 2-2. Clade 2-1 included ten haplotypes, of which H4 and H9 were shared by *C. roborovskii* and two or three other species, respectively. Clade 2-2 had 13 species-specific haplotypes, belonging to three species, *C. roborovskii*, *C. taklimakanense* and *C. yengisaricum*. In Clade 3, there were 12 haplotypes, all specific to *C. roborovskii*. The three clades were also supported by the phylogenetic analysis of the plastid DNA haplotypes (Supporting Information, Fig. S1).

Phylogenetic relationships and lineage divergence

Based on BI analysis, three main clades could be recognized in the tree (Supporting Information, Fig. S1), although lineages of the 43 identified haplotypes did not yield a species-level differentiation between the eight species. From the six species with two or more haplotypes, only haplotypes H1 and H2, belonging to *C. ebi-nuricum*, were well clustered together. The remaining species, including *C. gobicum*, *C. mongolicum*, *C. pumilum*, *C. roborovskii* and *C. yengisaricum*, were not reciprocally monophyletic in terms of the data available.

According to the estimates by BEAST (Fig. 3), the identified 43 haplotypes were dated to 1.97 Mya [95% highest posterior density (HPD): 2.95–0.99 Mya], i.e. in the early Pleistocene. Clades 1 and 3 were dated to 0.68 Mya (95% HPD: 1.01–0.34 Mya) and 0.95 Mya (95% HPD: 1.42–0.48 Mya), respectively. In Clade 1, *C. ebi-nuricum* diverged from the others at 0.40 Mya (95% HPD: 0.66–0.14 Mya). Divergence time between H1 and H2 was 0.06 Mya (95% HPD: 0.08–0.03 Mya). Clade 2 was divided into two parts. One was dated to 1.00 Mya (95% HPD: 1.49–0.51 Mya) and the other to 0.42 Mya (95% HPD: 0.63–0.21 Mya). In Clade 2, *C. taklimakanense* with haplotype H40 diverged from the other haplotypes at 1.00 Mya (95% HPD: 1.49–0.51 Mya). Divergence times among the eight *Calligonum* spp. were all in the early to late Pleistocene.

Table 1. The plastid DNA *rpl32-trnL* + *ycf6-psbM* haplotypes detected in the sampled populations of *Calligonum roborovskii* and its relatives in section *Medusa*

Species	Code	*N*	Haplotypes (individuals)	Species	Code	*N*	Haplotypes (individuals)
C. roborovskii	YKM	10	H9 (1), H13 (8), H14 (1)		DH	8	H4 (8)
	HS	8	H18 (8)		GZ	7	H4 (7)
	HJ	10	H19 (10)		YM	3	H43 (3)
	XNEZ	7	H19 (6), H20 (1)		JT	8	H4 (8)
	TMG	6	H21 (6)	*C. ebi-nurcum*	YAB	10	H1 (10)
	KEL	8	H21 (8)		YGC	7	H2 (7)
	LT	6	H21 (5), H22 (1)	*C. pumilum*	YLS	7	H3 (5), H4 (1), H5 (1)
	KC	8	H21 (7), H23 (1)		YWN	6	H4 (6)
	BCX	10	H23 (10)		FHXC	10	H6 (2), H7 (6), H8 (2)
	BC	10	H23 (10)		YLF	8	H8 (8)
	AKS	9	H23 (9)		FXT	9	H7 (9)
	AKSX	10	H24 (10)	*C. trifarium*	YHC	10	H9 (10)
	KP	9	H4 (9)	*C. mongolicum*	YQJ	8	H4 (7), H10 (1)
	BCK	10	H18 (10)		FMG	2	H4 (2)
	KS	10	H4 (10)		YTZ	8	H4 (8)
	SF	9	H4 (9)		FXX	10	H4 (10)
	YJSC	10	H27 (9), H28 (1)		YYW	10	H4 (5), H11 (5)
	YCL	10	H23 (1), H29 (1), H30 (1), H31 (4), H32 (1), H33 (2)		FHM	5	H9 (5)
	YCP	7	H23 (1), H31 (2), H34 (1), H35 (1), H36 (1), H37 (1)	*C. gobicum*	YQF	10	H12 (10)
	PS	8	H23 (2), H31 (3), H38 (1), H39 (2)		YG	10	H8 (7), H15 (2), H16 (1)
	LP	10	H31 (10)		YFG	6	H8 (6)
	YT	9	H31 (9)		FGC	8	H17 (8)
	RQX	9	H4 (9)		FHC	6	H16 (6)
	RQ	4	H41 (2), H42 (2)	*C. taklimakanense*	QM	10	H40 (10)
	GAKS	8	H4 (8)	*C. yengisaricum*	YJS	10	H24 (2), H25 (5), H26 (3)

Haplotypes correspond to those in Figures 1 and 2. *N*, number of sampled individuals.

Population genetic structure

For the four species (*C. gobicum*, *C. mongolicum*, *C. pumilum* and *C. roborovskii*) with a sample size larger than two populations, the AMOVA showed that most of the total variation occurred among populations (Supporting Information, Table S3): *C. gobicum* (96.77%), *C. mongolicum* (78.83%), *C. pumilum* (76.03%) and *C. roborovskii* (91.19%). The Mantel test based on plastid DNA data revealed a significant pattern of isolation-by-distance (Supporting Information, Table S3): *C. gobicum* ($r = 0.601$, $P \leq 0.001$); *C. mongolicum* ($r = 0.350$, $P \leq 0.01$); *C. pumilum* ($r = 0.787$, $P \leq 0.001$); and *C. roborovskii* ($r = 0.202$, $P \leq 0.001$). For these four species, each had a relatively high total genetic diversity (H_T) and lower within-population diversity (H_s) (Table 2). In the PERMUT analysis, N_{ST} was higher than G_{ST} for each species, and significant phylogeographical structure was detected in *C. mongolicum* and *C. roborovskii* (Table 2).

DISCUSSION

Plastid haplotype distribution in relation to *Calligonum* species

Forty-three haplotypes were identified from *C. roborovskii* and related species (Table 1, Fig. 1). Only haplotypes H4, H8 and H9 were shared by two or three species. The other 40 haplotypes (93%) are species-specific (Table 1, Fig. 1). Among the eight *Calligonum* spp., only the distribution ranges of *C. mongolicum* and *C. pumilum* are partly overlapping in eastern Xinjiang, whereas the others are distant from each other. The presence of strong geographical barriers, e.g. the Tianshan Mountains, the Gurbantuggut and Taklimakan deserts and the Tarim River, might have prevented the mixing of haplotypes between populations, contributing to allopatric divergence, which could explain why there are so many unique haplotypes. Among the 51 populations studied, 33 (65%) had only one haplotype

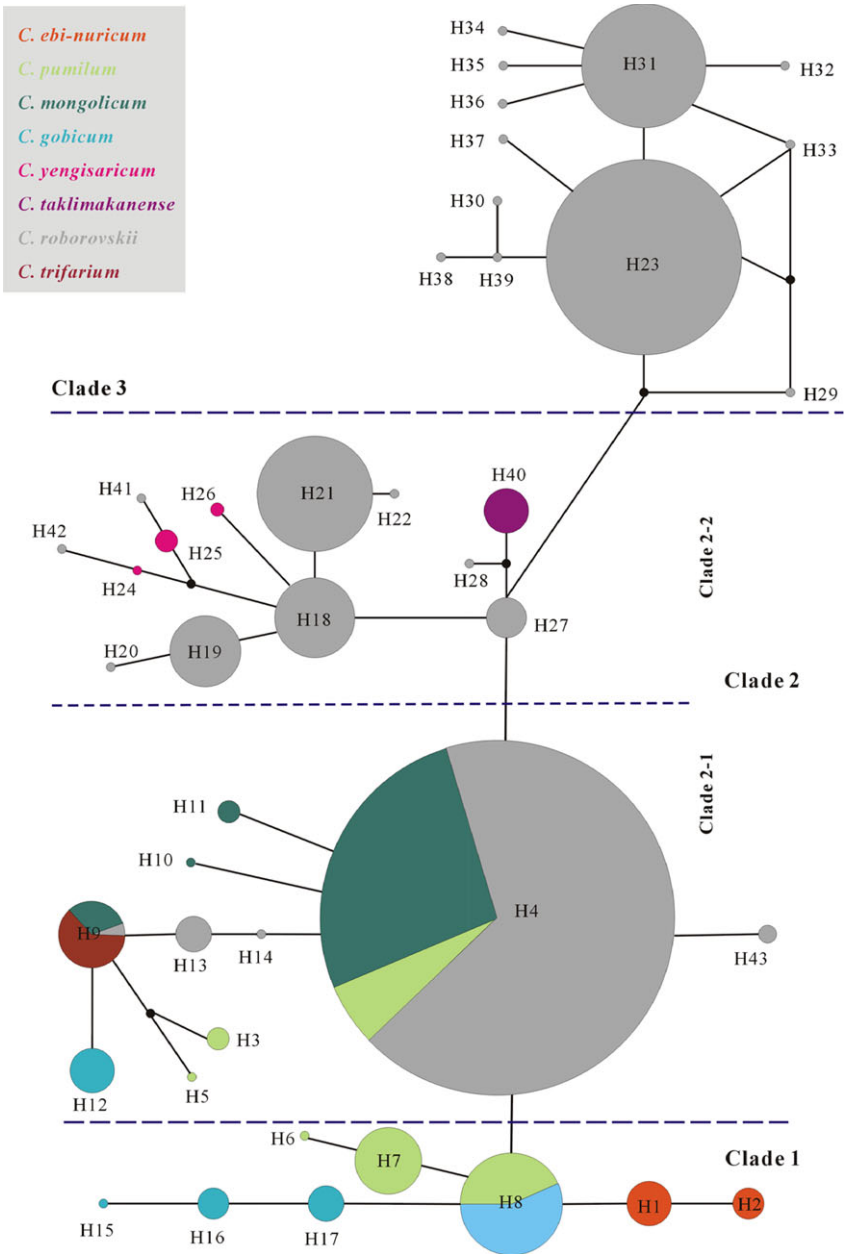

Figure 2. A network of plastid DNA haplotypes. Sizes of the circles are approximately proportional to the overall frequencies of the haplotypes.

(Table 1, Fig. 1), which could explain the low levels of within-population diversity for *C. gobicum*, *C. mongolicum*, *C. pumilum* and *C. roborovskii* (Supporting Information, Table S3). However, three populations (YCL, YCP and PS) located at the southern rim of the Tarim Basin together have 12 haplotypes (Table 1, Fig. 1) and account for 46% of those of *C. roborovskii* and 28% of the total. These three populations are surrounded by two seasonal rivers (Yarkant and Hotan), such that they are isolated from other populations, providing excellent opportunities for allopatric divergence. Quaternary climate change not only caused the variation occurring in the range of the Taklimakan Desert (Shi *et al.*, 2005), but also the water volume of the rivers (Zhang *et al.*, 2003), also causing changes to *Calligonum* habitats. These complex events must have contributed to the rapid speciation, resulting in these three populations having so many unique haplotypes. Therefore, we suggest that this area should be considered as a centre

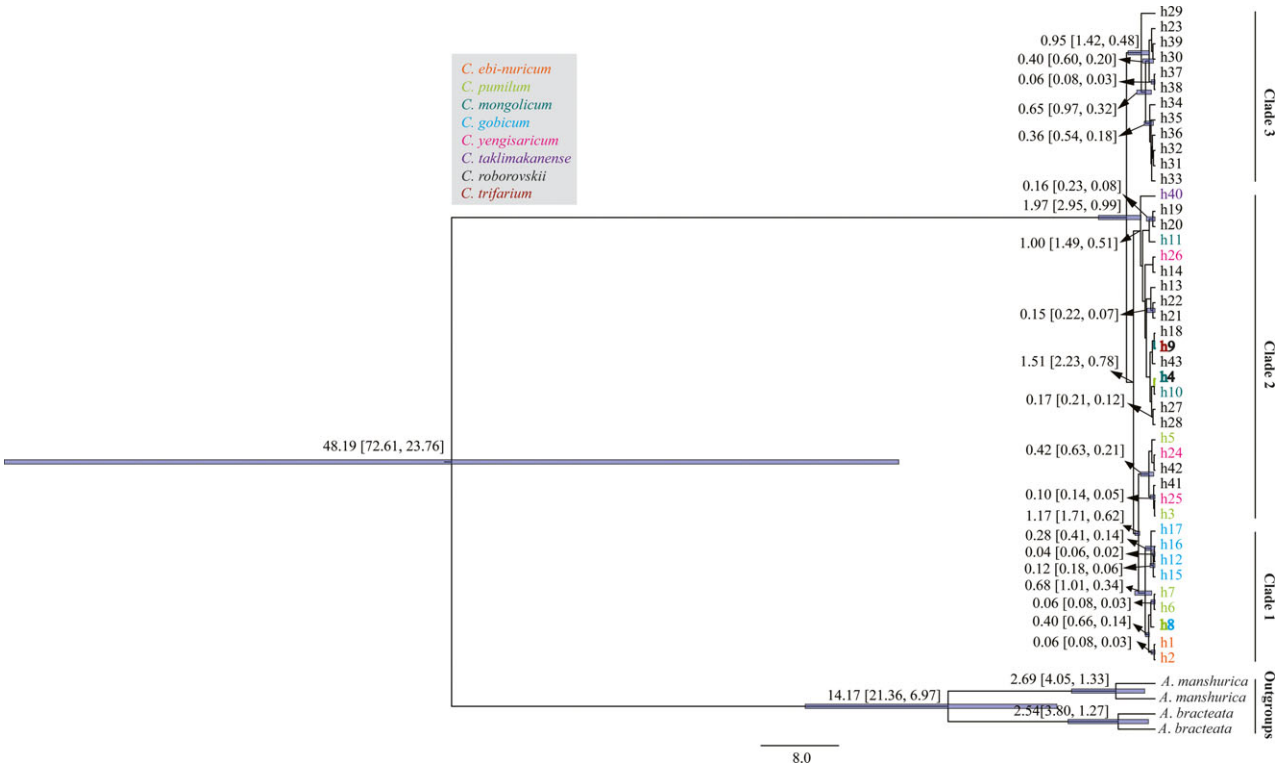

Figure 3. Bayesian divergence time estimates of *Calligonum roborovskii* and its relatives in section *Medusa* based on two plastid DNA sequences. Mean intervals of divergence (in Mya) are shown above branches. Blue bars at the nodes indicate 95% intervals. *Atraphaxis manshurica* and *A. bracteata* were employed as outgroups.

Table 2. Estimate of genetic diversity and population differentiation (± SE in parentheses) for *Callogonum roborovskii* and its relatives in section *Medusa*

Species	H_s	H_T	G_{ST}	N_{ST}
C. pumilum	0.229 (0.1412)	0.906 (0.0578)	0.747 (0.1657)	0.752 (NC)
C. mongolicum	0.134 (0.0936)	0.510 (0.1905)	0.737 (0.1857)	0.850 (0.1648)*
C. golicum	0.102 (0.1022)	0.920 (0.0533)	0.889 (0.1206)	0.971 (0.0379)
C. roborovskii	0.159 (0.0535)	0.874 (0.039)	0.818 (0.058)	0.892 (0.0402)**

H_s, average genetic diversity within population; H_T, total genetic diversity; G_{ST}, interpopulation haplotype differentiation; N_{ST}, interpopulation haplotype differentiation taking into account sequence difference; */**N_{ST} was significantly/very significantly different from G_{ST}; NC, not computed due to small sample size or low variation among populations.

of diversification for *C. roborovskii*, as mentioned previously (Taberlet & Cheddadi, 2002).

Genetic diversity and population differentiation

From the plastid DNA data sets, we detected a significant high level of total genetic diversity for *C. gobicum* (H_T = 0.920), *C. mongolicum* (H_T = 0.510), *C. pumilum* (H_T = 0.906) and *C. roborovskii* (H_T = 0.874), but within-population diversity for the four was low (Table 2), which has also been reported for several other arid land species (Ge *et al.*, 2011; Li *et al.*, 2012; Meng & Zhang, 2013; Su & Zhang, 2013, 2014; Xu & Zhang, 2015). There was a significant pattern of isolation-by distance for each of them (Table 2), but that for *C. mongolicum* and *C. roborovskii* was more positive because significant phylogeographical structures were also detected in both species (Table 2), indicating that gene flow is most likely to occur between neighbouring populations (Slatkin, 1993; Hutchison & Templeton, 1999). For maternally inherited plastid DNA, gene flow usually occurs by seed transmission (Nathan & Muller-Landau, 2000; Manzano & Malo, 2006). Although

fruits in section *Medusa* have bristles on the ribs, the possibility of seed dispersal dependent on animals might be limited because there are few other plants nearby, and almost all are inedible. Therefore, most seed dispersal is due to gravity, resulting in short-distance seed dispersal. These *Calligonum* spp. are naturally fragmented in their distributions in the desert and the scattered patchiness of their populations also seems to discourage both seed and pollen dispersal (Ghazoul, 2005). In addition, seed might be trapped or covered by stony or sandy desert surfaces. This restricted gene flow would result in high genetic differentiation among populations (Supporting Information, Table S3).

Phylogenetic implications for *Calligonum* species: most are reciprocally non-monophyletic

Based on BI analysis, the 43 identified haplotypes were split into three major clades, but not species-specific lineages (Supporting Information, Fig. S1). The result is similar to *Lagochilus* from arid north-western China, in which two main monophyletic clades representing an eastern region and a western region were detected from the combined *trnS-trnG* and *psbA-trnH* regions (Meng & Zhang, 2013). Apart from the two haplotypes H1 and H2 in *C. ebi-nuricum*, which formed a sister pair, the remaining *Calligonum* spp. with more than one haplotype (*C. gobicum*, *C. mongolicum*, *C. pumilum*, *C. roborovskii* and *C. yengisaricum*) were not reciprocally monophyletic. The three specific haplotypes (H24, H25 and H26) in *C. yengisaricum* were each found on a separate branch, and clustered with haplotypes from relatively remote populations belonging to *C. pumilum* and *C. roborovskii*. Thus, the non-monophyly probably results from incomplete lineage sorting. Haplotypes H4, H8 and H9 were shared by two or three species from *C. gobicum*, *C. mongolicum*, *C. pumilum* and *C. roborovskii*, which might have been caused by retention of ancestral polymorphisms during speciation and/or interspecific hybridization (Meng & Zhang, 2013). For *C. mongolicum* and *C. pumilum* with partially overlapping distributions and periods of flowering, a little gene flow would be expected.

The non-monophyly of two narrow endemic species, *C. pumilum* and *C. yengisaricum*, might be affected by the artificial classification. As we know, most current species, especially narrow endemic species, were described based on limited type specimens (Liu *et al.*, 2014). Shi *et al.* (2009) suggested that *C. pumilum* should be merged with *C. mongolicum* based on morphological databases from seven populations (three for *C. mongolicum* and four for *C. pumilum*), but this was not supported in other studies (Feng, Pan & Shen, 2008a). As identification of *Calligonum* spp. is sometimes difficult, it is necessary to collect genetic evidence based on multiple individuals (Liu *et al.*, 2014). To make the delimited or defined species testable and objective, these two narrow endemic species in section *Medusa* might have to be re-established based on genetic and morphological evidence at the population level.

There was a significant pattern of isolation-by-distance for each of them (Table 2). Allopatric speciation usually results in the origin of 'paraphyletic' species (Avise, 2000), because of incomplete lineage sorting. Reciprocal non-monophyly and shared haplotypes between sister species would be an expected transitional stage for diverging lineages (Tajima, 1983; Avise & Ball, 1990; Maddison, 1997). Our data show that plastid DNA lineage sorting is ongoing among these *Calligonum* spp. (Supporting Information, Fig. S1). Insufficient repeated extinctions and/or genetic drift in the fragmented habitats of the species might be likely to promote reciprocal non-monophyly (Orr, 1996). Nonetheless, further work on the sequencing of additional genomic regions, especially nuclear loci, is needed to make an in-depth conclusion about the phylogenetic relationships of the species.

Pleistocene divergence of *Calligonum* species in arid north-western China

According to the estimates by BEAST, the 43 haplotypes identified were dated to 1.97 Mya (95% HPD: 2.95–0.99 Mya), i.e. in the early Pleistocene, and diverged until the late Pleistocene (Fig. 3).

The formation of the Taklimakan Desert in the Tarim Basin and the Gurbantunggut Desert in the Junggar Basin had begun much earlier. The increased aridity and expansion might have occurred during the Pleistocene (Yang *et al.*, 2004, 2011), especially in the Taklimakan Desert, where migratory dunes formed during the late–early or early–mid-Pleistocene when the region became drier (Shi *et al.*, 2005). These *Calligonum* spp. inhabit gobi (stone desert) and/or sandy deserts and climate change caused the range of deserts to vary, also causing changes in their habitats (Sun *et al.*, 1998; Fang *et al.*, 2002a, b; Ding *et al.*, 2005; Shi *et al.*, 2005). Compared with sand, gobi is a relatively moist desert type, and is usually found at the periphery of sandy areas. Variation in the ranges of deserts caused by climate changes might have forced plants to exist in habitats experiencing greater drought and mobile dunes can easily bury seeds or plants. Thus, physiological adaptation to different desert habitats is implied in the divergence of

Calligonum spp., for example the C_4 photosynthesis pathway (Su, Xie & Zhou, 2011), assimilating shoots, a second flowering period in *C. ebi-nurcum*, *C. mongolicum* and *C. trifarium* (Li *et al.*, 1998), and the greater plant height of the tree-like *C. taklimakanense* (Sabirhazi *et al.*, 2010) compared with other *Calligonum* spp., an adaptation to avoiding burial by mobile dunes. This is similar to the example of two closely related species of *Pugionium* Gaertn. (Brassicaceae) from arid north-western China (Wang *et al.*, 2013). Besides the dynamics of sand movement, other geological processes, such as oasis formation and river course alterations caused by Pleistocene climate changes (Zhang *et al.*, 2003; Su & Zhang, 2014), also often affected population genetic structure and diversification (Garrick *et al.*, 2009; Ge *et al.*, 2011; Su & Zhang, 2014).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. The evolutionary relationships among plastid DNA haplotypes of *Calligonum roborovskii* and its relatives in section *Medusa*. Posterior probability is shown above branches. *Atraphaxis manshurica* and *A. bracteata* were employed as outgroups.

Table S1. Details of *Calligonum roborovskii* and its related species populations in *Calligonum* section *Medusa* used in the study.

Table S2. Distributions of variable locus of each haplotype for *Calligonum roborovskii* and its related species in *Calligonum* section *Medusa* in the *rpl32-trnL* and *ycf6-psbM* regions

Table S3. Analysis of molecular variance (AMOVA) for the populations of *Calligonum roborovskii* and related species partitioned by species, and within each species based on plastid DNA sequence data.