MOLECULAR ECOLOGY

Molecular Ecology (2016) **25**, 1192–1210 doi: 10.1111/mec.13538

A high frequency of allopolyploid speciation in the gymnospermous genus *Ephedra* and its possible association with some biological and ecological features

HUI WU,*† ZHEN MA,* MING-MING WANG,* AI-LI QIN,* JIN-HUA RAN* and XIAO-QUAN WANG*

*State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China, †University of Chinese Academy of Sciences, Beijing 100039, China

Abstract

The origin and evolution of polyploids have been studied extensively in angiosperms and ferns but very rarely in gymnosperms. With the exception of three species of conifers, all natural polyploid species of gymnosperms belong to *Ephedra*, in which more than half of the species show polyploid cytotypes. Here, we investigated the origin and evolution of polyploids of *Ephedra* distributed in the Qinghai–Tibetan Plateau (QTP) and neighbouring areas. Flow cytometry (FCM) was used to measure the ploidy levels of the sampled species that are represented by multiple individuals from different populations, and then, two single-copy nuclear genes (*LFY* and *DDB2*) and two chloroplast DNA fragments were used to unravel the possible origins and maternal donors of the polyploids. The results indicate that the studied polyploid species are allopolyploids, and suggest that allotetraploidy is a dominant mode of speciation in *Ephedra*. The high percentage of polyploids in the genus could be related to some of its biological attributes such as vegetative propagation, a relatively high rate of unreduced gamete formation, and a small genome size relative to most other gymnosperms. Significant ecological divergences between allotetraploids and their putative progenitors were detected by PCAs and ANOVA and Tukey's tests, with the exception of *E. saxatilis*. The overlap of geographical distributions and ecological niches of some diploid species could have provided opportunities for interspecific hybridization and allopolyploid speciation.

Keywords: ecological divergence, *Ephedra*, gymnosperm, hybridization, molecular phylogeny, polyploid speciation

Received 30 November 2014; revision received 17 December 2015; accepted 22 December 2015

Introduction

Polyploidy or whole-genome duplication (WGD) has long been recognized as an important process in plant evolution (Otto & Whitton 2000; Soltis *et al.* 2009). At least one round of WGD occurred before the divergence of seed plants (Jiao *et al.* 2011), and multiple rounds of WGD have been reported in angiosperms (Vision *et al.* 2000; Simillion *et al.* 2002; Bowers *et al.* 2003; also see review by Leitch & Leitch 2012). Polyploidy may have broad-scale effects on genomic repatterning, gene expression and genetic networks due to the inheritance of an additional set of chromosomes, and can produce immediate shifts in morphology, breeding system and ecological tolerances (Otto 2007; Soltis *et al.* 2010; Fawcett *et al.* 2013; Weiss-Schneeweiss *et al.* 2013), although some studies found no correlation between polyploidization rate and species richness (Wood *et al.* 2009; Mayrose *et al.* 2011). While it has been recognized that a polyploid species often has multiple origins (see review by Soltis *et al.* 2014a), it remains challenging to evaluate the direct effect of polyploidy on evolutionary success of the species (Madlung 2013; Soltis *et al.*

Correspondence: Xiao-Quan Wang, Fax: +86 10 62590843; E-mail: xiaoq_wang@ibcas.ac.cn

2014b). Moreover, although some studies indicate that ecological divergence is an important driver of polyploid speciation, especially allopolyploid speciation (Fawcett *et al.* 2009, 2013; Van de Peer *et al.* 2009; Ramsey 2011), it remains unresolved whether diploid and polyploid plants can share broad-scale climatic niches (Glennon *et al.* 2014).

In contrast to the high frequency of polyploids documented in angiosperms, polyploidy is exceedingly rare in gymnosperms excluding *Ephedra* (Khoshoo 1959; Ahuja 2005; Murray 2013; Wang & Ran 2014). When excluding three species of conifers, that is the two tetraploids *Juniperus chinensis* 'Pfitzeriana' and *Fitzroya cupressoides* and the hexaploid *Sequoia sempervirens* (Ahuja 2005), the remaining natural polyploids of gymnosperms all belong to the genus *Ephedra*, in which 50–65% of species show tetraploid or very rarely octoploid cytotypes (Khoshoo 1959; Huang *et al.* 2005). It is of interest to investigate why and how so many polyploids have evolved in this genus.

Ephedra comprises about 50 extant species that are mainly shrubs distributed in both temperate and subtropical arid environments in the Northern Hemisphere and South America (Kubitzki 1990; Ickert-Bond *et al.* 2009), with the basal-most lineages distributed in the Mediterranean area (Rydin & Korall 2009; Qin *et al.* 2013). These species originated by radiative speciation in the Cenozoic with a crown age of about 30 Ma (Ickert-Bond *et al.* 2009), although the earliest fossil record of the genus is from the Early Cretaceous (Yang & Wang 2013). As a secondary diversification centre, the Qinghai–Tibetan Plateau (QTP) and adjacent regions harbour approximately 16 species of *Ephedra* (Fu *et al.* 1999; Yang 2002; Yang *et al.* 2003), of which nine were reported to be polyploids or have polyploid cytotypes, including *E. distachya*, *E. equisetina*, *E. gerardiana*, *E. glauca*, *E. intermedia*, *E. likiangensis*, *E. monosperma*, *E. saxatilis* and *E. sinica*. All of the 16 species form a monophyletic clade with several other species from northern and western Asia and Horn of Africa (Huang & Price 2003; Rydin & Korall 2009) and are generally geographically isolated from the other species of the clade, whereas the nine polyploids are respectively located in three well supported subclades, southern QTP, eastern QTP and northern China (Qin *et al.* 2013). It was inferred that the subclade divergence occurred in the Miocene and was very likely linked to the uplift of the QTP and the Asian aridification (Qin *et al.* 2013). However, the origin of these polyploid species remains unknown.

The DNA sequence markers, single-/low-copy nuclear genes in particular, are increasingly and successfully used to study allopolyploid speciation in plants, such as in *Oryza* (Ge *et al.* 1999), *Paeonia* (Ferguson & Sang 2001), *Persicaria* (Kim *et al.* 2008), *Solanum* (Cai *et al.* 2012), *Nicotiana* (Kelly *et al.* 2013) and *Sequoia* (Yang *et al.* 2012b). Also, in recent years, the whole-genome or whole-transcriptome analysis has greatly advanced our knowledge of origin and evolution of polyploids in angiosperms (e.g. Roulin *et al.* 2012; Page *et al.* 2013; Renny-Byfield *et al.* 2013). However, genome sequencing of any gymnosperm remains a huge and expensive task, although a draft assembly of the genome has been generated for a couple of conifers, including Norway spruce (Nystedt *et al.* 2013), white spruce (Birol *et al.* 2013) and loblolly pine (Neale *et al.* 2014). Therefore, current phylogenetic analysis using single-/low-copy nuclear genes is still the best approach for exploring the evolution of polyploids in *Ephedra*.

The previous inference of several allotetraploids from karyomorphological data (Mehra 1946) and the presence of hybridogenic speciation in *Ephedra* (Cutler 1939; Wendt 1993) allow us to hypothesize that allopolyploid speciation might be common in the genus. This study aims to investigate the origin and evolution of polyploids of *Ephedra* distributed in the QTP and neighbouring areas. First, flow cytometry was used to measure ploidy levels of the sampled species, most of which were represented by multiple individuals from different populations. Then, two single-copy nuclear genes (*LFY* and *DDB2*) and two chloroplast DNA (cpDNA) fragments (*trn*T-*trn*F and *trn*S-*trn*fM) were used to investigate whether these polyploids are allopolyploids or autopolyploids and which species could be the maternal donors of the allopolyploids. Finally, based on a comprehensive analysis of biological attributes, phylogenetic relationships, geographical distributions and ecological factors of the habitats, we discussed the possible correlation between allopolyploid speciation and some biological and ecological features.

Materials and methods

Sampling

A total of 48 populations of twelve *Ephedra* species (one with two varieties) distributed in the QTP and adjacent regions were sampled for the measurement of ploidy levels, and the cpDNA and nuclear gene analyses (Table 1). Young branchlets were collected from the individuals that were at least 50 m apart from each other. The remaining four species that also occur in the QTP and neighbouring areas, namely *E. distachya*, *E. lomatolepis*, *E. rituensis* and *E. fedtschenkoae*, were not included in the study due to the controversy of their species status (Fu *et al.* 1999; Yang 2002; Yang *et al.* 2003), or a lack of enough population samples. Among them, *E. fedtschenkoae* seems unique in being

Table 1 The chlorotypes, nuclear gene alleles and ploidy levels detected in the sampled populations of the studied *Ephedra* species

Species	Pop.	Location	Lat. (N)	Long. (E)	Alt. (m)	N^a	N^b	Haplotypes (Individuals)	N^c	*LFY*			*DDB2*			N^d	Ploidy level
										C_A	C_B	C_C	C_A	C_B	C_C		
E. equisetina	JJS	Altay, XJ	47°49′	88°10′	1291	7	7	H11 (14)	1			2			2	5	2*x*
	SBG	Erdos, IM	39°40′	106°59′	1443	0	3	H12 (3)	1			2			2	3	2*x*
	SM	Alxa Zuoqi, IM	38°57′	105°52′	1924	5	10	H1 (1), H12 (14)	2			2			1	5	2*x*
	XHD	Zhangjiakou, HB	40°30′	115°20′	811	0	6	H12 (6)	1			2			2	5	2*x*
	XWT	Zhangjiakou, HB	39°55′	114°59′	1300	0	10	H12 (2), H15 (8)	1			2			2	5	2*x*
E. gerardiana	DZ	Tingri, Tibet	28°17′	86°48′	4737	16	0	H23 (16)	1			2			2	5	2*x*
	RCZ	Rikaze, Tibet	29°16′	88°51′	4000	24	0	H16 (24)	1		1	2		2	1	5	4*x*
	ZXG	Gar, Tibet	32°24′	79°44′	4700	24	0	H22 (24)	1			1			2	5	2*x*
E. glauca	WBB	Urumqi, XJ	43°37′	87°57′	1450	5	0	H1 (1), H3 (4)	1	2		2	1		1	5	4*x*
E. intermedia	GT	Minhe, QH	35°53′	102°48′	1866	5	0	H1 (5)	2	1		2	2		1	5	4*x*
	MNS	Manas, XJ	44°14′	86°20′	478	5	0	H10 (5)	2	2		2	1		1/2	5	4*x*
	QHH	Qinghai Lake, QH	36°33′	100°28′	3565	22	0	H1 (22)	1	1		2	1		1	5	4*x*
E. likiangensis	AN	Jinchuan, SC	31°17′	101°59′	2900	21	0	H12 (21)	1		1	2		1	1	5	4*x*
	GHZ	Lijiang, YN	27°07′	100°15′	3040	4	0	H13 (4)	1		1	1		2	1	4	4*x*
	MRK	Barkam, SC	31°54′	102°13′	2800	23	0	H12 (23)	1		2	1		1	1	5	4*x*
E. minuta	HZ	Huzhu, QH	36°53′	102°21′	2960	3	0	H12 (3)	1			1			1	3	2*x*
	QT	Ledu, QH	36°15′	102°15′	3358	20	0	H12 (20)	1			1			1	5	2*x*
E. monosperma	JFS	Baotou, IM	40°41′	110°45′	2198	0	28	H12 (28)	2			1			1	5	2*x*
	KBX	Zhangjiakou, HB	42°02′	114°47′	1691	0	15	H12 (8), H14 (7)	1			2			1	10	2*x*
	MGD	Mongolia	43°29′	104°03′	2279	0	4	H14 (4)	1			1			2	4	2*x*
	MGE	Mongolia	43°01′	106°04′	2200	0	4	H14 (4)	1			2			1	4	2*x*
	NMC	Damxung, Tibet	30°46′	90°52′	4800	8	0	H12 (8)	2			1			1	5	2*x*
	YMT	Delhi, QH	37°21′	98°07′	3473	17	0	H12 (17)	2			2			1	5	2*x*
	YX	Urumqi, XJ	43°19′	87°12′	2033	6	9	H12 (15)	2			1/2			1	5	2*x*
E. przewalskii	GB	Delhi, QH	37°16′	97°10′	2910	5	12	H1 (17)	1	1			1			5	2*x*
	GSE	Golmud, QH	36°13′	94°48′	3053	0	17	H1 (17)	1	1			1			5	2*x*
	KLMY	Karamay, XJ	45°33′	84°49′	360	0	16	H4 (1), H8 (15)	1	1			1			5	2*x*
	MGF	Mongolia	44°35′	101°42′	1597	0	12	H1 (6), H3 (6)	1	1			2			5	4*x*
	NCT	Golmud, QH	35°53′	94°22′	3716	0	12	H1 (12)	1	1			1			5	2*x*
	WB	Urumqi, XJ	43°33′	87°53′	1128	5	14	H3 (19)	1	2			1			5	4*x*
E. regeliana	BYG	Urumqi, XJ	43°25′	87°13′	1723	5	0	H2 (5)	1	2			1			5	2*x*
	XAB	Burqin to Altay, XJ	47°40′	86°48′	543	6	2	H4 (5), H9 (3)	1	3			2			5	4*x*
	XJJ	Urumqi, XJ	43°39′	87°39′	1130	5	3	H2 (8)	1	1			1			5	2*x*
	XZN	Shawan, XJ	43°57′	85°46′	1159	5	12	H2 (17)	1	2			1			5	2*x*
E. rhytidosperma	HLB	Alxa Zuoqi, IM	38°51′	105°50′	2150	6	0	H12 (6)	1	D1			D1			5	2*x*
	XK	Yinchuan, NX	38°36′	105°56′	1500	6	0	H12 (6)	1	D1			D1			5	2*x*
E. saxatilis	JC	Gyaca, Tibet	29°08′	92°35′	3249	25	0	H16 (5), H17 (20)	1		2	2		1	1	5	4*x*
	LX	Nang, Tibet	29°02′	93°04′	3100	29	0	H17 (29)	2		1	1/2		1	1/2	5	4*x*
	PM	Bomi, Tibet	29°51′	95°45′	2700	20	0	H17 (20)	1		2	2		1	2	5	4*x*
	YD	Yadong, Tibet	27°31′	88°56′	3600	25	0	H17 (25)	1		2	1		1	1	5	4*x*

Table 1 *Continued*

Species	Pop.	Location	Lat. (N)	Long. (E)	Alt. (m)	N^a	N^b	Haplotypes (Individuals)	N^c	*LFY*			*DDB2*			N^d	Ploidy level
										C_A	C_B	C_C	C_A	C_B	C_C		
E. saxatilis var. *mairei*	DC	Daocheng, SC	28°26′	100°21′	3936	12	0	H12 (3), H18 (4), H19 (5)	1		1	2		1	2	5	4*x*
	LY	Lijiang, YN	27°03′	100°11′	3744	24	0	H18 (24)	1		1	2		1	2	5	4*x*
	YJG	Kangding, SC	29°55′	102°00′	4100	28	0	H20 (23), H21 (5)	1		1	2		1	1	5	4*x*
E. sinica	KBD	Zhangjiakou, HB	42°02′	114°51′	1425	0	18	H5 (18)	1	2		1	1		1	10	4*x*
	KSKT	Hexigten Qi, IM	43°34′	117°10′	1350	13	0	H5 (13)	2	1		2	1		1/2	5	4*x*
	LWG	Baotou, IM	40°35′	110°39′	1028	0	31	H5 (31)	1	2		2	2		1	5	4*x*
	MGT	Erdos, IM	38°29′	107°30′	1350	0	40	H1 (32), H5 (6), H6 (1), H7 (1)	1	2		2	2		2	5	4*x*
	XHY	Zhangjiakou, HB	40°30′	115°20′	787	0	21	H5 (21)	2	2		1	1		2	10	4*x*
All species	48					740										248	
E. foeminea		Saudi Arabia					1	H24 (1)	1	1			1				

YN, Yunnan; SC, Sichuan; QH, Qinghai; NX, Ningxia; IM, Inner Mongolia; XJ, Xinjiang; HB, Hebei; Pop., population; Lat., latitude; Long., longitude; Alt., altitude. N^a, number of individuals sampled for analysis of cytoplasmic DNAs by Qin *et al*. (2013); N^b, number of individuals sampled for analysis of cytoplasmic DNAs in this paper; N^c, number of individuals sampled for analysis of nuclear genes; N^d, number of individuals sampled for analysis of flow cytometry. Numbers of alleles in C_A, C_B and C_C correspond, respectively, to those in clade A, clade B and clade C in the phylogenies of *DDB*2 and *LFY*, and D1 indicates that one allele was placed in clade D. 1/2, one individual has one allele and the other has two alleles.

monoecious, but Florin (1933) reported that monoecious individuals are common in *Ephedra*. The other three species are tetraploids based on previous studies (e.g. Leitch *et al.* 2001) and our preliminary investigation. Therefore, the exclusion of these species should not greatly affect our inference about the origins of other tetraploid species (mostly allotetraploids, see Results).

Information on the geographical variation of cytotypes is critical for studies of origin and evolution of polyploids. To determine the ploidy levels, we initially analysed 10 individuals from each of the three populations KBD, XHY and KBX, and did not find ploidy variation within populations. Consequently, we analysed five individuals from each of the other populations with the exception of four populations (SBG, GHZ, MGD and MGE), from which fewer than five individuals were available. To investigate variation patterns and evolutionary relationships of the maternally inherited cpDNA, sequences of two fragments (*trn*T-*trn*F and *trn*S-*trn*fM) were analysed for a total of 740 samples, including 306 individuals sampled in this study and 434 individuals reported in Qin *et al.* (2013).

Based on the analyses of ploidy and cpDNA variation, we further chose 58 individuals to explore nuclear gene relationships of the 12 species, one with two varieties. Also, one individual of the Saudi Arabian *E. foeminea* was sampled as outgroup based on the results of previous phylogenetic analyses (Ickert-Bond *et al.* 2009; Rydin & Korall 2009). The samples were consistently used in the three analyses (ploidy, cpDNA and nuclear genes), with the exception that different sample sizes were used.

Chromosome number counts

Chromosome numbers were counted for two species, *E. equisetina* (cultivated in the Beijing Botanical Garden) and *E. intermedia*. Fresh root tips were pretreated with 0.01% colchicine solution for 5–6 h and fixed in a mixture of ethanol/acetic acid (3:1) for 12 h at room temperature. After being macerated in 1N HCl at 60 °C for 5–10 min, the materials were stained with 1% carbolfuchsin, and then were squashed and observed under a light microscope. The chromosome number of each species was counted based on at least five cells.

Determination of ploidy levels

The flow cytometry (FCM) has made it convenient to detect the variation of DNA ploidy level in large samples from herbaria and silica gel-dried materials (Suda & Trávníček 2006; Schönswetter *et al.* 2007; Krejcikova *et al.* 2013; Vrána *et al.* 2014). In *Ephedra*, the leaves are reduced to small membranous sheaths. Therefore, we used the silica gel-dried young branchlets for the FCM measurement, mainly following the protocol of Suda & Trávníček (2006). Approximately 0.3 g silica gel-dried branchlets per individual was chopped with a razor blade in a Petri dish containing 1 mL of Otto I buffer (0.1 mol/L citric acid monohydrate, 0.5% (v/v) Tween-20, pH 2–3). After filtering through a 50-μm nylon mesh and centrifuging at 100 g for 8 min, the pellet was resuspended in 200 μL buffer of a 1:2 mixture of Otto I and Otto II (0.4 mol/L Na_2HPO_4 $12H_2O$) and stained with 50 μg/mL PI including 50 μg/mL RNase. The FCM measurements were taken using an Elite flow cytometer (BD FACSCalibur, USA). To guarantee the reliability of the measurements, several samples were reanalysed (up to four times) on different days to assess between-run fluctuations, and the results showed that the measurements are very consistent. If the coefficient of variation (CV) of the histogram peak exceeded 5%, the sample was discarded or remeasured. The DNA ploidy levels were inferred based on DNA contents measured in plants with known chromosome numbers. That is, based on chromosome number counts (see Results), the two species of *Ephedra*, *E. equisetina* (2n = 14, diploid) and *E. intermedia* (2n = 28, tetraploid), were used as external reference standards, with their DNA contents measured and shown in Fig. S1 (Supporting information).

DNA extraction, PCR amplification, cloning and sequencing

Total DNA was isolated from silica gel-dried young branchlets by the modified CTAB method (Rogers & Bendich 1985). Two cpDNA regions, *trn*T-*trn*F and *trn*S-*trn*fM, were amplified and sequenced following the protocols of Qin *et al.* (2013). The *LFY* gene was amplified with the forward primer *LFY*E2F2 (5′- GACAGTTGGTG CTTTAATAGG -3′) located at the second intron and the reverse primer *LFY*E3R1 (5′- CCTCATCTTTGGCTTGTT-TAT -3′) at the third exon, and the *DDB*2 gene was amplified with *DDB*2S2 at the second exon (5′- ACAG CCAGGTGATTGTTATGAG -3′) and *DDB*2A1 at the fifth exon (5′- TCTAAGGAGGTGACCCGTCTACT -3′).

The PCRs were conducted in a volume of 25 mL, containing 50–75 ng total DNA, 6.25 pmol of each primer, 200 mmol/L of each dNTP and 0.75 unit of Taq DNA polymerase (TaKaRa Biotech Co., China). PCR cycles were as follows: 4 min at 94 °C, 36 cycles of 30 s at 94 °C, 30 s at 58 °C and 1–2 min at 72 °C, with a final extension of 10 min at 72 °C. The PCR products were purified using a TIANgel Midi Purification Kit (TIANGEN, China) for the nuclear markers and then cloned with the pGEM-T Easy Vector System II

(Promega). For each individual, 6–20 clones (6–12 for diploids, and 8–20 for tetraploids and the outgroup) were sequenced using primer T7 or *LFY* E3R1. The sequencing products were separated on an ABI PRISM 3730XL DNA analyzer (Applied Biosystems). The sequences generated in this study are deposited in GenBank under accession numbers KT033384–KT033389 (*trn*S-*trn*fM), KT033390–KT033395 (*trn*T-*trn*F), KT033145–KT033275 (*LFY*) and KT033276–KT033383 (*DDB*2).

Data analyses

The DNA sequences were aligned and manually adjusted in BIOEDIT v.7.0.9 (Hall 1999). Haplotype networks were constructed with NETWORK 4.6.1.2 (Bandelt *et al.* 1999), and each indel was treated as a single mutation event. Phylogenetic trees of cpDNA haplotypes and the two nuclear genes were constructed by maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) methods, using PAUP*4.0b10 (Swofford 2002), PHYML3.0 (Guindon *et al.* 2010) and MRBAYES 3.1.2 (Ronquist & Huelsenbeck 2003), respectively. The gaps were treated as missing data.

The MP analysis used a heuristic search with 1000 random addition sequence replicates, tree-bisection–reconnection (TBR) and MULTREES on. Branch support was evaluated by a bootstrap analysis (Felsenstein 1985) of 1000 replicates using the same heuristic search settings, and a 50% majority-rule consensus was used. In the ML analysis, JMODELTEST 2 (Guindon & Gascuel 2003; Darriba *et al.* 2012) was used to determine the best-fit nucleotide substitution models under the Akaike Information Criterion (AIC), which were GTR+I for cpDNA, TVM+G for *LFY* and TVM+I for *DDB*2. As a starting point for the ML search, a BIONJ tree was used (Gascuel 1997). Branch support was estimated by bootstrap analysis with 1000 replicates. The BI analysis used the best-fit models determined also by JMODELTEST 2, including GTR+G for cpDNA [nst = 6, rates = gamma, Prset statefreqpr = dirichlet(1,1,1,1)], GTR+G for *LFY* [nst = 6, rates = gamma, Prset statefreqpr = dirichlet(1,1,1,1)] and HKY+G for *DDB*2 [nst = 2, rates = gamma, and Prset statefreqpr = dirichlet(1,1,1,1)]. One cold and three incrementally heated Markov chain Monte Carlo (MCMC) chains were run for 1 000 000 generations each, and trees were sampled every 100 generations with the first 300 samples discarded as burn-in.

To show explicitly the origin, particularly the putative progenitors, of the allopolyploid species, the program PADRE (Lott *et al.* 2009a,b) was used to generate a reticulate phylogenetic network of the studied species based on a collection of *LFY*, *DDB*2 and cpDNA trees under default settings. The input topologies were 50% majority-rule consensus MP trees that were generated from the reduced matrices comprising 13 species (including outgroup) and 19 representative individuals, which included one allele (diploids) or two alleles (polyploids) distributed in different main clades of the two nuclear gene phylogenies.

Ecological niche analysis

To investigate whether there was an association between speciation and climatic factors, ecological niche divergence among the species was also investigated. A total of 557 georeferenced occurrence records (herbarium collections at the Chinese Virtual Herbarium (CVH) http://www.cvh.org.cn/search; records in Freitag & Maier-Stolte 1994; and extensive field surveys by ourselves) were collected for 13 taxa of *Ephedra*, including 49 for *E. equisetina*, 12 for *E. gerardiana*, 9 for *E. glauca*, 180 for *E. intermedia*, 16 for *E. likiangensis*, 5 for *E. minuta*, 74 for *E. monosperma*, 63 for *E. przewalskii*, 76 for *E. regeliana*, 3 for *E. rhytidosperma*, 17 for *E. saxatilis*, 9 for *E. saxatilis* var. *mairei* and 44 for *E. sinica* (Fig. S2, Supporting information). We retrieved 19 bioclimatic layers (BIO1–BIO19) from Worldclim (http://www.worldclim.org; Hijmans *et al.* 2005) and the mean annual potential evapotranspiration (PET) from CGIAR-CSI (http://www.cgiar-csi.org; Trabucco *et al.* 2008) at a resolution of 30 arc seconds (about 1 km) based on the geocoordinates using ARCGIS 10.2. The 20 bioclim variables were examined for pairwise Pearson correlations, and 10 variables (BIO2, 3, 4, 8, 12, 14, 15, 18, 19 and PET, shown in Table S2, Supporting information) with correlation coefficients lower than 0.8 were finally selected.

To determine the ecological characteristics of all studied species in the QTP and neighbouring areas and investigate whether ecological divergence had driven polyploid speciation, we used two approaches. First, to identify the divergence of ecological niches, a principal component analysis (PCA) using 10 bioclim variables implemented in ADE4-R package (Dray *et al.* 2007) was conducted for all taxa, and allotetraploids vs. their putative progenitors, respectively. Then, to identify bioclim variables associated with speciation, a permutational analysis of variance (PERMANOVA) was performed to assess the variation of principal components (PC1 and PC2) and each bioclim variable among and within species using the LMPERM package (Wheeler 2010). The principal components and bioclim variables with significant differences indicated by the ANOVA were further assessed by the Tukey's honestly significant difference (HSD) test for every two taxa using the STATS package. The variation of these variables was shown by boxplots. The analyses of PCA, ANOVA and Tukey's HSD were

conducted in R version 3.1.2 (R Development Core Team, 2014).

Results

Variation in ploidy levels

To confirm the chromosome numbers of the two samples used as external standards for the FCM analysis, we observed mitotic cell divisions in root tips by light microscopy and found that the chromosome number was 2n = 14 for *E. equisetina* and 2n = 28 for *E. intermedia*. Therefore, the FCM fluorescence histograms of the two samples, as shown in Fig. S1 (Supporting information), were used to represent diploid (2*x*) and tetraploid (4*x*) nuclei, respectively. The peak ratio of *E. intermedia* to *E. equisetina* is 1.98. For all the *Ephedra* samples (12 species, 48 populations and 248 individuals) analysed by FCM, none of the CV values exceeded 5%. Based on the peak positions that indicate the relative DNA contents, the fluorescence histograms could be clearly divided into two cytotypes, corresponding to diploids and tetraploids, respectively. The peak ratios of diploids to *E. equisetina* ranged from 0.95 to 1.08, and those of tetraploids to *E. intermedia* ranged from 0.94 to 1.07. The FCM measurement indicated that three species (*E. gerardiana*, *E. przewalskii* and *E. regeliana*) harboured both diploid and tetraploid cytotypes, four species (*E. equisetina*, *E. minuta*, *E. monosperma* and *E. rhytidosperma*) showed only the diploid cytotype, and six taxa (*E. likiangensis*, *E. glauca*, *E. intermedia*, *E. saxatilis*, *E. saxatilis* var. *mairei* and *E. sinica*) exhibited only the tetraploid cytotype (Table 1).

In our survey, two ploidy levels (2n = 14, 28) were found in different populations of *E. przewalskii* and *E. regeliana* (Table 1), from which only diploids were previously reported (Kong *et al.* 2001; Jiang 2006; Wu *et al.* 2009). Moreover, for some species which were reported to have two or more cytotypes (Table S3, Supporting information), we only found a single cytotype, such as only diploids in *E. equisetina* and *E. monosperma* (Table 1).

Distributions and evolutionary relationships of cpDNA haplotypes

The *trn*T-*trn*F and *trn*S-*trn*fM sequences were obtained from all of the 740 samples, including 306 individuals determined in this study and 434 individuals reported in Qin *et al.* (2013) (Table 1). The new sequences generated are mainly from populations in northern China. The alignment of the combined two cpDNA fragments is 1115 bp in length, including 27 nucleotide substitutions and eight indels that were used to designate 24 haplotypes (H1-H23 for ingroups and H24 for outgroup, see Table S1, Supporting information), of which five (H6–8, H14, H15) were newly detected. The distributions of the ingroup haplotypes are shown in Fig. 1.

When the sequence of *E. foeminea* (H24) was used as outgroup, three main lineages (I–III) were consistently resolved in the network and phylogenetic tree of cpDNA haplotypes (Figs 2 and S3). Lineage I consisted of 10 haplotypes (H1–H10) that occurred in five species (*E. glauca*, *E. intermedia*, *E. przewalskii*, *E. regeliana* and *E. sinica*) mainly distributed in northern China, but only three of them were shared among species, including H1 among *E. glauca*, *E. intermedia*, *E. przewalskii* and *E. sinica*, H3 between *E. przewalskii* and *E. glauca*, and H4 between *E. przewalskii* and *E. regeliana*. Lineage II comprised four haplotypes (H12–H15), of which H12 was the most widely distributed, and was shared by six species (*E. equisetina*, *E. likiangensis*, *E. minuta*, *E. monosperma*, *E. rhytidosperma* and *E. saxatilis* var. *mairei*). Lineage III harboured eight haplotypes (H16–H23) that occurred in two species and a variety (*E. gerardiana*, *E. saxatilis* and *E. saxatilis* var. *mairei*) from the QTP. The haplotype H11 detected in the population JJS of *E. equisetina* was not grouped into any of the three lineages. Of the 48 *Ephedra* populations analysed, 37 (77%) harboured a single haplotype, 9 (19%) exhibited two haplotypes, and only two had more than two haplotypes (Fig. 1; Table 1).

Distributions and evolutionary relationships of the nuclear gene alleles

For each of the nuclear genes *LFY* and *DDB*2 that were PCR-amplified and cloned, 1–2 distinct clones (alleles) were obtained from each diploid individual, and 2–4 alleles were detected in each tetraploid individual, with the exception of two tetraploid individuals of *E. przewalskii* from populations MGF and WB, each of which contained only 1–2 alleles (Table 1). The distributions of the alleles of *LFY* and *DDB*2 are shown in Figs 3 and 4, respectively.

The *LFY* gene sequences (alleles) were 462–791 bp in length, and the sequence alignment contained 886 sites, of which 244 were variable and 167 were parsimony-informative. The *DDB*2 gene sequences ranged from 546 to 551 bp, and the sequence alignment contained 554 sites, of which 87 were variable and 52 were parsimony-informative.

The generated MP, ML and BI trees of each gene were highly congruent, and the *LFY* and *DDB*2 gene trees were also congruent in deep branches (Fig. S4, Supporting information). The simplified strict consensus MP trees of the two genes are shown in Fig. 5, both strongly supporting clades A, B, C and D. Clade A

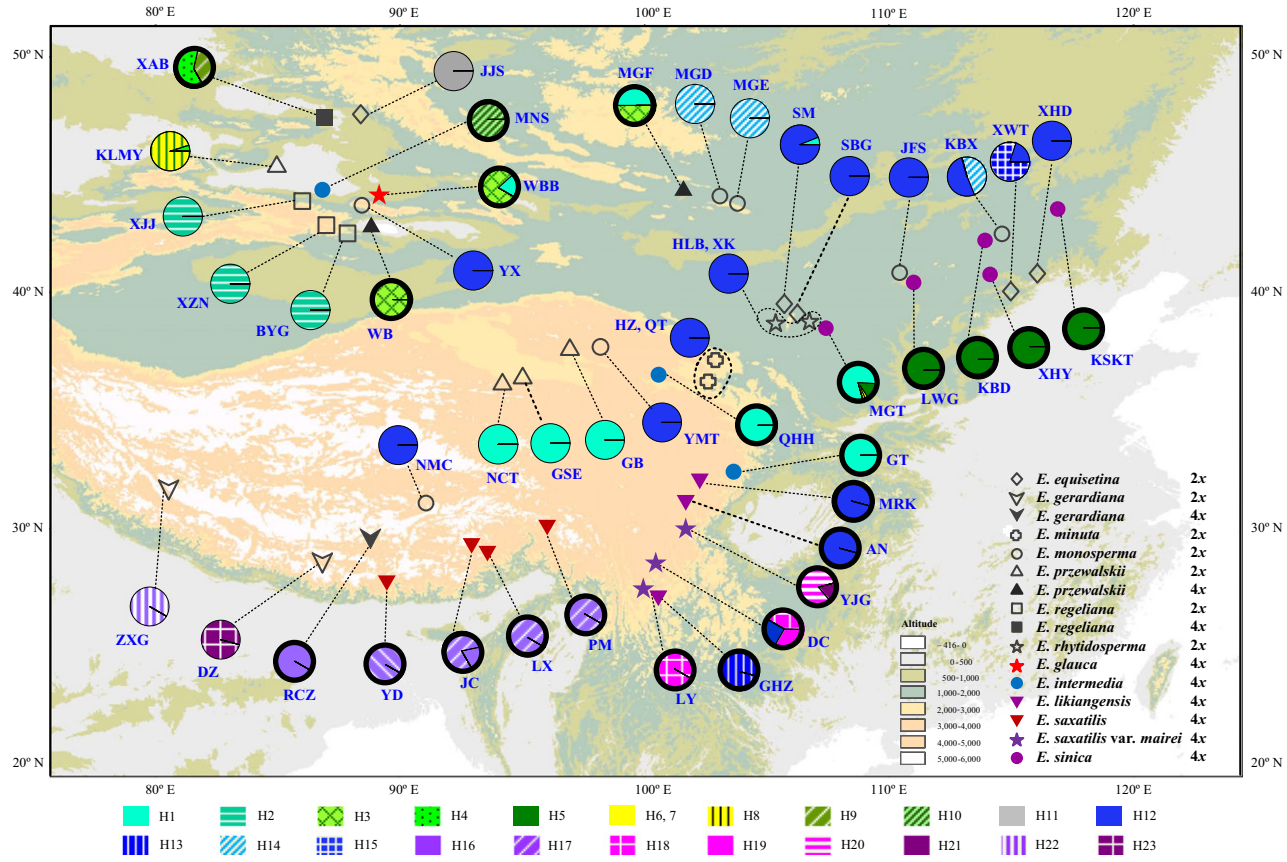


Fig. 1 Sampling locations and distribution frequencies of the cpDNA haplotypes detected in the studied 12 *Ephedra* species. Thick and thin outlines of the pie charts indicate tetraploids and diploids, respectively. Population names correspond to those in Table 1.

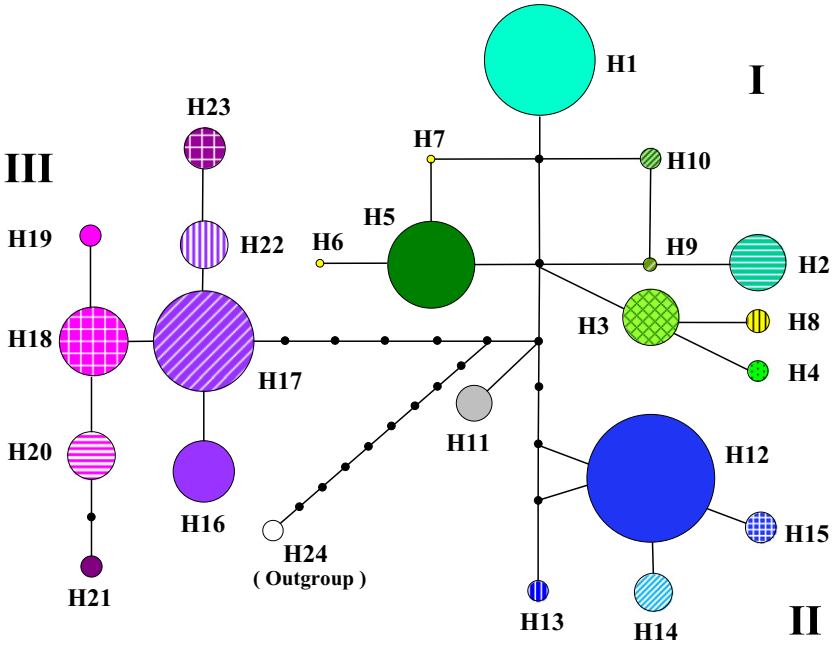


Fig. 2 A network of the cpDNA haplotypes constructed by NETWORK 4.6.1.2. The sizes of the circles in the network are proportional to the observed frequencies of the haplotypes.

(A-type) sequences (alleles) were from northern China and northern QTP, clade B (B-type) alleles occurred in southern and eastern QTP, and clade C (C-type) alleles had a very wide distribution (Figs 3 and 4). The clade D (type D) sequences were all from *E. rhytidosperma*, a species narrowly distributed in the Helan Mountain.

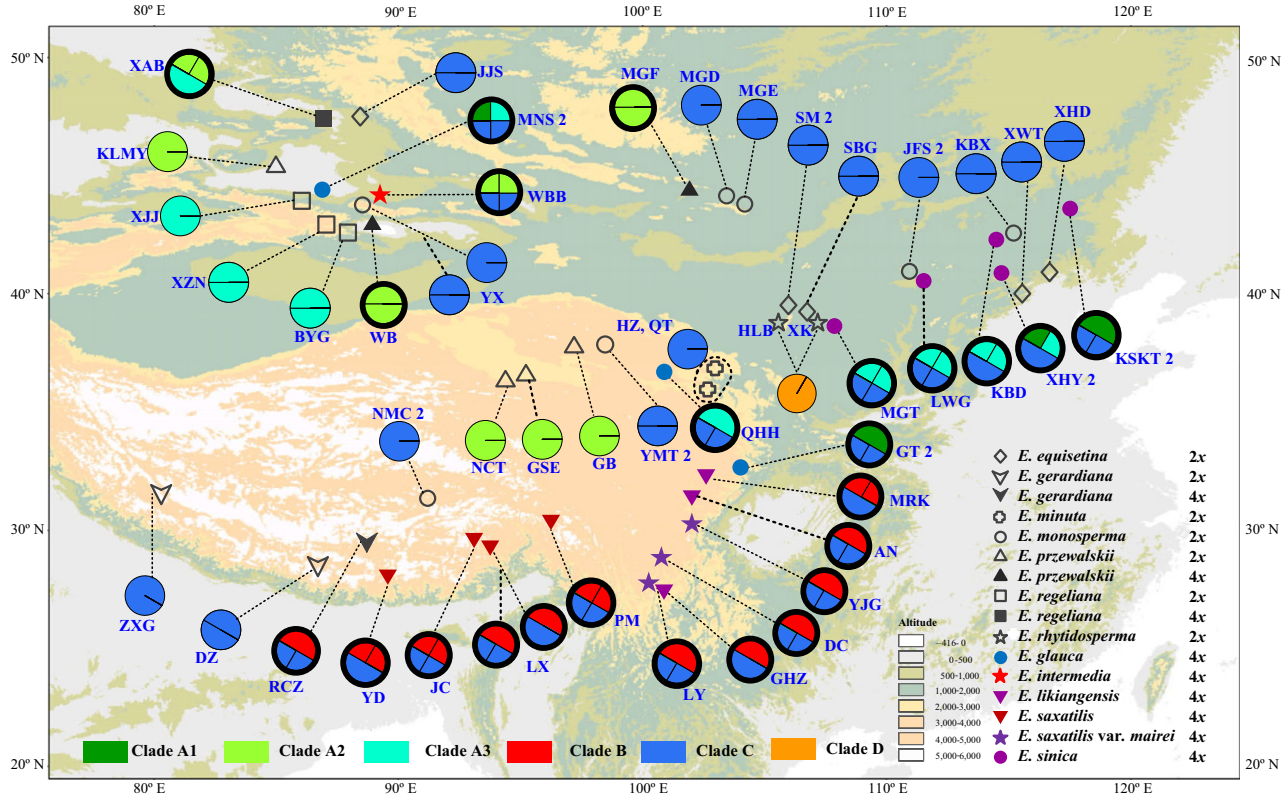


Fig. 3 Sampling locations and distributions of the *LFY* alleles detected in the studied *Ephedra* species. Pie charts show the proportions of alleles, and thick and thin outlines indicate tetraploids and diploids, respectively. The number 2 following a population name indicates that two individuals were studied. Different colours of the pie charts indicate the positions of the alleles in the gene phylogenies shown in Fig. 5. Population names correspond to those in Table 1.

The diploid species or populations contained only one type of sequences, type A (diploid populations of *E. przewalskii* and *E. regeliana*), type C (*E. equisetina*, *E. minuta*, *E. monosperma*, and diploid populations of *E. gerardiana*) or type D (*E. rhytidosperma*). In contrast, the tetraploid species contained two types of sequences, types A and C (*E. glauca*, *E. intermedia* and *E. sinica*) or types B and C (*E. likiangensis*, *E. saxatilis*, *E. saxatilis* var. *mairei*, and the tetraploid population of *E. gerardiana*). However, the tetraploid populations of *przewalskii* and *E. regeliana* only had A-type sequences (Fig. 5).

Reticulate network

A phylogenetic network generated from the integration of all three gene trees (cpDNA, *LFY* and *DDB2*) is shown in Fig. 6, from which eight allotetraploid taxa could be inferred, including *E. gerardiana*, *E. glauca*, *E. intermedia*, *E. likiangensis*, *E. regeliana*, *E. saxatilis*, *E. saxatilis* var. *mairei* and *E. sinica*. The diploid progenitors of these allotetraploids were not well resolved, but it seems that all studied diploid ingroup species, with the exception of *E. rhytidosperma*, could have been involved. For example, the three tetraploids *E. glauca*, *E. intermedia* and *E. sinica* possibly originated from hybridization with diploids most closely related to *E. przewalskii* in clade A as the maternal parents and diploids of clade C (*E. equisetina*, *E. minuta* and *E. monosperma*) as the paternal parents (see Discussion). The diploid *E. regeliana* does not share chlorotypes with any of the tetraploids, and therefore is not very likely to have acted as a maternal parent in the allotetraploid speciation.

Ecological differentiation

More than 10 georeferenced occurrence records were collected for each of the studied species, with the exception of *E. glauca*, *E. minuta* and *E. rhytidosperma* due to their narrow distributions. Results of the PCAs are shown in Figs 7 and 8, with the factor loadings shown in Table S2 (Supporting information).

For all of the 13 taxa, the PCA revealed two components that cumulatively explained 60.11% of variation, and the scatter plot showed that these taxa were clearly divided into two groups by PC1 and PC2 (Fig. 7).

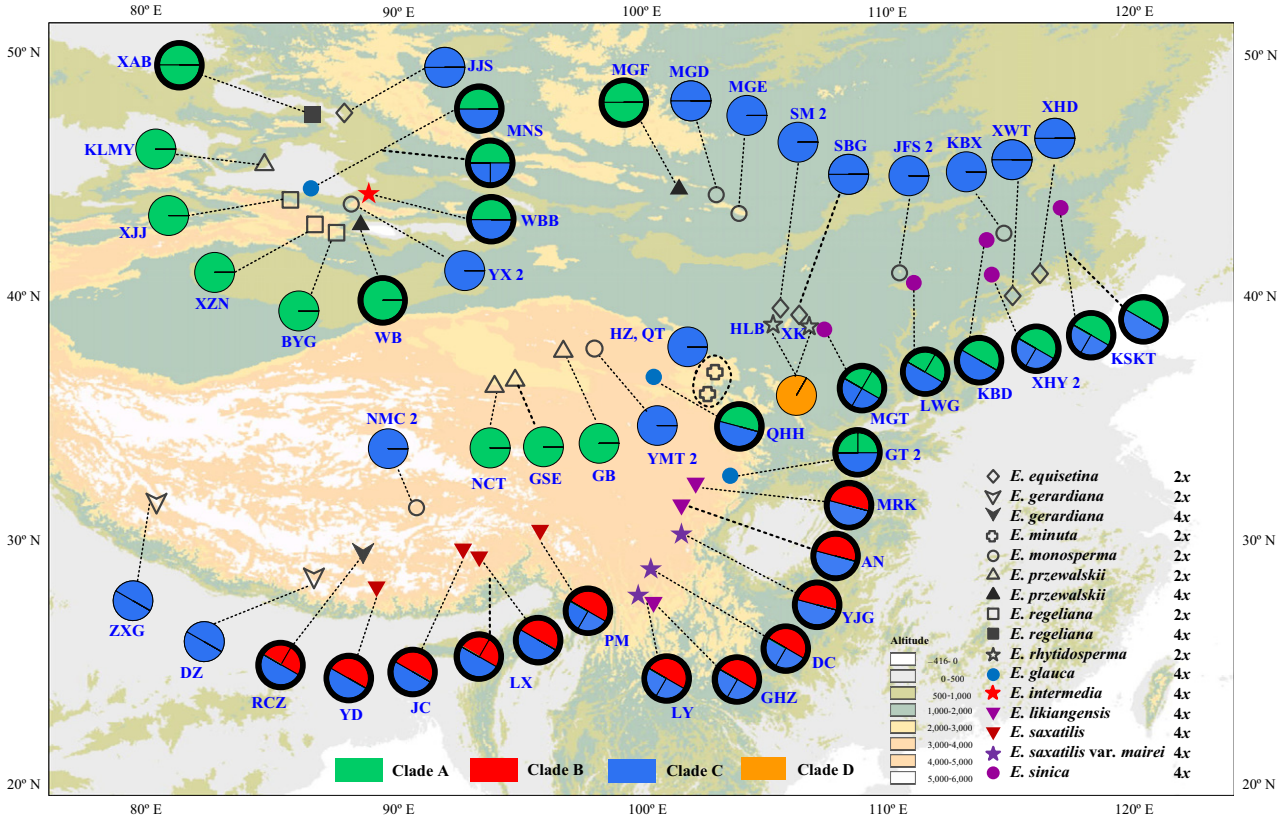


Fig. 4 Sampling locations and distributions of the *DDB2* alleles detected in the studied *Ephedra* species. Pie charts show the proportions of alleles, and thick and thin outlines indicate tetraploids and diploids, respectively. The number 2 following a population name indicates that two individuals were studied. Different colours of the pie charts indicate the positions of the alleles in the gene phylogenies shown in Fig. 5. Population names correspond to those in Table 1.

Group 1 include four taxa distributed in the south and east of QTP (*E. gerardiana*, *E. likiangensis*, *E. saxatilis* and *E. saxatilis* var. *mairei*), which show a distinct ecological niche with higher isothermality (BIO3) and precipitation in the warmest quarter (BIO18) and a lower temperature seasonality (BIO4), and group 2 comprise the remaining nine taxa. Although the ANOVAs detected significant differences of 10 bioclim variables among the 13 taxa, the Tukey's HSD tests indicated that only two bioclim variables (BIO3 and BIO4) were significantly differentiated between group 1 and group 2 species ($P < 0.01$; Table S4 and Fig. S5A, B, Supporting information), with the exception of a nonsignificant difference between group 1 species and *E. minuta* in BIO4. In addition, *E. likiangensis* and *E. saxatilis* var. *mairei* show significant differences from group 2 species in BIO12 and BIO18 ($P < 0.001$ for HSD; Fig. S5C, D, Supporting information). The two taxa occur in a moist climate with higher annual precipitation (BIO12) and precipitation of the warmest quarter (BIO18).

For comparisons between the allotetraploids and their putative progenitors, the PCA revealed two components (PC1 and PC2) that collectively explained 55.63–67.74% of variation (Table S2, Supporting information). All but one (*E. saxatilis*) of the allotetraploids show ecological divergence from their putative progenitors (Fig. 8). Two divergence patterns were found: (i) the allotetraploids, including *E. likiangensis* and *E. saxatilis* var. *mairei*, occupy separate ecological niches from their putative maternal progenitors; and (ii) the allotetraploids, including *E. glauca*, *E. intermedia* and *E. sinica*, show separate ecological niches from one of their putative paternal progenitors but have partially overlapped ecological niches with their other putative progenitors. For example, the niche of *E. glauca* is completely different from that of *E. minuta*, but is slightly overlapped with that of *E. monosperma* and more overlapped with those of *E. equisetina* and *E. przewalskii* (Fig. 8a); *E. intermedia* has a much wider niche than *E. minuta* and a separate niche from *E. equisetina*, and is clearly differentiated from *E. equisetina*, *E. monosperma* and *E. przewalskii* along PC2 (Fig. 8b). The allotetraploid *E. saxatilis* has a similar niche with its putative maternal progenitor *E. gerardiana* (Fig. 8d).

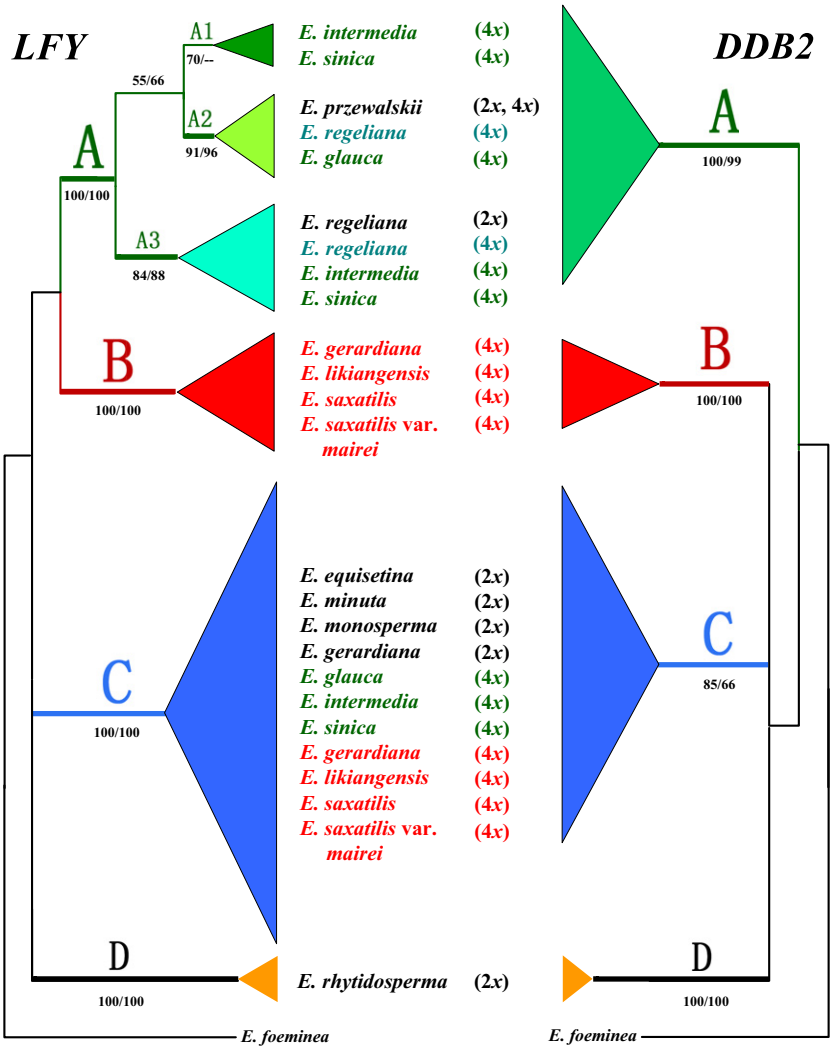


Fig. 5 Majority-rule consensus trees obtained from maximum parsimony analysis of the *LFY* and *DDB2* data sets. Numbers associated with branches are bootstrap percentages of MP and ML greater than 50%, respectively. Bold lines indicate Bayesian posterior probabilities greater than 0.90. Diploids and autotetraploids are in black, and allotetraploids are in colour.

Significant ecological divergences between the allotetraploids and their putative progenitors were also detected by the ANOVA and Tukey's tests of the two principal components (PC1, PC2) and the 10 bioclim variables (Tables 2 and S4). Results of the ANOVA indicate that the mean squares of all comparisons among species are higher than those within species with the exception of the comparison between *E. saxatilis* and *E. gerardiana*, and almost all interspecific divergences are significant ($P < 0.05$; Table S4, Supporting information). The Tukey's test detected significant niche divergence between the allotetraploids and their putative progenitors in 2–7 bioclim variables (Table 2). The divergence between *E. glauca* and *E. minuta* and between *E. glauca* and *E. monosperma* occurred, respectively, in seven and five bioclim variables, which is consistent with the results of the PCA. Compared to *E. minuta* and *E. monosperma*, *E. glauca* is higher in mean temperature of the wettest quarter (BIO8) and annual potential evapotranspiration (PET), intermediate in annual precipitation (BIO12), and lower in precipitation seasonality (BIO15) and precipitation of the warmest quarter (BIO18) (Fig. S5E–I, Supporting information). Relative to their putative progenitors, *E. intermedia* is lower in temperature seasonality (BIO4) and BIO8 but higher in BIO12 and precipitation of the coldest quarter (BIO19), with the exception of the comparison between it and *E. minuta* (Fig. S5J–M, Supporting information), and *E. likiangensis* is higher in isothermality (BIO3), BIO12, BIO18 and PET but lower in BIO4, with the exception of the comparison between it and *E. minuta* in BIO4 (Fig. S5N–R, Supporting information). Like *E. likiangensis*, *E. saxatilis* var. *mairei* is higher in BIO3, BIO12 and BIO18 except the comparison between it and *E. gerardiana* in BIO3 (Fig. S5S–U, Supporting information). The allotetraploid *E. sinica* also shows niche divergence from most of its putative progenitors and tends to occupy a niche with higher

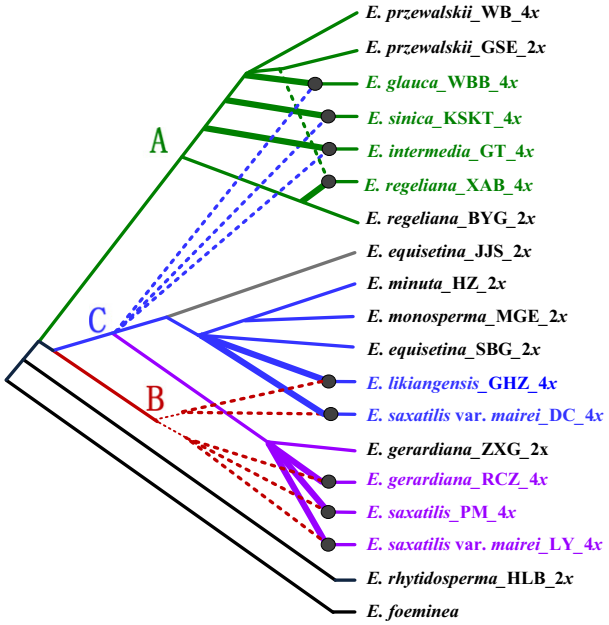


Fig. 6 A reticulate network constructed from the reduced 50% majority-rule consensus MP trees of *LFY*, *DDB2* and cpDNA using the program PADRE. Different colours of lines indicate the positions of the species in the cpDNA network (lineages I–III in Fig. 2) and the alleles in the two nuclear gene phylogenies (clades A–C in Fig. 5): green, lineage I, clade A; blue, lineage II, clade C; purple, lineage III, clade C; red, clade B; grey, the lineage of cpDNA haplotype H11. Bold solid and dashed lines represent putative maternal and paternal progenitors of the tetraploids, respectively. Diploids and autotetraploids are in black, and allotetraploids are in colour. Letters following species names are population names corresponding to those in Table 1.

BIO12, BIO15 and PET (Fig. S5V–X, Supporting information).

Discussion

A high frequency of allopolyploid speciation in Ephedra in the QTP and its vicinities

All tetraploid species of *Ephedra* from the QTP and adjacent regions originated by allopolyploid speciation. Phylogenetic analysis of single-/low-copy nuclear genes can be effective in revealing allopolyploid parental lineages (e.g. Ge *et al*. 1999; Ferguson & Sang 2001; Kim *et al*. 2008; Cai *et al*. 2012; Yang *et al*. 2012b; Kelly *et al*. 2013), when an allopolyploid species, particularly of recent origin, has paralogous sequences inherited from its two or more parental species. These paralogues can place an allotetraploid in different parental clades. In the present study, the FCM measurement of the ploidy levels in a number of individuals from different populations indicates that six (46%) of the studied 13 *Ephedra* taxa, including *E. glauca*, *E. intermedia*, *E. likiangensis*, *E. saxatilis*, *E. saxatilis* var. *mairei* and *E. sinica*, are tetraploids (Table 1). In both single-copy nuclear gene trees (*LFY* and *DDB2*), each of the six taxa contains two types of sequences that are distributed in different major clades A and C or B and C (Figs 5 and 6), strongly suggesting an allopolyploid origin involving diploid parents from these clades. Compared to clades A and C, clade B only contains tetraploids. This could be attributed to the extinction or lack of sampling of diploids in this clade.

The three tetraploid species *E. glauca*, *E. intermedia* and *E. sinica* exhibit nuclear gene alleles in both clades A and C (Fig. 5). These species are mainly distributed in northern China, and their chlorotypes belong to lineage I which is also confined to northern China (Figs 1 and 2). In particular, all of them share chlorotypes with diploid *E. przewalskii* rather than with diploid *E. regeliana* (Fig. 1). Therefore, the three tetraploid species possibly originated from hybridization with diploids most closely related to *E. przewalskii* (in clade A of Fig. 5 and lineage I of Fig. 2) from northern China as the maternal parents and diploids most closely related to the widespread *E. equisetina–E. minuta–E. monosperma* (in clade C of Fig. 5 and lineage II in Fig. 2) as the paternal parents (see the reticulate network in Fig. 6).

In contrast, the three tetraploid taxa *E. likiangensis*, *E. saxatilis* and *E. saxatilis* var. *mairei* exhibit nuclear gene alleles in both clades B and C (Fig. 5). The chlorotypes of *E. saxatilis* belong to lineage III (Fig. 2), and are narrowly distributed in southern QTP (Fig. 1), corresponding to the geographical distribution of this species. Hence, this species possibly originated by allopolyploidy with a maternal progenitor from southern QTP, very likely the diploid cytotype of *E. gerardiana* (in lineage III of Fig. 2 and clade C of Fig. 5), and a paternal progenitor from clade B (Fig. 6). However, as mentioned earlier, the ancient diploids in clade B could be currently extinct. The chlorotypes of *E. likiangensis* belong to lineage II with a relatively wide distribution (Figs 1 and 2), and thus, its maternal progenitor could be a diploid species in this lineage, such as *E. equisetina*, *E. minuta* and *E. monosperma* (with nuclear gene alleles in clade C), whereas its paternal progenitor should belong to clade B (Figs 5 and 6). The *E. saxatilis* var. *mairei* harbours a high frequency of chlorotypes of lineage III and a much lower frequency of chlorotypes of lineage II (Figs 1 and 2). Therefore, the maternal progenitor of this taxon could be diploid species from the two lineages such as *E. gerardiana* (2*x*), *E. equisetina*, *E. minuta* and *E. monosperma*, and its paternal progenitor could be from clade B (Figs 5 and 6).

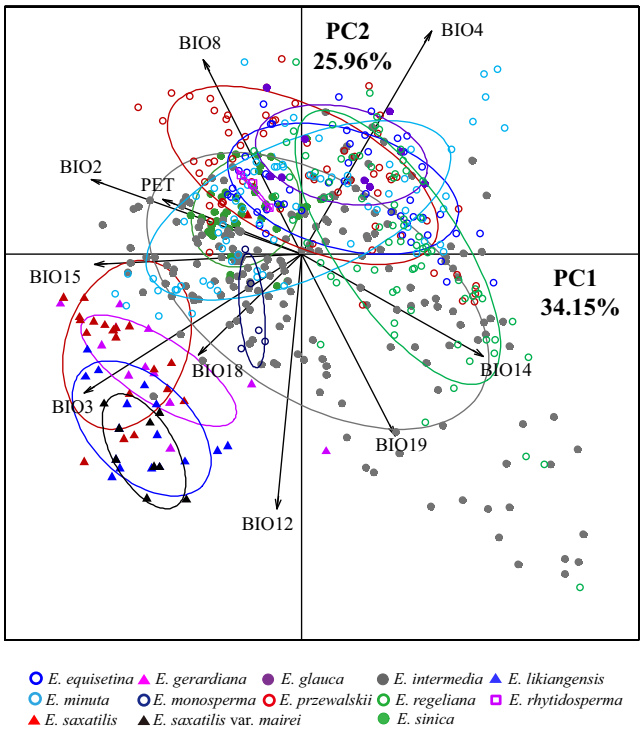


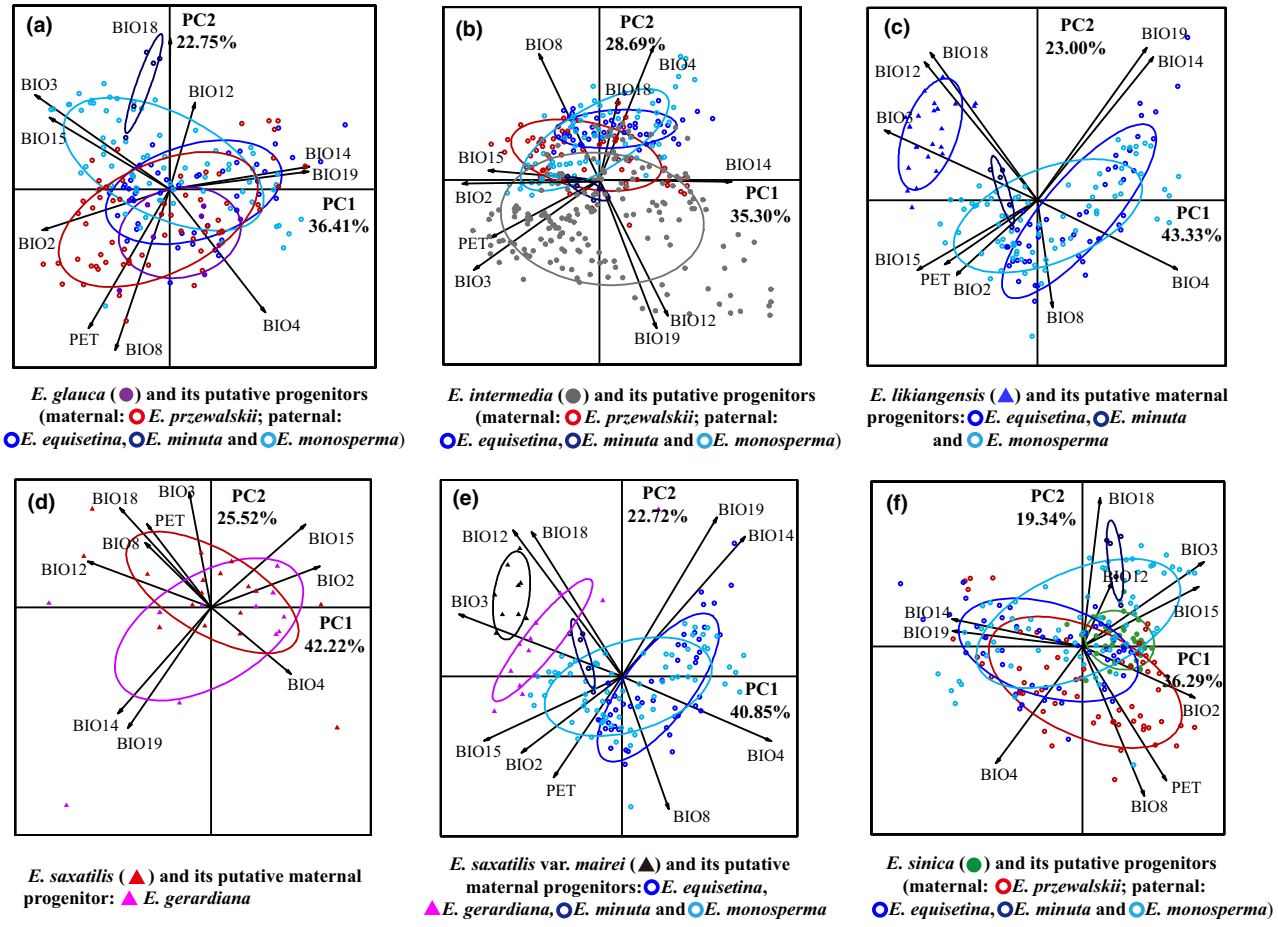
Fig. 7 Scatter plots of PC1 and PC2 showing ecological differentiation among the studied *Ephedra* species based on 10 bioclim variables at sampling locations.

As discussed above, the widespread diploids of *E. equisetina–E. minuta–E. monosperma* or their progenitors may have played important roles in allopolyploid speciation of *Ephedra* in the QTP and adjacent regions. Interestingly, most of the above six tetraploid taxa harbour two or more chlorotypes, even chlorotypes from different main lineages such as in *E. saxatilis* var. *mairei* (Figs 1 and 2). This may indicate multiple origins of a tetraploid taxon, divergence of chlorotypes subsequent to polyploidy, or interspecific chloroplast introgression.

Both diploid and tetraploid cytotypes are present in *E. gerardiana*, *E. przewalskii* and *E. regeliana* (Table 1). The tetraploids of *E. przewalskii* only have A2-type sequences of the nuclear gene *LFY* (in clade A, Fig. 5) as its diploids (Figs 3 and 4), but do not share all chlorotypes with the diploids (Table 1; Fig. 1), although their chlorotypes all belong to lineage I (Fig. 2). In particular, the chlorotype H3 is shared between the tetraploids of *E. przewalskii* and *E. glauca* (Table 1; Fig. 1). Therefore, the tetraploids of *E. przewalskii* may be autopolyploids or young allopolyploids with very closely related parental species. In the *LFY* tree (Fig. 5), the tetraploids of *E. regeliana* harbour two subtypes of sequences in clade A, including A2 with the diploids and tetraploids of *E. przewalskii* and the tetraploid *E. glauca*, and A3 with the diploid *E. regeliana* and two tetraploid species (*E. sinica* and *E. intermedia*). Notably, the diploids and tetraploids of *E. regeliana* do not share chlorotypes (Table 1; Fig. 1). Thus, the tetraploids of *E. regeliana* could be allopolyploids that might have originated by hybridization between the diploids of *E. regeliana* and *E. przewalskii* (Fig. 6), although an origin by hybridization between an autotetraploid of *E. regeliana* and a tetraploid of other species cannot be ruled out. In addition, there could be incomplete linage sorting or introgression at the tetraploid level. However, it is unknown why this tetraploid population (XAB) only exhibits private chlorotypes. The diploids and tetraploids of *E. gerardiana* occur in clade C and clade B+ clade C, respectively (Fig. 5), and they do not share chlorotypes (Table 1; Fig. 1). This may also suggest an allopolyploid origin or a complicated origin of the tetraploid cytotype like in the tetraploid *E. regeliana* (Fig. 6), although the two cytotypes of *E. gerardiana* do not show clear morphological difference. To understand the origin of the tetraploids of this species, more samples need to be studied in the future.

Polyploid evolution in Ephedra and its correlation with some biological and ecological features

Evolution of polyploids. Of the 36 *Ephedra* species that have been cytologically studied, 24 are polyploids or contain polyploid cytotypes (66% of species). Although intraspecific polyploidy has been documented in about half of the species, the results of some early cytological studies need to be checked carefully as mentioned earlier, and more population samples are necessary to



E. glauca (●) **and its putative progenitors (maternal: ○*E. przewalskii*; paternal: ○*E. equisetina*, ○*E. minuta* and ○*E. monosperma*)**

E. intermedia (●) **and its putative progenitors (maternal: ○*E. przewalskii*; paternal: ○*E. equisetina*, ○*E. minuta* and ○*E. monosperma*)**

E. likiangensis (▲) **and its putative maternal progenitors: ○*E. equisetina*, ○*E. minuta* and ○*E. monosperma***

E. saxatilis (▲) **and its putative maternal progenitor: ▲*E. gerardiana***

E. saxatilis* var. *mairei (▲) **and its putative maternal progenitors: ○*E. equisetina*, ▲*E. gerardiana*, ○*E. minuta* and ○*E. monosperma***

E. sinica (●) **and its putative progenitors (maternal: ○*E. przewalskii*; paternal: ○*E. equisetina*, ○*E. minuta* and ○*E. monosperma*)**

Fig. 8 Scatter plots of PC1 and PC2 (a–f) showing ecological differentiation between allotetraploids and their putative progenitors based on 10 bioclim variables at sampling locations.

study the ploidy levels of a species. Notably, all polyploids in the 24 species are tetraploids with the exception of an octoploid cytotype reported in *E. funerea* and *E. gerardiana* (Table S3, Supporting information). According to the available information (Table S3, Supporting information), tetraploids are present in about 75% of the Old World species and 62.5% of the New World species. Our present study found that all tetraploid *Ephedra* species from the QTP and neighbouring areas, excluding the species with both diploid and tetraploid populations, are allotetraploids. Therefore, we may conclude that allotetraploidy is a dominant mode of speciation in *Ephedra*, although the origin of other polyploids in this genus, especially from Europe and America, needs to be further studied. In fact, all of the remaining three polyploid species from other gymnosperm lineages were also deduced to be allopolyploids by Yang *et al.* (2012b).

The biological features related to polyploid speciation. The high percentage of polyploids in *Ephedra* could be related to some attributes of the genus such as a shrub habit, vegetative propagation, a relatively high rate of unreduced gamete formation, and a relatively low basic chromosome number and small genome size (at diploid level) for a gymnosperm (Leitch & Leitch 2012). Previous studies in angiosperms indicate that perennial herbaceous plants with clonality are more likely to form polyploids, which can persist for long periods of time until a suitable mate is found (Otto & Whitton 2000; Leitch & Bennett 2007; Otto 2007; Husband *et al.* 2013; Weiss-Schneeweiss *et al.* 2013). Grif (2000) reported that plant taxa with a small DNA value per genome have a high percentage of polyploidy and show higher ploidy levels, and Wood *et al.* (2009) found that the generic base count (the minimum number of chromosomes reported in a genus) is negatively associated with polyploid incidence in angiosperms. In particular, the union of unreduced gametes has been considered as the most likely way of polyploid formation in plants (Harlan & de Wet 1975; de Wet 1980; Soltis *et al.* 2010; Brownfield & Köhler 2011), and the different rates of unreduced

Table 2 Statistical differences of ecological differentiation between allotetraploids and their putative progenitors based on the Tukey's HSD test for the two principal components revealed by the PCA and the 10 bioclim variables

Allotetraploids	Putative progenitors	PC1	PC2	BIO2	BIO3	BIO4	BIO8	BIO12	BIO14	BIO15	BIO18	BIO19	PET
E. glauca	*E. equisetina*	ns	ns	—	ns	ns	ns	**	ns	ns	***	—	ns
	E. minuta	ns	***	—	*	*	**	***	ns	*	***	—	**
	E. monosperma	ns	***	—	ns	ns	*	*	ns	**	***	—	**
	E. przewalskii	ns	ns	—	ns	ns	ns	ns	ns	ns	ns	—	ns
E. intermedia	*E. equisetina*	—	***	—	***	***	***	***	ns	ns	***	***	***
	E. minuta	—	ns	—	ns	ns	ns	ns	ns	ns	***	**	**
	E. monosperma	—	***	—	ns	***	***	***	ns	***	***	***	***
	E. przewalskii	—	***	—	**	***	***	***	ns	ns	ns	***	ns
E. likiangensis	*E. equisetina*	***	***	—	***	***	**	***	ns	***	***	ns	*
	E. minuta	*	ns	—	***	ns	ns	***	ns	ns	***	ns	**
	E. monosperma	***	***	—	***	***	ns	***	ns	ns	***	ns	***
E. saxatilis var. *mairei*	*E. equisetina*	***	***	ns	***	***	**	***	ns	*	***	—	ns
	E. gerardiana	ns	ns	ns	ns	ns	ns	***	ns	ns	***	—	ns
	E. minuta	ns	ns	ns	**	ns	ns	***	ns	ns	***	—	ns
	E. monosperma	***	***	ns	***	***	ns	***	ns	ns	***	—	ns
E. sinica	*E. equisetina*	***	ns	ns	ns	ns	ns	***	***	***	*	***	**
	E. minuta	ns	***	ns	ns	*	***	ns	ns	ns	ns	ns	**
	E. monosperma	**	ns	ns	ns	ns	***	***	ns	***	ns	ns	***
	E. przewalskii	*	***	ns	ns	ns	ns	***	ns	***	***	*	ns

ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; —, $P > 0.05$ in the ANOVAS.

gamete formation could influence the establishment of polyploids in different lineages (Bretagnolle & Thompson 1996; Ramsey & Schemske 1998, 2002; Ramsey 2006; Younis *et al.* 2014). According to the karyomorphological study of *Ephedra* based on pollen germination, three of five tetraploid species exhibited unreduced pollen grains that accounted for 2–5% of the total amount (Mehra 1946). Also, dimorphism of pollen size in the same herbarium specimen has been reported from seven species of *Ephedra*, including *E. alata*, *E. americana*, *E. aphylla*, *E. breana*, *E. chilensis*, *E. ochreata*, *E. torreyana*, *E. trifurca* and *E. tweediana* (Beug 1956; Kedves 1987; Ickert-Bond *et al.* 2003). Hence, a relatively high rate of unreduced gamete formation in *Ephedra* has very likely contributed to the high frequency of polyploids in the genus.

The ecological differentiation associated with polyploid speciation. Polyploids possess the potential for evolving into new species with evolutionary novelty, and some previous studies suggest that ecological divergence may play a prominent role in polyploid speciation (Brochmann *et al.* 2004; Hijmans *et al.* 2007; Dušková *et al.* 2010; McIntyre 2012; Theodoridis *et al.* 2013). In this study, we also found that ecological divergence was associated with the speciation or divergence of polyploids in *Ephedra*.

Significant ecological divergences between the allotetraploids and their putative progenitors were detected by the PCAs and the ANOVA and Tukey's tests, with the exception of *E. saxatilis* (Figs 8 and S5). The allotetraploids have separate ecological niches from their putative maternal progenitors, or occupy separate ecological niches from one of their putative paternal progenitors but have partially overlapped ecological niches with their other putative progenitors (Figs 8 and S5). For example, *E. likiangensis* and *E. saxatilis* var. *mairei* endemic to the QTP inhabit dense grass swards or dwarf-shrub communities and prefer a moister climate with higher annual precipitation and precipitation of the warmest quarter than their putative progenitors *E. equisetina*, *E. minuta* and *E. monosperma* (Table S5; Fig. S5P, Q, T, U, Supporting information); *E. intermedia* favours conditions that have lower temperature seasonality and mean temperature of the wettest quarter and favours higher annual precipitation and precipitation of the coldest quarter, compared to its putative maternal progenitor *E. przewalskii* and paternal progenitors *E. equisetina* and *E. monosperma* (Table 2; Figs 8b and S5J–M).

The allotetraploid *E. sinica* has a vast distribution from northwestern China northward to Mongolia and Russia and eastward up to the Gulf of Bohai. Similarly, the allotetraploid *E. intermedia* is widely distributed in Irano-Turanian and central Asian floristic regions. Both these species widely occur in open areas such as zonal steppe, desert steppe or coarse-textured-skeletal-sandy soils (Our unpublished observations; Freitag & Maier-

Stolte 1994; Fu *et al.* 1999). However, their putative progenitors *E. equisetina*, *E. minuta* and *E. monosperma* are usually subordinate components of acrophytia with narrow distributions in dry rocky slopes (Table S5; Fig. S2, Supporting information). It is particularly interesting that most of the allotetraploid taxa we found, including *E. intermedia*, *E. likiangensis*, *E. saxatilis* var. *mairei* and *E. sinica*, have adapted to a moister climate with a higher annual precipitation than their putative progenitors (Fig. S5L, P, T, V, Supporting information), considering that the genus *Ephedra* generally occurs in dry habitats.

The ecological divergence associated with allotetraploidy or its divergence could be associated with new habitats triggered by the fast uplift of the QTP and the Asian aridification in the Middle to Late Miocene (An *et al.* 2001; Guo *et al.* 2002; Spicer *et al.* 2003; Dupont-Nivet *et al.* 2007; Jiang & Ding 2008; Royden *et al.* 2008), during which the tetraploid *Ephedra* species originated (Qin *et al.* 2013). Such a scenario has been argued for angiosperm polyploids (Wen *et al.* 2014), for example *Aconitum* subgenus *Lycoctonum* (Yuan & Yang 2006), *Anaphalis* (Meng *et al.* 2010), *Buddleja* (Chen *et al.* 2007), *Leontopodium* (Meng *et al.* 2012), *Meconopsis* (Yang *et al.* 2012a), *Melampodium* (Rebernig *et al.* 2010), *Rheum* (Liu *et al.* 2010), *Rhodiola* (Zhang *et al.* 2014) and *Silene* (Luo *et al.* 2011).

Both geographical distributions and ecological niches of some diploid species, such as *E. equisetina*, *E. monosperma* and *E. regeliana* (2*x*), mostly or partially overlap (Figs 7 and S2), which could have provided opportunities for the hybridization between these species, giving rise to allotetraploid species by allopolyploid speciation. To investigate whether ecological divergence had driven polyploid speciation of *Ephedra* in the QTP and neighbouring areas, more molecular markers or phylogenomic approaches should be used to resolve the parental species of these polyploids, and the molecular mechanisms underlying the adaptation to a specific ecological factor could be explored in future studies.

Acknowledgements

We thank Drs. Yu-Zhi Cun and Fu-Sheng Yang, and Mr. Cai-Yuan Qiao for their help in sample collection; Ms. Wan-Qing Jin for her assistance in DNA sequencing. We also thank the Royal Botanic Garden, Edinburgh, for providing the sample of *Ephedra foeminea* for DNA analysis. We appreciate the Subject Editor and the three anonymous reviewers for their insightful comments and suggestions on the manuscript. This study was supported by the National Natural Science Foundation of China (Grant Nos 31330008, 31170197 and 30730010) and the Chinese Academy of Sciences (KJZD-EW-L07 and the CAS/SAFEA International Partnership Program for Creative Research Teams).

References

Ahuja MR (2005) Polyploidy in gymnosperms: revisited. *Silvae Genetica*, **54**, 59–69.

An Z, Kutzbach JE, Prell WL, Porter SC (2001) Evolution of Asian monsoons and phased uplift of the Himalaya-Tibetan plateau since Late Miocene times. *Nature*, **411**, 62–66.

Bandelt HJ, Forster P, Rohl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, **16**, 37–48.

Beug HJ (1956) Pollendimorphismus bei *Ephedra*. *Naturwissenschaften*, **43**, 332–333.

Birol I, Raymond A, Jackman SD *et al.* (2013) Assembling the 20 Gb white spruce (*Picea glauca*) genome from whole-genome shotgun sequencing data. *Bioinformatics*, **29**, 1492–1497.

Bowers JE, Chapman BA, Rong JK, Paterson AH (2003) Unravelling angiosperm genome evolution by phylogenetic analysis of chromosomal duplication events. *Nature*, **422**, 433–438.

Bretagnolle F, Thompson JD (1996) An experimental study of ecological differences in winter growth between sympatric diploid and autotetraploid *Dactylis glomerata*. *Journal of Ecology*, **84**, 343–351.

Brochmann C, Brysting AK, Alsos IG *et al.* (2004) Polyploidy in arctic plants. *Biological Journal of the Linnean Society*, **82**, 521–536.

Brownfield L, Köhler C (2011) Unreduced gamete formation in plants: mechanisms and prospects. *Journal of Experimental Botany*, **62**, 1659–1668.

Cai DY, Rodriguez F, Teng YW *et al.* (2012) Single copy nuclear gene analysis of polyploidy in wild potatoes (*Solanum* section *Petota*). *BMC Evolutionary Biology*, **12**, 70.

Chen G, Sun WB, Sun H (2007) Ploidy variation in *Buddleja* L. (Buddlejaceae) in the Sino-Himalayan region and its biogeographical implications. *Botanical Journal of the Linnean Society*, **154**, 305–312.

Cutler HC (1939) Monograph of the North American species of the genus *Ephedra*. *Annals of the Missouri Botanical Garden*, **26**, 373–428.

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, **9**, 772.

Dray S, Dufour AB, Chessel D (2007) The ade4 package-II: two-table and K-table methods. *R News*, **7**, 47–52.

Dupont-Nivet G, Krijgsman W, Langereis CG, Abels HA, Dai S, Fang XF. (2007) Tibetan plateau aridification linked to global cooling at the Eocene-Oligocene transition. *Nature*, **445**, 635–638.

Dušková E, Kolář F, Sklenář P *et al.* (2010) Genome size correlates with growth form, habitat and phylogeny in the Andean genus *Lasiocephalus* (Asteraceae). *Preslia*, **82**, 127–148.

Fawcett JA, Maere S, Van de Peer Y (2009) Plants with double genomes might have had a better chance to survive the Cretaceous-Tertiary extinction event. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 5737–5742.

Fawcett JA, Van de Peer Y, Maere S (2013) Significance and biological consequences of polyploidization in land plant evolution. In: *Plant Genome Diversity 2: Physical Structure, Behaviour and Evolution of Plant Genomes*(eds Leitch IJ, Greilhuber J, Doležel J, Wendel JF), pp. 277–293. Springer, Wien.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791.

Ferguson D, Sang T (2001) Speciation through homoploid hybridization between allotetraploids in peonies (*Paeonia*). *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 3915–3919.

Florin CR (1933) Über einige neue oder wenig bekannte asiatische *Ephedra*-Arten der Sektion *Pseudobaccatae* Stapf.-Kongl. Svenska Vetenskapsakad. *Handle*, **12**, 1–49.

Freitag H, Maier-Stolte M (1994) Ephedraceae. In: *Chorology of Trees and Shrubs in Southwest Asia and Adjacent Regions 10*(ed. Browicz K), pp. 5–16, 39–52. Polish Scientific Publishers, Poznan.

Fu L, Yu Y, Riedl H (1999) Ephedraceae. In: *Flora of China*(eds Wu CY, Raven P), pp. 97–101. Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis.

Gascuel O (1997) BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. *Molecular Biology and Evolution*, **14**, 685–695.

Ge S, Sang T, Lu BR, Hong DY (1999) Phylogeny of rice genomes with emphasis on origins of allotetraploid species. *Proceedings of the National Academy of Sciences of the United States of America*, **49**, 14400–14405.

Glennon KL, Ritchie ME, Segraves KA (2014) Evidence for shared broad-scale climatic niches of diploid and polyploid plants. *Ecology Letters*, **17**, 574–582.

Grif VG (2000) Some aspects of plant karyology and karyosystematics. *International Review of Cytology*, **196**, 131–175.

Guindon S, Gascuel O (2003) A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. *Systematic Biology*, **52**, 696–704.

Guindon S, Dufayard J, Lefort V, Anisimova M, Hordijk W, Gascuel O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*, **59**, 307–321.

Guo ZT, Ruddiman WF, Hao QZ *et al.* (2002) Onset of Asian desertification by 22 Myr ago inferred from loess deposits in China. *Nature*, **416**, 159–163.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95–98.

Harlan JR, de Wet JMJ (1975) On Ö. Winge and a prayer: the origins of polyploidy. *The Botanical Review*, **41**, 361–390.

Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A (2005) Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, **25**, 1965–1978.

Hijmans RJ, Gavrilenko T, Stephenson S, Bamberg J, Salas A, Spooner DM (2007) Geographical and environmental range expansion through polyploidy in wild potatoes (*Solanum* section *Petota*). *Global Ecology and Biogeography*, **16**, 485–495.

Huang J, Price RA (2003) Estimation of the age of extant *Ephedra* using chloroplast *rbcL* sequence data. *Molecular Biology and Evolution*, **20**, 435–440.

Huang J, Giannasi DE, Price RA (2005) Phylogenetic relationships in *Ephedra* (Ephedraceae) inferred from chloroplast and nuclear DNA sequences. *Molecular Phylogenetics and Evolution*, **35**, 48–59.

Husband BC, Baldwin SJ, Suda J (2013) The incidence of polyploidy in natural plant populations: major patterns and evolutionary processes. In: *Plant Genome Diversity 2: Physical Structure, Behaviour and Evolution of Plant Genomes*(eds Leitch IJ, Greilhuber J, Doležel J, Wendel JF), pp. 255–276. Springer, Wien.

Ickert-Bond SM, Skvarla JJ, Chissoe WF (2003) Pollen dimorphism in *Ephedra* L. (Ephedraceae). *Review of Palaeobotany and Palynology*, **124**, 325–334.

Ickert-Bond SM, Rydin C, Renner SS (2009) A fossil-calibrated relaxed clock for *Ephedra* indicates an Oligocene age for the divergence of Asian and New World clades and Miocene dispersal into South America. *Journal of Systematics and Evolution*, **47**, 444–456.

Jiang HY (2006) Study on the genetic relationship analysis and determination of main medical composition for *Ephedra*. Bachelor's degree Thesis, Gansu Agricultural University, Lanzhou, China.

Jiang H, Ding Z (2008) A 20 Ma pollen record of East-Asian summer monsoon evolution from Guyuan, Ningxia, China. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **265**, 30–38.

Jiao YN, Wickett NJ, Ayyampalayam S *et al.* (2011) Ancestral polyploidy in seed plants and angiosperms. *Nature*, **473**, 97–100.

Kedves M (1987) LM and EM studies on pollen grains of recent *Welwitschia mirabilis* Hook. and *Ephedra* species. *Acta Botanica Hungarica*, **33**, 81–103.

Kelly LJ, Leitch AR, Clarkson JJ, Knapp S, Chase MW (2013) Reconstructing the complex evolutionary origin of wild allopolyploid tobaccos (*Nicotiana* section *Suaveolentes*). *Evolution*, **67**, 80–94.

Khoshoo TN (1959) Polyploidy in gymnosperms. *Evolution*, **13**, 24–39.

Kim ST, Sultan SE, Donoghue MJ (2008) Allopolyploid speciation in *Persicaria* (Polygonaceae): insights from a low-copy nuclear region. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 12370–12375.

Kong H, Chen Q, Ma J (2001) A study on karyotypes of two species in *Ephedra*. *Journal of Lanzhou University*, **37**, 101–103.

Krejcikova J, Sudova R, Lucanova M *et al.* (2013) High ploidy diversity and distinct patterns of cytotype distribution in a widespread species of *Oxalis* in the Greater Cape Floristic Region. *Annals of Botany*, **111**, 641–649.

Kubitzki K (1990) *The Families and Genera of Vascular Plants*. Springer, Berlin.

Leitch IJ, Bennett MD (2007) Genome size and its uses: the impact of flow cytometry. In: *Flow Cytometry with Plant Cells: Analysis of Genes, Chromosomes and Genomes*(eds Dolezel J, Greilhuber J, Suda J), pp. 153–176. Wiley, Weinheim.

Leitch AR, Leitch IJ (2012) Ecological and genetic factors linked to contrasting genome dynamics in seed plants. *New Phytologist*, **194**, 629–646.

Leitch IJ, Hanson L, Winfield M, Parker J, Bennett MD (2001) Nuclear DNA C-values complete familial representation in gymnosperms. *Annals of Botany*, **88**, 843–849.

Liu R, Wang A, Tian X, Wang D, Liu J (2010) Uniformity of karyotypes in *Rheum* (Polygonaceae), a species-rich genus in the Qinghai-Tibetan Plateau and adjacent regions. *Caryologia*, **63**, 82.

Lott M, Spillner A, Huber KT, Moulton V (2009a) PADRE: a package for analyzing and displaying reticulate evolution. *Bioinformatics*, **25**, 1199–1200.

Lott M, Spillner A, Huber KT, Petri A, Oxelman B, Moulton V (2009b) Inferring polyploid phylogenies from multiply-labeled gene trees. *BMC Evolutionary Biology*, **9**, 216.

Luo D, Liu D, Xu B, Nie ZL, Sun H, Li ZM. (2011) A karyological study of six species of *Silene* L. (Caryophyllaceae) from the Hengduan Mountains, SW China. *Caryologia*, **64**, 10–13.

Madlung A (2013) Polyploidy and its effect on evolutionary success: old questions revisited with new tools. *Heredity*, **110**, 99–104.

Mayrose I, Zhan SH, Rothfels CJ *et al.* (2011) Recently formed polyploid plants diversify at lower rates. *Science*, **333**, 1257.

McIntyre PJ (2012) Polyploidy associated with altered and broader ecological niches in the *Claytonia perfoliata* (Portulacaceae) species complex. *American Journal of Botany*, **99**, 655–662.

Mehra PN (1946) A study of the karyotypes and the occurrence of diploid male gametophytes in some species of the genus *Ephedra*. *Proceedings of the National Academy of Sciences of the United States of America*, **16**, 259–286.

Meng Y, Sun H, Yang YP, Nie ZL (2010) Polyploidy and new chromosome counts in *Anaphalis* (Asteraceae: Gnaphalieae) from the Qinghai-Tibet Plateau of China. *Journal of Systematics and Evolution*, **48**, 58–64.

Meng Y, Nie ZL, Sun H, Yang YP (2012) Chromosome numbers and polyploidy in *Leontopodium* (Asteraceae: Gnaphalieae) from the Qinghai-Tibet Plateau of SW China. *Caryologia*, **65**, 87–93.

Murray BG (2013) Karyotype variation and evolution in gymnosperms. In: *Plant Genome Diversity 2: Physical Structure, Behaviour and Evolution of Plant Genomes*(eds Leitch IJ, Greilhuber J, Doležel J, Wendel JF), pp. 231–244. Springer, Wien.

Neale DB, Wegrzyn JL, Stevens KA *et al.* (2014) Decoding the massive genome of loblolly pine using haploid DNA and novel assembly strategies. *Genome Biology*, **15**, R59.

Nystedt B, Street NR, Wetterbom A *et al.* (2013) The Norway spruce genome sequence and conifer genome evolution. *Nature*, **497**, 579–584.

Otto SP (2007) The evolutionary consequences of polyploidy. *Cell*, **131**, 452–462.

Otto SP, Whitton J (2000) Polyploid incidence and evolution. *Annual Review of Genetics*, **34**, 401–437.

Page JT, Huynh MD, Liechty ZS *et al.* (2013) Insights into the evolution of cotton diploids and polyploids from whole-genome re-sequencing. *G3: Genes, Genomes, Genetics*, **3**, 1809–1818.

Qin AL, Wang MM, Cun YZ *et al.* (2013) Phylogeographic evidence for a link of species divergence of *Ephedra* in the Qinghai-Tibetan Plateau and adjacent regions to the Miocene Asian aridification. *PLoS One*, **8**, e56243.

R Development Core Team (2014) *A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna. URL http://www.R-project.org/.

Ramsey J (2006) Unreduced gametes and neopolyploids in natural populations of *Achillea borealis* (Asteraceae). *Heredity*, **98**, 143–150.

Ramsey J (2011) Polyploidy and ecological adaptation in wild yarrow. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 7096–7101.

Ramsey J, Schemske DW (1998) Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics*, **29**, 467–501.

Ramsey J, Schemske DW (2002) Neopolyploidy in flowering plants. *Annual Review of Ecology and Systematics*, **33**, 589–639.

Rebernig CA, Weiss-Schneeweiss H, Schneeweiss GM *et al.* (2010) Quaternary range dynamics and polyploid evolution in an arid brushland plant species (*Melampodium cinereum*, Asteraceae). *Molecular Phylogenetics and Evolution*, **54**, 594–606.

Renny-Byfield S, Kovarik A, Kelly LJ *et al.* (2013) Diploidization and genome size change in allopolyploids is associated with differential dynamics of low- and high-copy sequences. *The Plant Journal*, **74**, 829–839.

Rogers SO, Bendich AJ (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Molecular Biology*, **5**, 69–76.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.

Roulin A, Auer PL, Libault M *et al.* (2012) The fate of duplicated genes in a polyploid plant genome. *The Plant Journal*, **73**, 143–153.

Royden LH, Burchfiel BC, van der Hilst RD (2008) The geological evolution of the Tibetan plateau. *Science*, **321**, 1054–1058.

Rydin C, Korall P (2009) Evolutionary relationships in *Ephedra* (Gnetales), with implications for seed plant phylogeny. *International Journal of Plant Sciences*, **170**, 1031–1043.

Schönswetter P, Lachmayer M, Lettner C *et al.* (2007) Sympatric diploid and hexaploid cytotypes of *Senecio carniolicus* (Asteraceae) in the Eastern Alps are separated along an altitudinal gradient. *Journal of Plant Research*, **120**, 721–725.

Simillion C, Vandepoele K, Van Montagu MCE, Zabeau M, Van de Peer Y (2002) The hidden duplication past of *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 13627–13632.

Soltis DE, Albert VA, Leebens-Mack J *et al.* (2009) Polyploidy and angiosperm diversification. *American Journal of Botany*, **96**, 336–348.

Soltis DE, Buggs RJA, Doyle JJ, Soltis PS (2010) What we still don't know about polyploidy. *Taxon*, **59**, 1387–1403.

Soltis DE, Segovia-Salcedo MC, Jordon-Thaden I *et al.* (2014a) Are polyploids really evolutionary dead-ends (again)? A critical reappraisal of Mayrose et al. (2011). *New Phytologist*, **202**, 1105–1117.

Soltis DE, Visger CJ, Soltis PS (2014b) The polyploidy revolution then...and now: stebbins revisited. *American Journal of Botany*, **101**, 1057–1078.

Spicer RA, Harris NBW, Widdowson M *et al.* (2003) Constant elevation of southern Tibet over the past 15 million years. *Nature*, **421**, 622–624.

Suda J, Trávníček P (2006) Reliable DNA ploidy determination in dehydrated tissues of vascular plants by DAPI flow cytometry—new prospects for plant research. *Cytometry Part A*, **69**, 273–280.

Swofford DL (2002) *PAUP*: Phylogenetic Analysis Using Parsimony (and Other Methods) 4.0 Beta*. Sinauer Associates, Sunderland, Maryland.

Theodoridis S, Randin C, Broennimann O, Patsiou T, Conti E (2013) Divergent and narrower climatic niches characterize polyploid species of European primroses in *Primula* sect. *Aleuritia*. *Journal of Biogeography*, **40**, 1278–1289.

Trabucco A, Zomer RJ, Bossio DA, Van Straaten O, Verchot LV (2008) Climate change mitigation through afforestation reforestation: a global analysis of hydrologic impacts with four case studies. *Agriculture, Ecosystems & Environment*, **126**, 81–97.

Van de Peer Y, Fawcett JA, Proost S, Sterck L, Vandepoele K (2009) The flowering world: a tale of duplications. *Trends in Plant Science*, **14**, 680–688.

Vision TJ, Brown DG, Tanksley SD (2000) The origins of genomic duplications in *Arabidopsis*. *Science*, **290**, 2114–2117.

Vrána J, Cápal P, Bednářová M, Doležel J (2014) Flow cytometry in plant research: a success story. *Applied Plant Cell Biology*, **22**, 395–430.

Wang XQ, Ran JH (2014) Evolution and biogeography of gymnosperms. *Molecular Phylogenetics and Evolution*, **75**, 24–40.

Weiss-Schneeweiss H, Emadzade K, Jang TS, Schneeweiss GM (2013) Evolutionary consequences, constraints and potential of polyploidy in plants. *Cytogenetic and Genome Research*, **140**, 137–150.

Wen J, Zhang JQ, Nie ZL, Zhong Y, Sun H (2014) Evolutionary diversifications of plants on the Qinghai-Tibetan Plateau. *Frontiers in Genetics*, **5**, 4.

Wendt T (1993) A new variety of *Ephedra torreyana* (Ephedraceae) from west Texas and Chihuahua, with notes on hybridization in the *E. torreyana* complex. *Phytologia*, **74**, 141–150.

de Wet JM (1980) Origins of polyploids. In: *Polyploidy: Biological Relevance*(ed. Lewis WH), pp. 3–15. Plenum Press, New York.

Wheeler B (2010) lmPerm: Permutation tests for linear models. *R package version*, 1.

Wood TE, Takebayashi N, Barker MS, Mayrose I, Greenspoon PB, Rieseberg LH. (2009) The frequency of polyploid speciation in vascular plants. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 13875–13879.

Wu JL, Li S, Jiang HY, Gao YH, Niu JY (2009) Staining and slide-preparing technique of mitotic chromosomes and application in karyotype determination of *Ephedra*. *China Journal of Chinese Materia Medica*, **34**, 2725–2729.

Yang Y (2002) *Systematics and Evolution of Ephedra L. (Ephedraceae) from China*. Chinese Academy of Sciences, Beijing.

Yang Y, Wang Q (2013) The earliest fleshy cone of *Ephedra* from the early Cretaceous Yixian formation of northeast China. *PLoS One*, **8**, e53652.

Yang Y, Fu DZ, Zhu GH (2003) A new species of *Ephedra* (Ephedraceae) from China. *Novon*, **13**, 153–155.

Yang FS, Qin AL, Li YF, Wang XQ (2012a) Great genetic differentiation among populations of *Meconopsis integrifolia* and its implication for plant speciation in the Qinghai-Tibetan Plateau. *PLoS One*, **7**, e37196.

Yang ZY, Ran JH, Wang XQ (2012b) Three genome-based phylogeny of Cupressaceae *s.l.*: further evidence for the evolution of gymnosperms and Southern Hemisphere biogeography. *Molecular Phylogenetics and Evolution*, **64**, 452–470.

Younis A, Hwang YJ, Lim KB (2014) Exploitation of induced 2n-gametes for plant breeding. *Plant Cell Reports*, **33**, 215–223.

Yuan Q, Yang QE (2006) Polyploidy in *Aconitum* subgenus *Lycoctonum* (Ranunculaceae). *Botanical Journal of the Linnean Society*, **150**, 343–353.

Zhang JQ, Meng SY, Wen J, Rao GY (2014) Phylogenetic relationships and character evolution of *Rhodiola* (Crassulaceae) based on nuclear ribosomal ITS and plastid *trn*L-F and *psb*A-*trn*H sequences. *Systematic Botany*, **39**, 441–451.

X.Q.W. designed the study. H.W. and Z.M. performed the laboratory work. M.M.W. and A.L.Q. contributed plant materials. H.W., X.Q.W., Z.M. and J.H.R. analysed the data. X.Q.W. and H.W. wrote the article.

Data accessibility

DNA sequences: GenBank accessions KT033145–KT033395.

Sequence alignments, tree files and climate data: Dryad (doi:10.5061/dryad.kb508).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1. Histograms of fluorescence intensity in diploid *Ephedra equisetina* (I) and tetraploid *E. intermedia* (II).

Fig. S2. Sampling locations for the statistic analysis of ecological differentiation.

Fig. S3. The ML tree of *Ephedra* constructed by the cpDNA haplotypes.

Fig. S4. Majority-rule consensus trees obtained from maximum parsimony analyses of the *LFY* and *DDB2* data sets.

Fig. S5. Boxplots showing variation of bioclim variables among the studied *Ephedra* species.

Table S1. The cpDNA haplotypes detected in the studied *Ephedra* species and their GenBank accessions.

Table S2. A summary of the principal component analysis.

Table S3. The chromosome numbers in *Ephedra* L.

Table S4. Results of the ANOVA and Tukey's HSD tests for the two principal components revealed by the PCA and the 10 bioclim variables.

Table S5. Morphological characteristics, habitat preference and distribution of *Ephedra* species from the QTP and adjacent regions.