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Genetic Diversity and Population Structure Analysis of Wild *Cymbidium tortisepalum* Based on Chloroplast DNA in Yunnan Province of China

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Abstract. ***Cymbidium tortisepalum*** **is a primary orchid species in Yunnan Province, China, and has an extremely high ornamental and economic value. To reveal the levels and distribution of genetic variation and structure of wild *C. tortisepalum* resources, sequence variations of six chloroplast DNA intergenic spacers (psbM-trnD, trnV-trnA, accD-psal, rrn23, trnk-rps16, and ycf1) were analyzed in 404 wild individuals from 28 populations in the three river area in Yunnan Province, China. The results showed that the six chloroplast DNA sequences were aligned with 61 polymorphic sites, including 50 indels and 11 haplotypes in 404 individuals, which revealed a low level of genetic diversity (total genetic diversity = 0.240, and the average value of nucleotide diversity = 0.00024). In addition, a fairly low genetic differentiation [coefficients for genetic differentiation among populations (G_{ST}) = 0.099, number of substitution (N_{ST}) = 0.081] was found among the studied populations, and N_{ST} value was less than G_{ST}, which indicated that no significant phylogeographic structure existed in those populations. Furthermore, analysis of molecular variance revealed that great genetic variance (91%) came from individuals within the populations, which indicated that there was no clear genetic differentiation among populations. On the basis of these findings, a conservation plan was proposed to sample or preserve fewer populations but with more individuals from each population.**

Cymbidium tortisepalum is a perennial herb, which belongs to Orchidaceae family (Du Puy and Cribb, 1988). Wild *C. tortisepalum* mainly grows in the river valleys or under secondary forest of *Pinus yunnanensis*, *Abies fabri*, and *Quercus aquifolioides* in the middle of the mountains, with a range of 1000 to 2700 m, 70% to 80% shading intensities, and 60% to 80% air humidity, and where the soil is moist all year with a high nutrient content and pH between 5.5 and 6.5 (Xue et al., 2007). Because this plant has an extremely narrow geographic spread, high dependence on ecological environment, and poor natural reproduction ability, its wild resources are rare, being scattered only within Taiwan Province, the southwest of Sichuan Province, and the northwest of Yunnan Province, China (Lin et al., 2016; Peng, 2003). It is primarily distributed in the three parallel river region (Jinsha, Lancang, and Nujiang rivers) of the northwestern Yunnan Province, at latitude 24°N to 28.4°N and longitude 98°E to 101°E. *C. tortisepalum* is famous for its graceful shape, varied flowers, and beautiful color, which gives it a high ornamental and economic value, and it has become one of the most sought-after orchid species in Yunnan Province (Du Puy and Cribb, 1988). The *C. tortisepalum* industry has reached a value of more than 100 million yuan in the Yunnan (Qi, 2004). With increased market, the price of one wild *C. tortisepalum* can reach up to tens of thousands and even millions of yuan. Consequently, the wild resources of *C. tortisepalum* in Yunnan Province are being diminished by overexploitation and habitat loss (Rao et al., 2008; Xue et al., 2007). Therefore, the research on germplasm resources, and the evaluation of the genetic diversity and structure of wild *C. tortisepalum* in Yunnan Province, could provide valuable knowledge to the protection of wild resources. However, current studies of *C. tortisepalum*, both within China and abroad, are mainly focused on cultivation management (He et al., 2019), tissue culture, and rapid propagation (Huan et and Tanake, 2004; Teixeira da Silva et al., 2006), and cross-breeding (Li et al., 2009, 2010; Zhao et al., 2019). Reports of genetic diversity and genetic structure of wild *C. tortisepalum* are

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Table 1. Details of sampling sites of 28 populations of wild *Cymbidium tortisepalum* in Yunnan Province, China. From 2010 to 2011, we collected 404 individuals from 28 naturally distributed populations of *C. tortisepalum* in northwestern Yunnan. Approximately 15 to 24 individuals were selected from each population with a distance at least 20 m.

Region[z]	Population	Location name	N	Habitat[y]	Latitude (N)	Longitude (E)	Altitude (m)
NW	BD	Badi Township, Weixi County, Diqing Tibetan Autonomous Prefecture	15	Pine forest, mountain slope	27°54′04.37″	98°56′46.47″	1908
	BJX	Baijixun Township, Weixi County, Diqing Tibetan Autonomous Prefecture	17	Oak forest, escarpment	27°21′19.76″	99°06′28.42″	1882
	WD	Weideng Township, Weixi County, Diqing Tibetan Autonomous Prefecture	17	Pine and oak mixed forest, vale	27°04′08.04″	99°10′49.88″	1945
	ZL	Zhonglu Township, Weixi County, Diqing Tibetan Autonomous Prefecture	18	Pine and oak mixed forest, mountain slope	27°09′28.58″	99°09′32.00″	1715
	CK	Cikai Township, Gongshan County, Nujiang Lisu Autonomous Prefecture	15	Bamboo grove, escarpment	27°41′46.61″	98°41′57.31″	1609
	SD	Shideng Township, Lanping County, Nujiang Lisu Autonomous Prefecture	13	Pine and oak mixed forest, mountain slope	26°45′19.18″	99°09′45.31″	2142
	ZP	Zhongpai Township, Lanping County, Nujiang Lisu Autonomous Prefecture	14	Pine and oak mixed forest, valley	26°55′37.15″	99°11′12.06″	1719
	HS	Huangshan Township, Yu Long County, Lijiang City	18	Pine and oak mixed forest, mountain slope	26°50′12.29′	100°10′51.28″	2557
MD	YP	Yingpan Township, Lanping County, Nujiang Lisu Autonomous Prefecture	14	Pine and oak mixed forest, mountain slope	26°24′02.33″	99°06′43.62″	2290
	LJ	Lajing Township, Lanping County, Nujiang Lisu Autonomous Prefecture	14	Pine and oak mixed forest, mountain slope	26°30′11.69″	99°15′13.60″	2220
	PH	Pihe Township, Fugong County, Nujiang Lisu Autonomous Prefecture	11	Pine forest, foot of a hill	26°33′04.48″	98°54′29.55″	1576
	LK	Liuku Township, Lushui County, Nujiang Lisu Autonomous Prefecture	12	Pine and oak mixed forest, mountain slope	25°50′18.63″	98°48′34.77″	1912
	LZ	Luzhang Township, Lushui County, Nujiang Lisu Autonomous Prefecture	17	Oak forest, valley	25°56′48.28″	98°46′18.81″	2142
	CX	Changxin Township, Yunlong County, Dali Bai Autonomous Prefecture	14	Pine and oak mixed forest, mountain slope	26°03′50.08″	99°26′30.24″	1949
	BC	Biaocun Township, Yunlong County, Dali Bai Autonomous Prefecture	11	Oak forest, escarpment	26°64′43.36″	99°07′38.72″	1385
	JZ	Jiuzhou Township, Yunlong County, Dali Bai Autonomous Prefecture	11	Oak forest, valley	25°45′02.33″	99°13′40.58″	1380
	JC	Jiancao Township, Yunlong County, Dali Bai Autonomous Prefecture	16	Oak forest, vale	26°09′03.65″	99°17′27.17″	2160
	BF	Baofeng Township, Yunlong County, Dali Bai Autonomous Prefecture	14	Oak forest, mountain slope	25°48′28.97″	99°22′15.96″	1638

(Continued on next page)

Table 1. (Continued)

Region[z]	Population	Location name	N	Habitat[y]	Latitude (N)	Longitude (E)	Altitude (m)
	MJ	Minjian Township, Yunlong County, Dali Bai Autonomous Prefecture	11	Pine and oak mixed forest, mountain slope	25°42′01.83″	98°58′01.24″	2094
	TJ	Tuanjie Township, Yunlong County, Dali Bai Autonomous Prefecture	13	Pine and oak mixed forest, escarpment	25°44′39.02″	99°34′41.09″	2066
	XS	Xishan Township, Eryuan County, Dali Bai Autonomous Prefecture	17	Pine and oak mixed forest, valley	25°57′35.53″	99°40′20.84″	2422
	XZ	Xizhou Township,Dali County-level City, Dali Bai Autonomous Prefecture	13	Pine and oak mixed forest, escarpment	25°51′01.51″	100°07′50.65″	1980
	XT	Xiangtu Township, Jianchuan County, Dali Bai Autonomous Prefecture	12	Pine and oak mixed forest, mountain slope	26°16′10.81″	99°32′03.19″	2492
SW	WM	Wama Township, Longyang District, Baoshan City	18	Pine and oak mixed forest, mountain slope	25°33′29.18″	98°56′13.39″	1953
	TP	Taiping Township, Longyang District, Baoshan City	15	Pine and oak mixed forest, valley	24°48′21.04′	99°01′04.77″	1923
	PP	Pupiao Township, Longyang District, Baoshan City	14	Oak forest, mountain slope	24°34′48.73″	99°10′41.68″	1871
	XG	Xiaguan County, Dali County-level City, Dali Bai Autonomous Prefecture	16	Pine and oak mixed forest, mountain slope	25°34′58.51″	100°13′31.88″	1993
	YW	Youwang Township, Shidian County, Baoshan City	14	Pine forest, mountain slope	24°47′44.73″	99°06′30.96″	1540

[z]NW = northern region of three parallel rivers in the western Yunnan Province; MD = middle region of three parallel rivers in the western Yunnan Province; SW = Southern region of three parallel rivers in the western Yunnan Province.
[y]Pine (*Pinus yunnanensis*), oak (*Quercus aquifolioides*), and bamboo (*Fargesia pleniculmis*).

limited to morphological diversity (Suo et al., 2016; Ye et al., 2015), nuclear simple sequence repeats [nSSR (Zhao et al., 2017)] and direct amplification of length polymorphism [DALP (Jia et al., 2012)]. Moreover, population samples in other studies represented less than 10% of the wild *C. tortisepalum* found in Yunnan Province and were lower than the sample size in this study. Furthermore, there is no report about the genetic diversity and structure of wild *C. tortisepalum* base on chloroplast DNA (cpDNA). Therefore, it is highly important to further explore the genetic diversity of populations and the genetic structure of wild *C. tortisepalum* based on cpDNA in Yunnan Province to adopt appropriate references for the formation of effective conservation strategies.

The chloroplast genome, as an independent genetic unit not subjected to genetic recombination and whose selection pressure is small, can directly reflect the genetic variation accumulated with a plant species during long-term evolution (Jiang et al., 2019; Ning et al., 2020; Schaal et al., 1998). It is highly suitable for investigating the genetic relationships and genetic diversity of the species in a geographic location (Liu et al., 2012) and has been widely used (Petit et al., 2005; Shaw et al., 2007; Sun et al., 2019; Yang et al., 2013).

In our study, 404 wild individuals from 28 populations in Yunnan Province were used as experimental materials, and their genetic structure and their lineage differentiation were investigated by cpDNA sequence analysis. The purpose of our study is to provide a theoretical basis for the formation of a protection strategy and to provide technical support for the industrial development of wild *C. tortisepalum* in Yunnan Province of China.

Methods

Experimental material. On the basis of two investigations of the wild *C. tortisepalum* resources in Yunnan Province before sampling, 28 sampling sites located in the western area of the Yunnan Province were determined. Approximately 15 to 24 individuals were selected from each population with a distance of at least 20 m. Finally, 404 individuals from 28 populations of wild *C. tortisepalum* were collected between 2010 and 2011. Afterward, 1 g of leaves were sampled from each plant, sealed in a bag, and placed in a sealing box with desiccant. Prepared materials were stored at –80 °C in the Southwest China Germplasm Bank of Wild Species (Kunming, China). The collection area name, latitude, longitude, altitude, and habitat were recorded for each sample. Detailed locations and geographic distribution of the populations are shown in Table 1 and Fig. 1.

Total DNA extraction. Genomic DNA was extracted from a single individual using the cetyltrimethylammonium bromide (CTAB) method (Doyle, 1986) with some modifications (Tang, 2014). The quality and quantity of the DNA were checked on 1% agarose gels. The extracted DNA was diluted to 50 $ng \cdot \mu L^{-1}$ and stored at –20 °C for future use.

Polymerase chain reaction amplification and sequencing. On the basis of complete chloroplast genome sequence of the

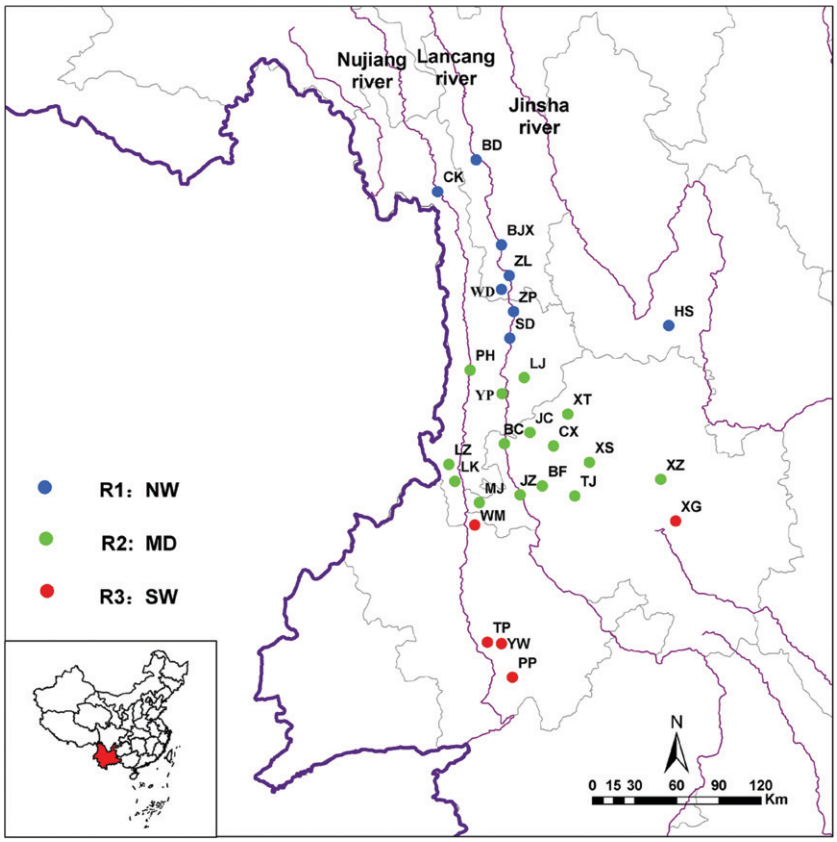

Fig. 1. Simplified map showing the geographic distribution of the 28 sampling sites of *Cymbidium tortisepalum* in Yunnan Province, China. Blue marks represent populations in the northern region of three parallel rivers in the western Yunnan Province (NW); green marks markers represent those in the middle region of three parallel rivers in the western Yunnan Province (MD), and red marks represent those in the southern region of three parallel rivers in the western Yunnan Province. The abbreviations of the sites refer to Table 1.

genus *C. tortisepalum* by our research group (Yang et al., 2013), we selected 43 regions for designing polymerase chain reaction (PCR) primers. Of these, six chloroplast fragments had variable sites (psbM-trnD, trnV-trnA, accD-psal, rrn23, trnk-rps16, and ycf1) among 10 tested samples in the pilot study, which were finally used to sequence all samples. Sequences were deposited at GenBank with accession numbers KY621375-KY621467.

PCR amplification was performed using a thermal cycler (GeneAmp PCR System 9700; PerkinElmer, Waltham, MA). Primer information is shown in Table 2. PCR reactions were 25 μL in volume and contained 2.5 μL 10 × PCR buffer, 2.5 μL $MgCl_2$ (25 mM), 2.0 μL dNTP mixture (2.5 mM), 1.0 μL primer F (5 μM), 1.0 μL primer R (5 μM) DNA, 0.125 μL Taq polymerase (5 $U.\mu L^{-1}$), 2 μL Template DNA (50 $ng{\cdot}\mu L^{-1}$), and then adding double-distilled water (ddH_2O) to a total volume of 25 μL. The cycling parameters were as follows: 3 min of denaturation at 94 °C; followed by 35 cycles of 1 min denaturation at 94 °C, 1.5 min annealing at 56 °C, and 2 min extension at 72 °C; with a final extension of 72 °C for 10 min. PCR products were visualized using gel electrophoresis on an agarose gel (0.8% to 1%). Sequencing of PCR products was accomplished using a DNA analyzer (ABI 3730; Thermo Fisher Scientific, Waltham, MA).

Data analysis. Sequencher version 4.1.4 software (Larkin et al., 2014) was used for sequence splicing. Geneious version 4.8 (Drummond et al., 2011) was used for sequence alignment and calibration. DnaSP version 5.0 software (Librado and Rozas, 2009) was used to perform haplotype statistics and sites with gaps were not considered in our analysis. ArcMap software (version 9.3; ESRI, Redlands, CA) was adopted to construct the geographic information map of all haplotypes within each population (Gmbh, 2004). Network version 4.6 software (Forster et al., 2007) was used to construct a haplotype network diagram. Arlequin version 3.5 (Excoffier and Lischer, 2010) was used to detect the genetic structure and perform analysis of molecular variance (AMOVA). GenALEx version 6.5 (Peakall and Smouse, 2006) was used to calculate the geographic distance, and the Mantel test (Mantel, 1967) was used to test the correlation between the two matrices. Genetic diversity (H_S), the total genetic diversity (H_T), and the genetic differentiation coefficients (G_{ST} and N_{ST}) were calculated using Permut version 2.0 software (Pons and Petit, 1996), G_{ST} and N_{ST} were compared via the U statistical method (1000 repeated permutation test) (Nei, 1987; Tajima, 1993). We used MEGA5 (Tamura et al., 2011) to construct a phylogenetic tree with neighbor-joining (NJ) method. Arlequin version 3.5 (Excoffier and Lischer, 2010) was used to perform mismatch analyze and calculate the Tajima's D (Tajima, 1993, 1996) and Fu's parameters (Fu, 1997) based on the infinite alleles model. DnaSP version 5.0 (Librado et al., 2009) was employed to construct mismatch distribution diagram. Our results suggested that although

Table 2. Primer sequences for the six genetic loci of wild *Cymbidium tortisepalum* in Yunnan Province, China. In our previous study (Yang et al., 2013), 43 primer sequences were designed based on complete chloroplast genome sequence and six chloroplast fragments had variable sites among 10 tested samples in the pilot study, which were finally used to sequence all samples. The sequence has been deposited at GenBank with accession numbers KY621375-KY621467.

Region	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
trnk-rps16	TCCACCGATGAGTTAGCA	ATGTTGAGGAAGGCAGAAT
psbM-trnD	GTCATCTAAACCCGAAAAGT	ATTTGAAGAGCGACCATACT
trnV-trnA	TGGGCTCTTTCATCAACT	CAGGGTCTCCATCACTTC
accD-psal	ACGATGAAGATAAGGATGGGAGA	TGCGATTGCTGGAAAGACTA
rrn23	ACGGTAAACGCTGGGTAG	AGTTCGGGATGGATTGGT
ycf1	TATTTCGGTATTCTTTAGGTA	ACTTATCTTCCCTGTCCC

Table 3. Distribution frequencies of the 11 haplotypes in the 28 populations of wild *Cymbidium tortisepalum* in Yunnan Province, China.

Population	Location	No.	Haplotype distribution	*Hd*	π^z ($\times 10^{-2}$)
BD	Badi Township, Weixi County, Diqing Tibetan Autonomous Prefecture	15	H1(7), H3(8)	0.600	0.051
BJX	Baijixun Township, Weixi County, Diqing Tibetan Autonomous Prefecture	17	H1(15), H3(2)	0.221	0.028
WD	Weideng Township, Weixi County, Diqing Tibetan Autonomous Prefecture	17	H1(11), H3(6)	0.522	0.046
ZL	Zhonglu Township, Weixi County, Diqing Tibetan Autonomous Prefecture	18	H1(18)	0.000	0.000
CK	Cikai Township, Gongshan County, Nujiang Lisu Autonomous Prefecture	15	H1(13), H2(1), H3(1)	0.371	0.067
SD	Shideng Township, Lanping County, Nujiang Lisu Autonomous Prefecture	13	H1(12), H5(1)	0.154	0.006
ZP	Zhongpai Township, Lanping County, Nujiang Lisu Autonomous Prefecture	14	H1(12), H3(1), H4(1)	0.396	0.059
HS	Huangshan Township, Yu Long County, Lijiang City	18	H1(18)	0.000	0.000
YP	Yingpan Township, Lanping County, Nujiang Lisu Autonomous Prefecture	14	H1(10), H2(1), H3(3)	0.505	0.089
LJ	Lajing Township, Lanping County, Nujiang Lisu Autonomous Prefecture	14	H1(14)	0.000	0.000
PH	Pihe Township, Fugong County, Nujiang Lisu Autonomous Prefecture	11	H1(8), H4(1), H8(2)	0.473	0.044
LK	Liuku Township, Lushui County, Nujiang Lisu Autonomous Prefecture	12	H1(11), H5(1)	0.318	0.013
LZ	Luzhang Township, Lushui County, Nujiang Lisu Autonomous Prefecture	17	H1(13), H4(2), H6(1), H7(1)	0.507	0.083
CX	Changxin Township,Yunlong County, Dali Bai Autonomous Prefecture	14	H1(10), H2(2), H3(1), H5(1)	0.495	0.119
BC	Biaocun Township, Yunlong County, Dali Bai Autonomous Prefecture	11	H1(10), H2(1)	0.345	0.122
JZ	Jiuzhou Township, Yunlong County, Dali Bai Autonomous Prefecture	11	H1(11)	0.000	0.000
JC	Jiancao Township, Yunlong County, Dali Bai Autonomous Prefecture	16	H1(14), H3(1), H4(1)	0.242	0.031
BF	Baofeng Township, Yunlong County, Dali Bai Autonomous Prefecture	14	H1(12), H4(2)	0.264	0.076
MJ	Minjian Township, Yunlong County, Dali Bai Autonomous Prefecture	11	H1(10), H3(1)	0.345	0.031
TJ	Tuanjie Township, Yunlong County, Dali Bai Autonomous Prefecture	13	H1(13)	0.282	0.012
XS	Xishan Township, Eryuan County, Dali Bai Autonomous Prefecture	17	H1(15), H3(1), H5(1)	0.331	0.030
XZ	Xizhou Township,Dali County-level City, Dali Bai Autonomous Prefecture	13	H1(11), H2(2)	0.295	0.197
XT	Xiangtu Township, Jianchuan County, Dali Bai Autonomous Prefecture	12	H1(12)	0.000	0.000
WM	Wama Township, Longyang District, Baoshan City	18	H1(18)	0.000	0.000
TP	Taiping Township, Longyang District, Baoshan City	15	H1(13), H2(1), H11(1)	0.371	0.107
PP	Pupiao Township, Longyang District, Baoshan City	14	H1(10), H9(3), H10(1)	0.495	0.339
XG	Xiaguan County, Dali County-level City, Dali Bai Autonomous Prefecture	16	H1(16)	0.000	0.000
YW	Youwang Township, Shidian County, Baoshan City	14	H1(14)	0.000	0.000
Mean				0.269	0.055

[z] *Hd* = haplotype diversity; π = nucleotide diversity.

these methods and indices were commonly to infer population expansion, they vary in sensitivity and in their adaptability to data.

Results

Sequence characteristics and haplotype distribution. A sequence with a total length of 2702 bp was obtained from the six chloroplast fragments of the 404 individuals of wild *C. tortisepalum* from the Yunnan Province. Sixty-one mutation sites were detected, which included 50 indels and 11 haplotypes based on 11 base substitution sites. The distribution of each haplotype with the population is shown in Table 3 and Fig. 2.

Among the six sequences, the sequence length of the chloroplast trnk-rps16 fragment was the longest, which was 786 bp, and two base substitutions were detected. The sequence length of the chloroplast trnV-trnA fragment was the second longest at 704 bp, with two base substitutions. The sequence length of the chloroplast fragments psbM-trnD, accD-psal, rrn23, and ycfl, were 171, 283, 486, and 272 bp, respectively. There were only three, one, two, and one polymorphic loci detected in these fragments, respectively.

Nine of the 28 wild populations of *C. tortisepalum* sampled contained only one haplotype (32% of the population is within a single haplotype group). The remaining 19 populations showed polymorphisms, which included two to four haplotypes. Haplotypes H6, H7, H10, and H11 existed within only one individual, which were private haplotypes belonging to some of the populations. H6 and H7, two kinds of private haplotypes, both were distributed in the Luzhang (LZ) population. The haplotypes H10 and H11 were only found in the Pupiao (PP) population. Among the 11 haplotypes, H1 was the most widely distributed, and was present in all 28 populations. The H3 haplotype was the second most widely distributed.

Genetic diversity and genetic structure. Results revealed that the genetic diversity of wild *C. tortisepalum* at the species level ($H_T = 0.204$, $\pi = 0.00024$) and population level were low. In regard to genetic differentiation, there was no significant differentiation among populations ($G_{ST} = 0.099$, $N_{ST} = 0.081$). The higher G_{ST} value than N_{ST} value, indicates that the probability of the presence of haplotypes with close relation in the same or similar populations is low and there is no significant phylogeographic structure within the wild *C. tortisepalum* populations (Tables 4 and 5).

AMOVA revealed that the greatest genetic variance (≈91%) came from individuals within the population of wild *C. tortisepalum*, and the remaining 9.001% of the genetic variation existed among the populations, which indicated there is no clear genetic differentiation among populations (Table 6).

The results of the Mantel test analysis showed that the genetic distance of the wild *C. tortisepalum* had no significant correlation with geographical distance ($r = 0.279$, $P = 0.020$). The NJ adjacency tree constructed by the nonforeign group is shown in Fig. 3. Results revealed that 11 haplotypes

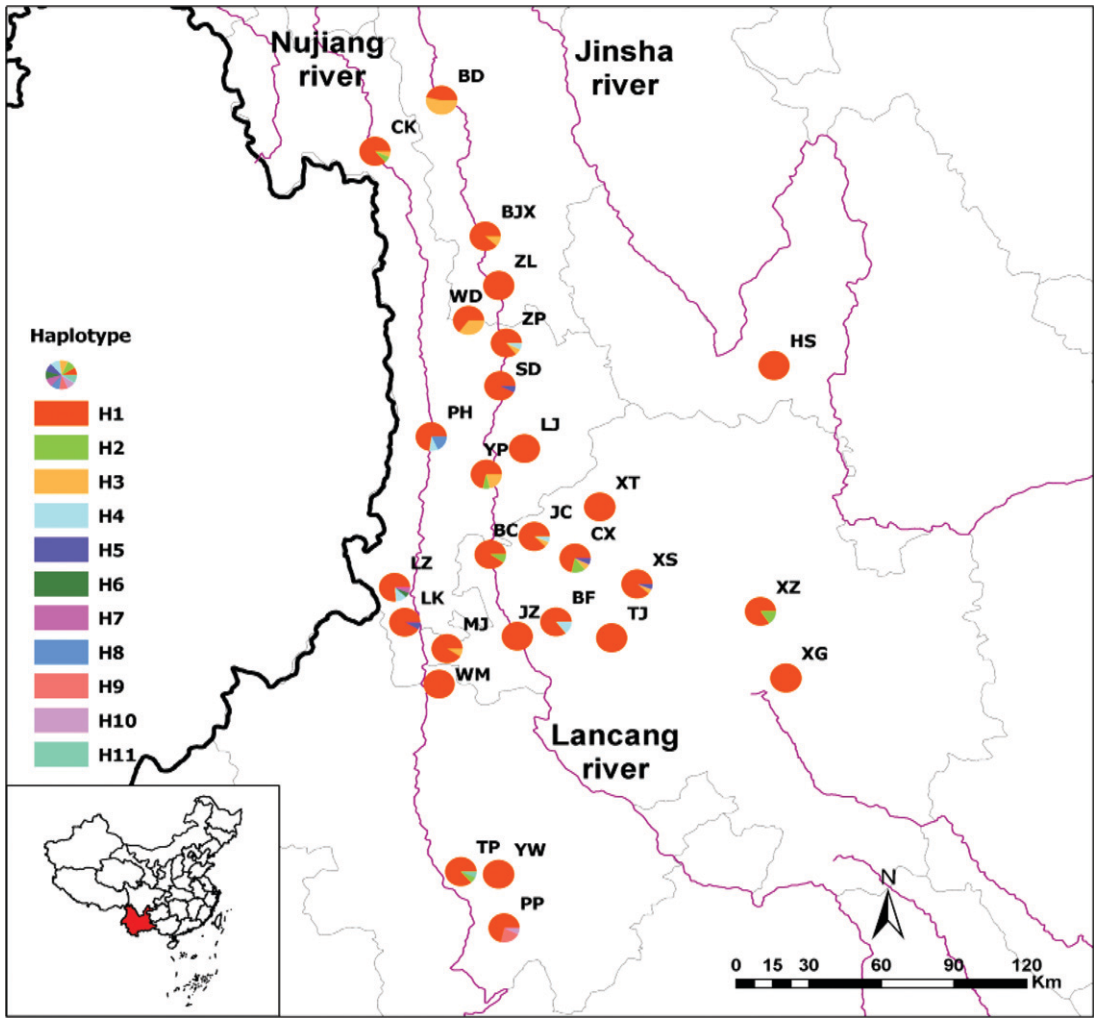

Fig. 2. Geographic distribution of the chloroplast DNA haplotypes of wild *Cymbidium tortisepalum* in Yunnan Province, China. Different colors in the label box represent different haplotypes, and the pie charts represent the distribution proportion of each haplotype in the populations.

of the wild *C. tortisepalum* formed six major branches with a 75% bootstrap value and six haplotypes (H2, H3, H6, H9, H10, and H11) constituted an independent branch. In addition, the remaining five haplotypes formed the collateral independent branch. The chloroplast haplotype network diagram constructed using the network of wild *C. tortisepalum* is shown in Fig. 4. All the haplotypes constituted a relatively simple linear relationship.

Mismatch analysis and neutral test. We examined the expansion history of wild *C. tortisepalum* using the tools of mismatch analysis and neutral test (Table 5, Fig. 5). According to the results of mismatch analysis, the

Table 4. Total genetic diversity of wild *Cymbidium tortisepalu*m based on chloroplast DNA in Yunnan Province, China. Average genetic diversity within populations (H_S), the total genetic diversity (H_T), the genetic differentiation coefficients (G_{ST}) and number of substitution (N_{ST}) were calculated using Permut version 2.0 software (Pons and Petit, 1996), G_{ST} and N_{ST} were compared via the U statistical method [1000 repeated permutation test (Nei, 1987; Tajima, 1993)].

Region[z]	*Hs*	H_T	G_{ST}	N_{ST}	*P*
			Mean (SE)		
Total	0.216 (0.0334)	0.240 (0.0402)	0.099 (0.0391)	0.081 (0.0233)	0.0000**
NW	0.241 (0.0695)	0.299 (0.0886)	0.196 (NC)	0.153 (0.0533)	0.0006**
MD	0.227 (0.4652)	0.234 (0.0459)	0.029 (NC)	0.026 (NC)	0.0362*
SW	0.150 (0.0994)	0.164 (0.1016)	0.082 (NC)	0.148 (NC)	0.1273

[z]NW = northern region of three parallel rivers in the western Yunnan Province; MD = middle region of three parallel rivers in the western Yunnan Province; SW = southern region of three parallel rivers in the western Yunnan Province.
*, **Significant at $P < 0.05$ or 0.01, respectively; data with no asterisks indicate that no significant difference found.

Table 5. Genetic diversity and neutral test of wild *Cymbidium tortisepalum* based on chloroplast DNA in Yunnan Province, China.

Groups[z]	Ns[y]	π	H_d	SSD	HRag	D	F*
Total	404	0.00024	0.241	0.06159	0.27860	−0.51328	0.19961
NW	127	0.00018	0.285	0.05533	0.25750	−0.29468	0.27766
MD	200	0.00031	0.241	0.02168	0.16689	−0.86229	0.33932
SW	77	0.00044	0.150	0.07484	0.17983	−0.07948	0.85512

[z]NW = northern region of three parallel rivers in the western Yunnan Province; MD = middle region of three parallel rivers in the western Yunnan Province; SW = southern region of three parallel rivers in the western Yunnan Province.

[y]Ns = number of sequences; π = nucleotide diversity; H_d = haplotype diversity; SSD = sum of squared deviation under expansion model; HRag = Harpending's raggedness index; D = Tajima's D statistic; F = Fu and Li's F* test statistic.

Harpending's raggedness (HRag) index of *C. tortisepalum* changed as a curve with a single peak both in the overall level or the three division areas and the observed values were in good agreement with the expected values, which indicated that the populations might have experienced rapid expansion, or it may have been caused by high mobility among neighboring groups accompanied with rapid population expansion. However, this inference was not supported by the neutral test because no significant differences were found among Tajima's *D* value and Fu and Li's value.

Discussion

Population genetic diversity and genetic differentiation. Chloroplast DNA is characterized by the small genome, maternal inheritance of angiosperm, and small selection pressure and rapid variation of its nonencoding area (Wolfe et al., 1989). Therefore, it is highly suitable for studies on natural population genetic structure, the system evolution of plants. This study reports the largest survey of molecular diversity of native *C. tortisepalum*to date, with 404 individiuals from 28 populations sequenced at six cpDNA fragments. The total genetic diversity of wild *C. tortisepalum* was low. The reason for this result may be firstly related to the properties of the chloroplast genome. The chloroplast genome is a haploidisation of the cytoplasmic genome, and its evolutionary rate is low. Compared with the nuclear genome, the effective population size reflected by the chloroplast genome is much smaller, which is about half of the nuclear genes (Birky, 1988). In addition, long-term human activities that exert pressure on the population cause a sharp reduction in the number of groups in the distribution of the population. According to our investigations in recent years, the distribution of wild *C. tortisepalum* in Dali, Nujiang, Baoshan, and other places has been in declining state due to overutilization of the species. The effective population has been severely reduced and the random genetic drift intensified, which has caused the loss of genetic diversity (Tang, 2014). Furthermore, as the effective size of the populations decrease, the effects of random genetic drift and the bottleneck effect on chloroplast genome are more severe than those on the nuclear genome. The loss of genetic diversity, reflected by the chloroplast genome, is also amplified (Jordano and Godoy, 2000; Xun et al., 2011).

Alternatively, the results of this study may be related to the narrow geographic distribution and breeding system of this species. Wild resources of *C. tortisepalum* are mainly concentrated in the Dali Bai Autonomous Region, the Nujiang Lisu Nationality Autonomous Prefecture, Baoshan City, Lijiang City, and the Diqing Tibetan Autonomous Prefecture in the Yunnan Province. In general, species with a small distribution tend to have lower genetic diversity than those with a wide range distribution.

AMOVA of the cpDNA sequence results showed that the genetic differentiation of wild *C. tortisepalum* primarily existed within the populations (91%), and genetic differentiation among populations (F_{SC} = 8.4%) and divergence among all populations (F_{ST} = 0.09001) were relatively low, which was consistent with the theory that 90% of variation exists within populations of cross-pollinated plants (Hamrickn and Godt,1990). A similar result of 89.25% molecular variation within populations was obtained in our previous investigation involving AMOVA of SSR data of these plants (Tang, 2014; Zhao et al., 2017), which, combined with the present results, indicate that the genetic background of wild *C. tortisepalum* is relatively simple, and there are no complex lineage differentiations. Low genetic differentiation of wild *C. tortisepalum* may be due to its unique life history, its highly specialized floral traits, its long evolutionary history, and severe bottlenecks, which the species may have experienced during the last ice age, as well as the fragmentation of the population distribution region with effects on seed and pollen flow.

The nucleotide polymorphism (Hd = 0.241) and haplotype polymorphism ($\pi = 0.24 \times 10^2$) of *C. tortisepalum* at the species level were low. The genetic diversity of this species was primarily concentrated within populations (H_S = 0.216), which was consistent with the results of a study by Jia et al., (2012). At the species level, the N_{ST} value was less than the G_{ST} value ($P > 0.05$), which indicated that the probability of closely related haplotypes in the same or similar populations was low and that- there was no clear spectrum structure of the 28 populations in this study. This result was consistent with that of our previous study on SSR (Tang, 2014), which was in agreement with the theory that there is no significant correlation between geographic distance and genetic distance among outcrossing species, especially species with a small geographic distribution (Willyard et al., 2007).

Table 6. Analyses of molecular variance of wild *Cymbidium tortisepalum* based on chloroplast DNA in Yunnan Province, China.

Loci	Source of variation	df	Sum of squares	Variance component	Fixation indices[z]
Six	Among groups	2	1.311	0.00130Va	F_{CT} = 0.00598
Loci	Among populations	25	11.793	0.01874Vb	F_{SC} = 0.08454
	Within groups				
	Within populations	376	76.287	0.20289Vc	F_{ST} = 0.09001
Total		403	89.391	0.22293	

[z]F_{CT} = genetic diversity between regions; F_{SC} = differentiation among individuals among populations; F_{ST} = divergence among all populations.

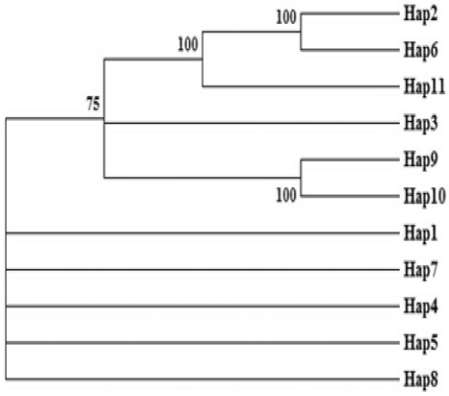

Fig. 3. Neighbor-joining trees obtained by analysis of 11 chloroplast DNA haplotypes of wild *Cymbidium tortisepalum* in Yunnan Province, China. Hap1–Hap11 represent 11 haplotypes, respectively. Bootstrap values (>50%) are based on 1000 replicates and are indicated above branches.

The level of genetic diversity within a species depends on the breeding system, especially the number of effective populations and their variation among generations, which has significance to the future evolution and survival of the species. Endemic, rare, or endangered species are generally considered to have low levels of genetic diversity, due to low population numbers, the isolation of populations, and their adaptation to a single habitat (Barrett and Kohn, 1991). The genetic diversity of the endangered and their nonendangered species were compared by Frankham (1995) and Spielman et al. (2004), which demonstrated that the genetic diversity of most endangered species was lower than that of corresponding nonendangered species. However, results

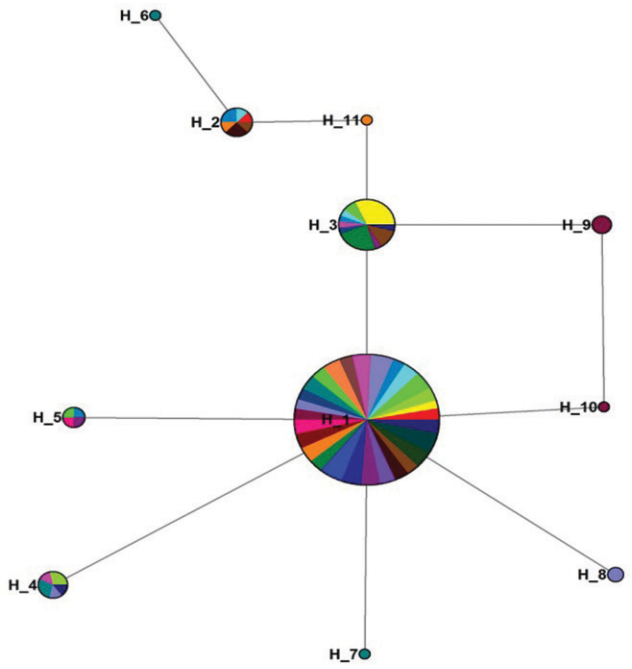

Fig. 4. The haplotype network of 11 chloroplast DNA haplotypes detected in 28 populations of wild *Cymbidium tortisepalum* in Yunnan Province, China. The size of circles corresponds to the frequency of each haplotype, H1–H11, represent 11 haplotypes, respectively, with each solid line representing one step mutation.

of another study revealed that the genetic diversity level was still high for some endangered species, such as *Iris aphylla* (Wroblewska et al., 2003), *Suzukia luchuensis* (Maki et al., 2003), and *Sarracenia leucophylla* (Wang et al., 2004).

Chloroplast haplotype analysis. When interpreting the haplotype network diagram, the newly derived haplotype is often located at the tip of the clade, while the ancestral haplotypes are usually found in interior node (Crandall and Templeton, 1993; Schaal et al., 1998; Templeton, 1998). The most widely distributed haplotype, H1, located in the central position, was the ancestral haplotype, and H5, H8, H4, and the unique haplotypes H6 and H7, located at the end of the network, were the newly derived haplotypes.

Moreover, the haplotype diversity of the Bady (BD) population located in Badi Township in Diqing Weixi County was the highest. The number of haplotypes (four) was the highest in the Changxin (CX) population located in Dali and the LZ population located in the NuJiang region. The private haplotypes H10 and H11 found in PP and Taiping (TP) populations located in Pupiao and Taiping town of Baoshan Longyang District, respectively. Populations in a refuge should have a higher genetic diversity than those in a nonrefuge, and they should contain the ancestral (or ancient) haplotype (Hewitt, 1999, 2004; Lascoux et al., 2004; Petit et al., 2005). It can be inferred that BD, CX, Lushui (LS), and the towns of PP and TP in Baoshan may have been a refuge for wild *C. tortisepalum* during the last ice age, and these areas may have richer resources of wild *C. tortisepalum*.

Conservation and future studies on wild *C. tortisepalum*. Orchidaceae is one of the largest families of flowering plants, although most are rare and endangered plants. All wild orchid species are included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora. The main goal for protecting an endangered species is to conserve its genetic diversity because the species may only survive and adapt to the environmental changes by maintaining sufficient genetic variation within and among populations (Barrett and Kohn, 1991). The results of this study showed that the total genetic diversity and genetic deviation of wild *C. tortisepalum*, as analyzed by cpDNA, were low. Therefore, this species may have a relatively low tolerance to ecologic or random climatic events and natural phenomena. On the basis of our investigation, we considered that the main cause in impending danger to wild *C. tortisepalum* resources was not due to its genetic basis and biological characteristics but to habitat fragmentation and the rapid decrease in the number of populations and the number of individuals within populations, which is caused by habitat destruction and overutilization by humans. We found that the survey region for wild *C. tortisepalum* had experienced severe habitat destruction, which made it difficult to find wild *C. tortisepalum* resources in its reported distribution areas. Most of the populations were small, and the individuals in the population were scattered. With time, the loss of genetic diversity would further exacerbate the endangered status of this species. Therefore, to maintain the long-term survival of this species, the protection of the habitat of wild *C. tortisepalum* is critical. As possible refuges for resources of wild *C. tortisepalum*, BD, CX, LS, PP, and TP towns should be given priority for protection and future studies.

The results of this study also showed that the vast majority of genetic variation in wild *C. tortisepalum* was located within populations. The haplotype frequency had a large difference within populations, and low frequency haplotypes accounted for the

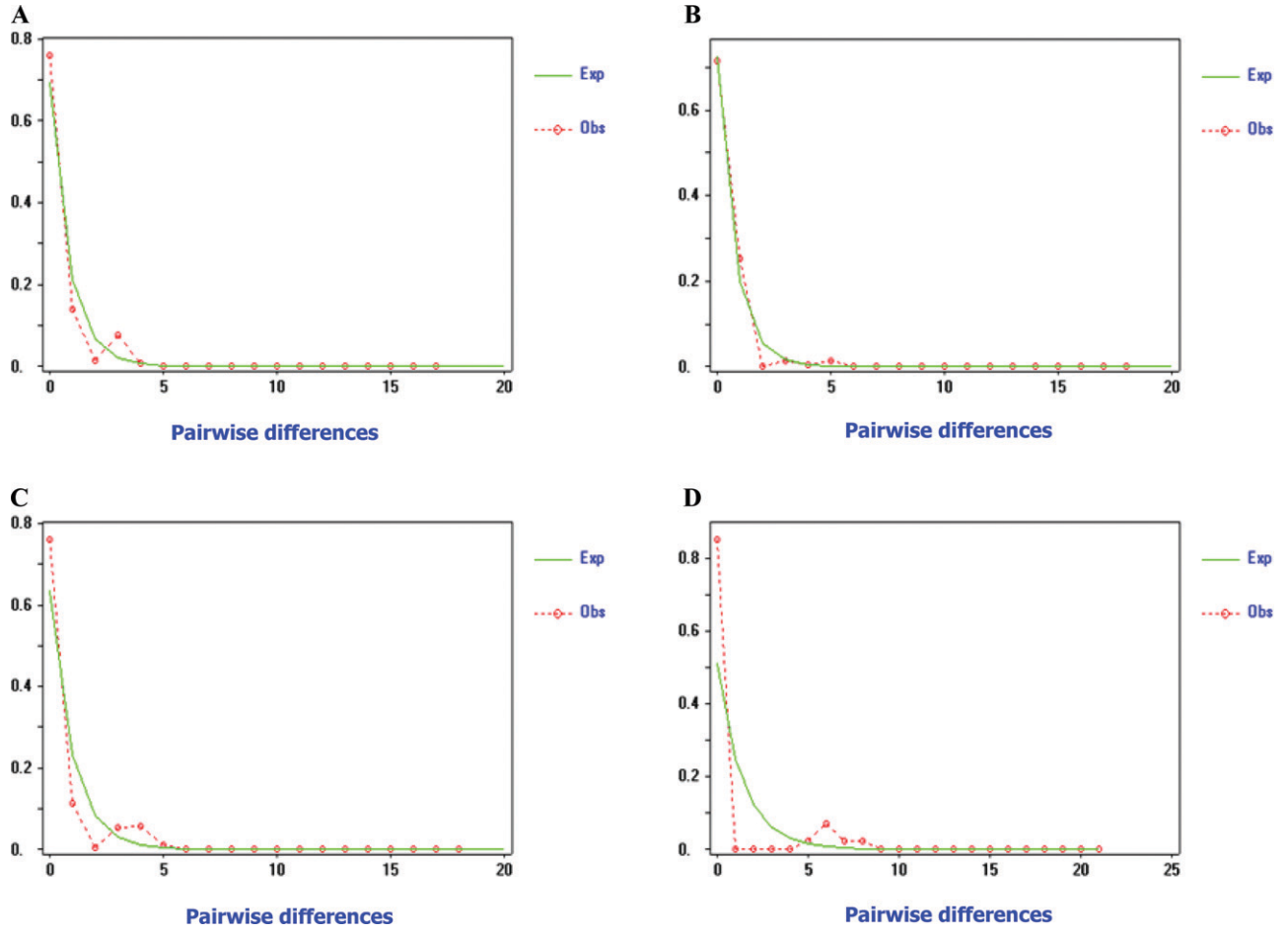

Fig. 5. Mismatch distribution analysis based on chloroplast DNA of wild *Cymbidium tortisepalum* in Yunnan Province (**A**), in northern region of three parallel rivers in the western Yunnan Province (**B**), in middle region of three parallel rivers in the western Yunnan Province (**C**), and in southern region of three parallel rivers in the western Yunnan Province (**D**). Solid lines indicate expected value (Exp), dotted lines reflect observed value (Obs).

majority of the populations. The low frequency of haplotypes was likely caused by decreases in the populations and random genetic drift and also the result of declining genetic diversity within populations. Therefore, we suggest that the number of populations could be reduced and that individuals from each population should be increased for conservation or sampling. In particular, this protection strategy should be preferred in populations with high genetic diversity and low frequency haplotype groups.

Future studies should be based on the investigation of large-scale resources, with the collection of as many representative population samples as possible from areas with less human activity and more simple ecological climates, such as the Diqing Tibetan Autonomous Region. The diversity and genetic differentiation of wild *C. tortisepalum* should be studied by the method of single-copy nuclear gene sequence and cpDNA sequences of a certain length, which would provide a more reliable basis for further elucidation of the genetic diversity and the population genetic differentiation patterns of wild *C. tortisepalum*.

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