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MORPHOLOGICAL DIVERSITY OF LEAF AND ITS GEOGRAPHIC DIFFERENTIATION OF *CITRUS CAVALERIEI* FROM YUNNAN

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Abstract

The orange species *Citrus cavaleriei* is endemic to China and its unifoliate compound leaves are characterized by the well-developed wing leaf. Nine populations from Yunnan Province were studied, based on ten leaf characters, including the total length of leaf (TLL), the length of wing leaf (LWL), the width of wing leaf (WWL), the length of leaflet (LL), the width of leaflet (WL), LWL/WWL, LL/WL, LWL/TLL, LWL/LL, and WWL/WL. The results show that the maximums of most leaf characters are in the Weixin Population while the minimums are in the Yangbi Population; the variation of most leaf characters in Yangbi Population is highest and that in Suijiang Population and Eshan-1 Population is lowest; the variations of WWL in all populations were higher, while that of LWL/TLL is lower; the variance of every character among populations is very significant; the correlation between the most leaf characters is very positively or negatively significant. Meanwhile the longitude has a very significant correlation with all leaf characters; the latitude with LL, WL, TLL, and LL/WL has a very significant positive correlation, that with WWL has a significant positive correlation, and that with LWL/TLL, LWL/LL, WWL/WL, and LWL/WWL has a very significant negative correlation; the correlation between the altitude and LWL/TLL, LWL/LL, WWL/WL, and LWL/WWL are positively significant and that between the altitude and LL, WL, TLL, WWL, LWL, and LL/WL were extremely negatively significant. The clustering of ten leaf characters from nine populations is in accordance with the geographical distribution, especially the longitude.

Key words: *Citrus cavaleriei*; Geographic differentiation; Leaf; Morphological diversity.

Introduction

Genetic variation is the results of the evolution, and is the basic precondition for species surviving, adapting and expanding (Soltis & Soltis, 1991; Ge, 1997). Variation can be revealed on the level of the population, individual, tissue or molecule (Moritz & Hillis, 1996). For a species there are different morphological characters with the different patterns and levels, among the different individuals or populations, and all variation is the product of difference of the strength and direction of kinds of biological and abiological factors; namely, the diversity of the species is the results comprehensively from the genetic and the environmental diversity (Gu, 2004). Therefore, it is an effective method to check the genetic variation based on the morphology or characters (Schaal *et al.*, 1991; Ge & Hong, 1994). In the natural populations, most morphological characters are quantitative and determined by the polygenes, which has certain significance for adaption and evolution. To reveal the relationship between the plants and the environments is helpful to explore the patterns and the mechanisms of plants adapting and evolving, as well as the influencing factors, and to profoundly understand the natural choices, the gene flows and the genetic drifts (Schaal *et al.*, 1991; Ge & Hong, 1994; Ge, 1997). For example, many micro-morphological characters of seeds had been used to assess the evolutionary relationship among the different families (Abid *et al.*, 2014).

Many wild and semi-wild *Citrus* species and its relative genera grow in the southern areas of China, especially Yunnan and its adjacent areas, including India and Burma (Gmitter & Hu, 1990). As an important wild orange, *C. cavaleriei* is endemic to China, and maybe one of parents of modern many cultivated *Citrus* (Mabberley, 2002, 2004). The species is found in the most extensive areas, mainly in the southern areas to the southern slope of the Qinling Mountain, but it can't grow well when introduced in the southern areas of Tropic of Cancer (Huang, 1997). It grows on the stony areas such as river valleys, stream banks, steep slopes, or cliffs (Gou, 1985; Wu *et al.*, 2010; Chen *et al.*, 2012a, 2012b). At present, the living situation of *C. cavaleriei* in the fields is getting worse and worse, and the number of wild populations are dramatically decreasing in recent years. This species can reach the most northern distribution of the natural *Citrus* and can be found up to an elevation of 2 500 m. It can grow well at the temperature of -11.5ºC and resist the low temperature of -15ºC, which means that the species is able to resist cold, infertility and shade, and can be used for the resistance breeding, as well as dwarf breeding.

As a special *Citrus* species, many morphological characters of *C. cavaleriei* are between *Citrus* subgen. *Papeda* and *C.* subgen. *Citrus* (Swingl, 1943; Swingle & Reece, 1967; Pang *et al.*, 2007). It is closely ralated to the *Papeda* because its unifoliate compound leaves have well-developed wing leaves while it is similar to the *Citrus* based on the simple flowers and the filaments fused into a bunch. The species *Citrus cavaleriei* is so important and controversial that many previous studies mainly discussed its systematic position, namely the species belonging to the original *Papeda* (Swingle & Reece, 1967; Zeng, 1990; Ye *et al.*, 1982; Zhu, 1988; Fang *et al.*, 1993; Zhong & Ye, 1993; Zhong, 1992; Xiong, 2002; Liang *et al.*, 2007) or evolutional *Citrus* (Tanaka, 1969; Jiang, 1983; Xie *et al.*, 2008), even the derivants from *Papeda* (Nicolosi *et al.*, 2000; Abkenar *et al.*, 2004; Bayer *et al.*, 2009). Due to the wide distribution and different habitats of this species, it is characterized with many variable morphological characters, such as leaves, flowers, fruits, and seeds. In Yunnan Province, the natural environment is diverse and a several types of *C. cavaleriei* grow here. For example, the type from west Yunnan, such as Yangbi and Tengchong, is distinctly different from that from northeast Yunnan near Sichuan Province (Bao *et al.*, 1991; Huang, 1997), and the latter is more closed to the typical plant of this species from other areas. In this paper, ten leaf morphological characters of *C. cavaleriei* from nine populations were studied, and the features and the rules of morphological variation of leaves among and in the populations will be discussed here. The rules of geological differentiation, as well as the relationship between the variation degrees or variation patterns of leaf morphology and its ecological environments, can provide the scientific basis for further discussing the biological and geographic origin and the migratory route of *C. cavaleriei*.

Materials and Methods

Materials: The leaves of *Citrus cavaleriei* are unifoliate and the wings at the both sides of the common petiole are very developed, namely the developed wing leaves (Fig. 1). The detailed distribution information of *C. cavaleriei* in Yunnan Province was confirmed after researching the specimens and references. From June 2014 to March 2015, the resources researching and data collecting in the natural distribution had been done and the information about the natural populations were listed in the Table 1.

Sampling and measurement: By the GPS instrument, the concise location of every population was positioned, and the information of the longitude, the latitude and the altitude were listed (Table 1, Fig. 2). A total of 138 plants were sampled from nine populations in Yunnan Province. In every population, the fresh adult leaves from at least 20 plants or all plants (when less than 20 plants) were collected and numbered, and five indexes of leaf morphological characters were measured and recorded, including the total length of leaf (TLL), the length of wing leaf (LWL), the width of wing leaf (WWL), the leaf length (LL), the width of leaf (WL). The accuracy of measurement is within 0.01 cm. More than one voucher specimens were collected in every population and kept in the herbarium (SWFC).

Data analysis: By Excel 2007and SPSS19.0, all data of *Citrus cavaleriei* from nine populations were calculated based on ten indexes, including TLL, LWL, WWL, LL, WL, LWL/WWL, LL/WL, LWL/TLL, LWL/LL, and WWL/WL. The minimum, the maximum, the average, the standard deviation and the coefficient of variation from ten indexes were calculated, and the variation of leaf characters in every population were measured based on the coefficient of variation. The variation levels in each population and between populations were tested by the one-way ANOVA. The correlation between the leaf morphological characters, and between the leaf characters and the geographic indexes were analyzed by the bi-variate correlation analysis. The data were clustered based UPGMA by NTSYS2.1.

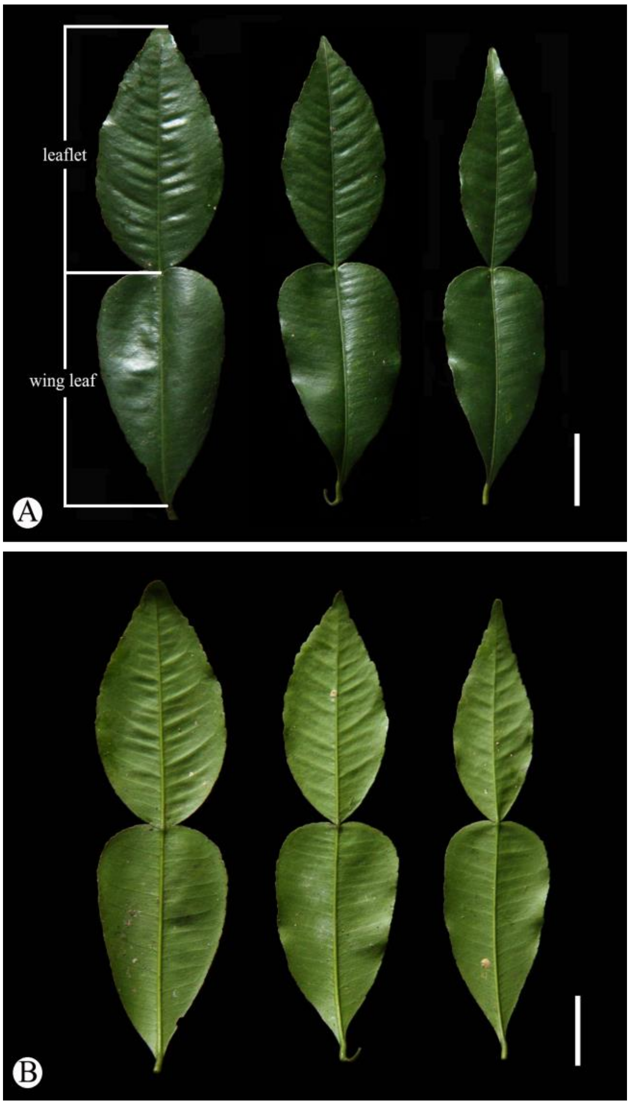

Fig. 1. Leaves of *Citrus cavaleriei* from Yuanjiang Population (Bar scale: 2 cm).

Table 1. Geographical coordinates and habitats of *Citrus cavaleriei* from Yunnan Province.

Population	Location	Sample	Longitude	Latitude	Altitude (m)	Habitat	Time of sampling
TC	Tengchong, Mingguang	20	98.5333	25.4833	2016	West slope, sunny, dry, shrub	2014/10/25
YB	Yangbi, Cangshanxi	21	99.9500	25.6167	2108	Sunny, fertile, wet, open forest	2014/10/27
YJ	Yuanjiang, Wadie	20	102.2667	23.9667	2102	Rocky hill, wet, jungle	2015/03/04
ES-1	Eshan, Chahe	5	102.2833	24.3167	2224	Sunny, fertile, wet, open forest	2015/03/02
ES-2	Eshan, Chahe	20	102.3000	24.3000	2232	Rocky hill, shady, jungle	2015/03/02
AN	Anning, Wenquan	13	102.4500	25.0000	1981	On both sides of the ditch, fertile, wet, jungle	2014/10/06
SJ	Suijiang, Banli	12	104.0667	28.3833	969	Shady wet, jungle	2014/06/11
ZX	Zhenxiong, Chishuiheyuan	7	104.9333	27.5500	1664	Rocky hill, sunny, shrub	2014/08/05
WX	Weixin, Zhaxi	20	105.0167	27.8500	1568	Rocky hill, stream bank	2014/08/04

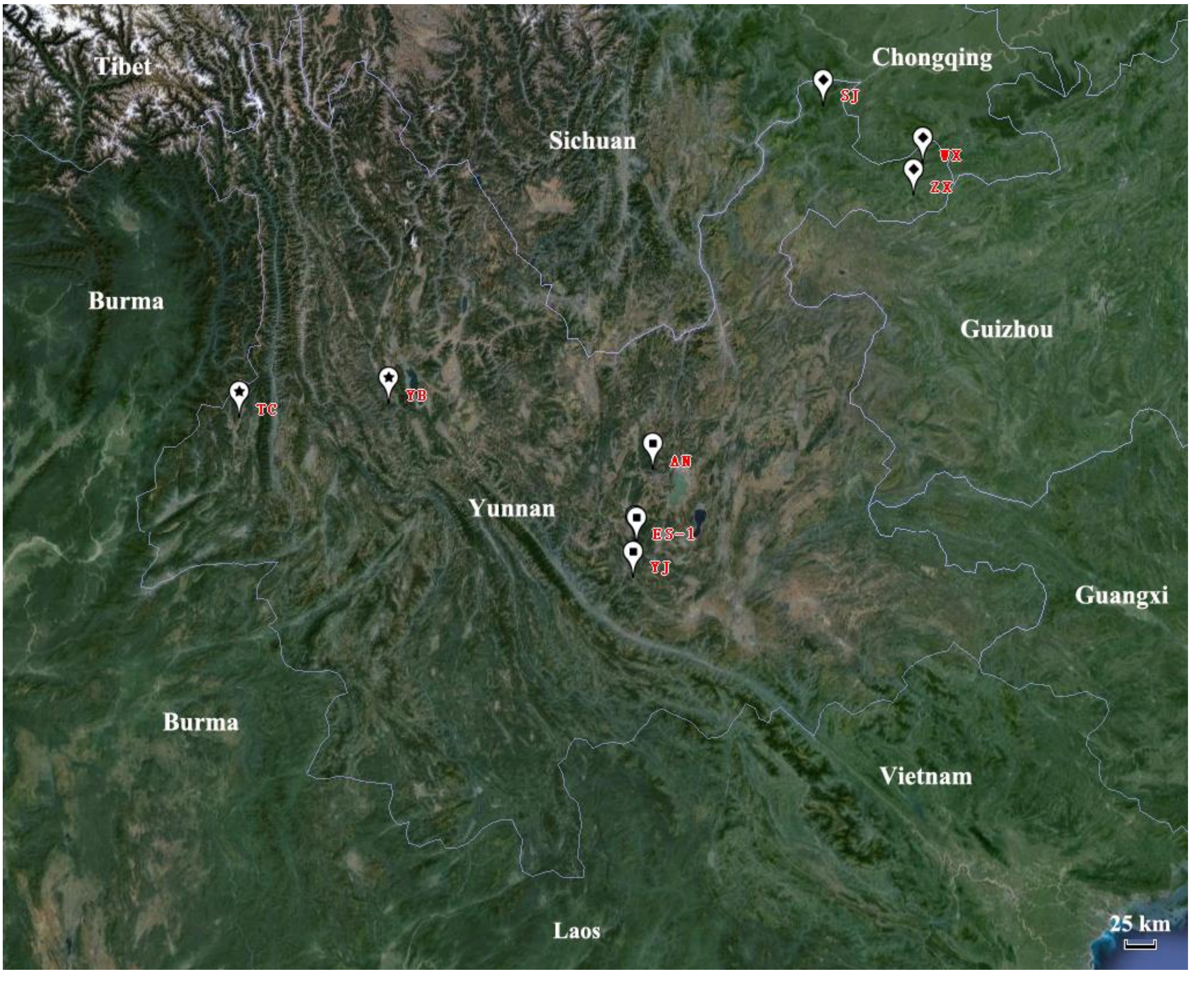

Fig. 2. Sampling information of *Citrus cavaleriei* from Yunnan Province.

Results

Variation of leaf morphology: The maximum of TLL, LWL and LL are 19.2 cm (WX), 9.5 cm (WX), 10.8 cm (WX, SJ) respectively (Table 2) and the minimum are 4.3 cm (YB), 2.1 cm (YB), 2.2 cm (YB); the maximum of WLW and LW are 5.3 cm (WX) and 4.9 cm (SJ) and the minimum are 1.0 cm (YB) and 0.9 cm (YB); the maximum and the minimum of LWL/WWL are 3.33 (WX) and 1.35 (AN), these of the LL/WL is 5.22 (YB) and 1.51 (ES-2), these of the LWL/TLL are 0.59 (ES-2) and 0.34 (SJ), these of the LWL/LL are 1.38 (TC) and 0.52 (SJ), and these of the WWL/WL are 1.57 (TC) and 0.61 (SJ). The maximum of most leaf characters are found in the Weixin Population while the minimum of many characters are in the Yangbi Population.

For the same leaf character, the variation in the different populations of *Citrus cavaleriei* is significant (Table 3). For example, the coefficient of variation (*CV*) of TLL, LL and WL in Yangbi Population are the maximum (26.53%, 27.65%, 30.78%), those in Suijiang Population are the minimum (14.30%, 15.13%, 15.57%); the CV of LWL in Yangbi Population is the maximum (27.38%) and that in Eshan1 Population is the minimum (15.80%); the CV of WWL and LWL/LL are the maximum in Zhenxiong Population (31.32%, 17.00%) and those in Eshan-1 Population are the minimum (15.8%, 9.62%). It can be inferred that the variation of most leaf characters in the Yangbi Population are the maximum, those in Suijiang and Eshan-1 Population are the minimum and the plants in these two populations are more relatively stable.

The variations of leaf characters of *Citrus cavaleriei* in the same population show obvious differences too (Figs. 3-6). In Tengchong, Anning and Weixin Population, CV of WWL are the maximum (21.51%, 24.29%, 28.05% respectively), and that of LWL/TLL is the minimum (6.44%, 8.21%, 8.95% respectively). The results show that the variations of the wing leaf width are larger in all populations and the leaf trait is adapt to be influenced by the environments, while those of LWL/TLL are lesser; being a relatively stable characters, the index LWL/TLL can be used as an important referring index when identifying this species.

In total, the difference of ten leaf characters from nine populations are very significant (Table 3), and the order is: LL > LWL > TLL > LWL/LL > TLL > WWL/WL > WWL > LWL > LL/WL > LWL/WWL. Based on many leaf characters, it is inferred that the diversity of leaf morphology from all populations in Yunnan Province is very high.

Table 2. Maximum and minimum of ten leaf characters from nine populations of *Citrus cavaleriei*.

Character	Index	TC	YB	ES-1	ES-2	AN	YJ	SJ	ZX	WX
TLL (cm)	Min	6.00	4.30	8.75	7.10	6.00	8.35	9.20	6.00	6.80
	Max	15.20	15.5	14.20	13.80	17.6	15.5	17.5	13.15	19.20
LWL (cm)	Min	2.80	2.10	4.25	3.30	3.00	4.25	3.60	2.50	2.80
	Max	7.50	8.00	6.90	7.60	8.75	7.50	7.80	7.05	9.50
WWL (cm)	Min	1.55	1.00	2.20	1.55	1.50	2.10	1.80	1.08	1.50
	Max	3.80	3.90	3.90	3.70	4.80	4.25	4.10	4.00	5.30
LL (cm)	Min	3.20	2.20	4.25	3.65	3.00	4.05	5.05	3.50	4.00
	Max	8.10	8.70	7.40	7.10	9.10	8.90	10.8	7.10	10.8
WL (cm)	Min	1.50	0.90	2.10	1.60	1.45	1.75	2.20	1.60	1.55
	Max	3.30	3.60	3.50	3.70	4.70	4.00	4.90	3.80	4.80
LWL/WWL	Min	1.54	1.43	1.61	1.54	1.35	1.45	1.55	1.46	1.39
	Max	2.96	3.00	2.19	2.71	2.44	2.57	2.37	3.33	2.64
LL/WL	Min	1.95	1.53	1.80	1.51	1.64	1.61	1.76	1.68	1.85
	Max	3.60	5.22	2.42	3.05	2.85	2.91	3.00	2.76	3.31
LWL/TLL	Min	0.43	0.39	0.43	0.41	0.38	0.43	0.34	0.37	0.36
	Max	0.58	0.54	0.52	0.59	0.56	0.56	0.51	0.54	0.56
LWL/LL	Min	0.74	0.65	0.76	0.69	0.61	0.74	0.52	0.59	0.57
	Max	1.38	1.17	1.08	1.46	1.26	1.28	1.04	1.16	1.25
WWL/WL	Min	0.81	0.81	0.89	0.83	0.76	0.88	0.61	0.64	0.81
	Max	1.57	2.44	1.29	1.28	1.28	1.52	1.29	1.20	1.55

Note: TLL (total length of leaf), LWL (length of wing leaf), WWL (width of wing leaf), LL (length of leaflet), WL (width of leaflet)

Table 3. F-test of one-factor analysis of variance.

Characters	F	P	Character	F	P
TLL (cm)	26.240**	0.000	LWL/TLL	36.843**	0.000
LWL (cm)	11.995**	0.000	LWL/LL	34.498**	0.000
WWL (cm)	12.571**	0.000	LWL/WWL	6.761**	0.000
LL (cm)	43.841**	0.000	LL/WL	11.210**	0.000
WL (cm)	35.037**	0.000	WWL/WL	21.957**	0.000

Note: * Significant difference ($p<0.05$), ** Very significant difference ($p<0.0 1$)

The correlation between the leaf characters: The correlation between the most leaf characters is very positively or negatively significant; the correlation between TLL and WWL/WL is negatively significant; those of a few characters are not significant (Table 4). The results from the correlation analysis show that all leaf morphology is in agreement with each other to keep the stability of leaf in *Citrus cavaleriei*.

Correlation between the leaf and geographic factors: It is showed that the distribution longitude of population has a very significant correlations with ten leaf characters (Table 5). The longitude with WL, LL, TLL, WWL, and LWL has a very significant positive correlation respectively (R=0.468, 0.431, 0.348, 0.298, 0.182), from which it is concluded that the length of the unifoliate, the leaflet, and the wing leaf and the width of the leaflet and the wing leaf increase when the value of the longitude increasing. The longitude has a very significant negative correlations with LWL/TLL, WWL/WL, LWL/LL, LWL/WWL, and LL/WL respectively (R=-0.384, -0.361, -0.373, -0.246, -0.129), and it is inferred that the length and the width of the wing leaf and the leaflet all decreased from the west to the east. In a word, the wing leaf and the leaflet from the west populations are longer and wider than that in the east populations.

The latitude with LL, WL, TLL, and LL/WL all has a very significant positive correlation (R=0.442, 0.312, 0.291, 0.190), that with WWL has a significant positive correlation (R=0.114), and that with LWL/TLL, LWL/LL, WWL/WL, and LWL/WWL has a very significant negative correlation (R=-0.545, -0.531, -0.315, -0.128) (Table 4). It can be included that the wing leaf and the wing leaf of C. *cavaleriei* from areas with higher latitude are more longer and wider while the values of LWL/TLL, LWL/LL, WWL/WL, and LWL/WWL in lower latitude are more higher.

Ten leaf characters are significantly correlated with the altitude (Table 5). The correlation of between the altitude and LWL/TLL, LWL/LL, WWL/WL, and LWL/WWL are positively significant (R=0.577, 0.558, 0.402, 0.132) and that between the altitude and LL, WL, TLL, WWL, LWL, and LL/WL are extremely negatively significant (R=-0.572, -0.458, -0.422, -0.210, -0.156, -0.141). The data of correlation show that the value of LL, WL, TLL, ELW, LWL, and LL/WL in the population at higher altitude are lower than that at lower altitude; but the value of LWL/TLL, LWL/LL, WWL/WL, and LWL/WWL are larger.

Clustering of leaf characters: The results of UPGMA clustering analysis based on the average of ten leaf characters from nine populations show that nine populations of *Citrus cavaleriei* are divided into five clades. The first clade consisted of three populations, namely Suijiang, Zhenxiong and Weixin Population; the second one comprises Eshan-2, Anning and Yuanjiang Population; others are Eshan-1, Yangbi and Tengchong Population (Fig. 7). The clustering results of the leaf characters keep high accordance with the geographical distribution, specially the longitude, which illustrate the variation rules of leaf characters with geographic variation.

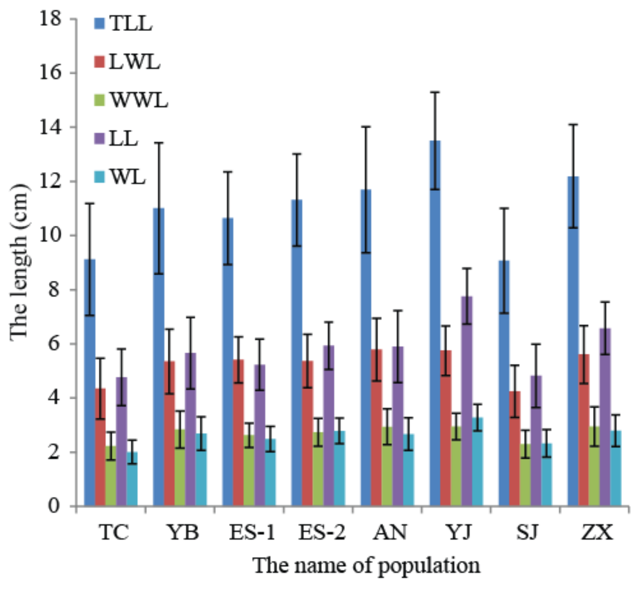

Fig. 3. Average and standard deviation of TLL, LWL, WWL, LL, and WL of *Citrus cavaleriei* from nine populations.

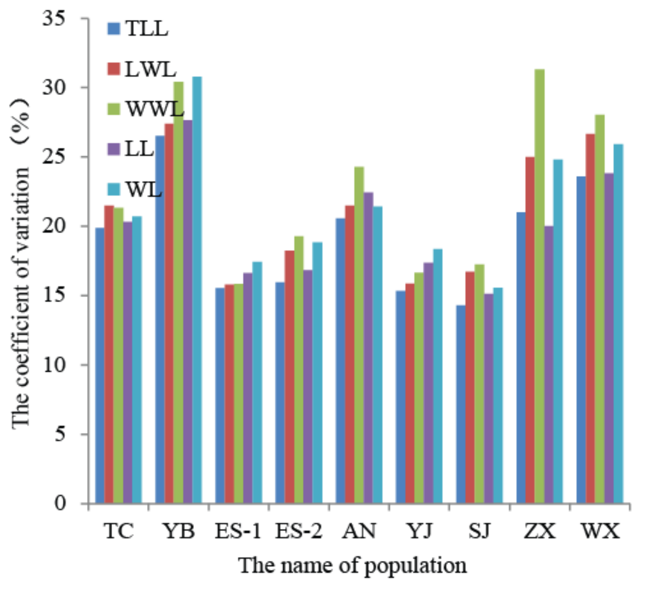

Fig. 5. Coefficients of variation of TLL, LWL, WWL, LL, and WL of *Citrus cavaleriei* from nine populations.

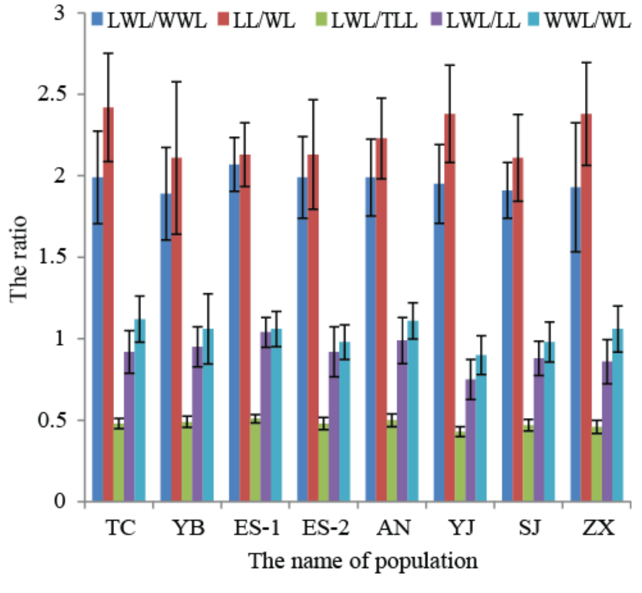

Fig. 4. Average and standard deviation of LWL/WWL, LL/WL, LWL/TLL, LWL/LL, and WWL/WL of *Citrus cavaleriei* from nine populations.

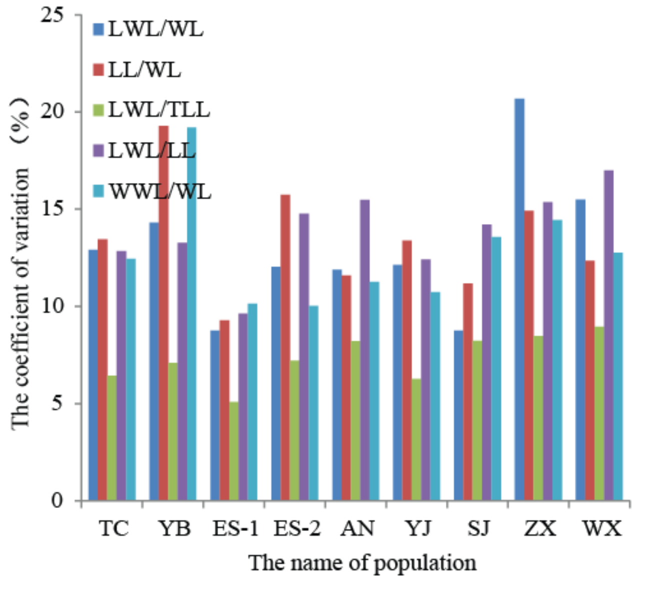

Fig. 6. Coefficients of variation of LWL/WWL, LL/WL, LWL/TLL, LWL/LL, and WWL/WL of *Citrus cavaleriei* from nine populations.

Table 4. Correlation coefficient between ten leaf characters of *Citrus cavaleriei*.

Character	LWL (cm)	WWL (cm)	LL (cm)	WL (cm)	LWL/ WWL	LL/ WL	LWL/ TLL	LWL/ LL	WWL/ WL
TLL (cm)	0.892**	0.831**	0.941**	0.667**	-0.014	0.177**	-0.199**	-0.066	-0.108*
LWL (cm)		0.841**	0.686**	0.557**	0.136**	0.011	0.231**	0.204**	0.150**
WWL (cm)			0.706**	0.69**	-0.399**	-0.193**	0.015	0.015	0.141**
LL (cm)				0.656**	-0.125	0.277**	-0.508**	-0.26**	-0.287**
WL (cm)					-0.325**	-0.247**	-0.340**	-0.19**	-0.288**
LWL/WWL						0.409**	0.348**	0.305**	-0.001
LL/WL							-0.254**	0.051	0.253**
LWL/TLL								0.994**	0.589**
LWL/LL									0.454**

Note: * Significantly correlated at α = 0.05, **Highly significantly correlated at α = 0.01.

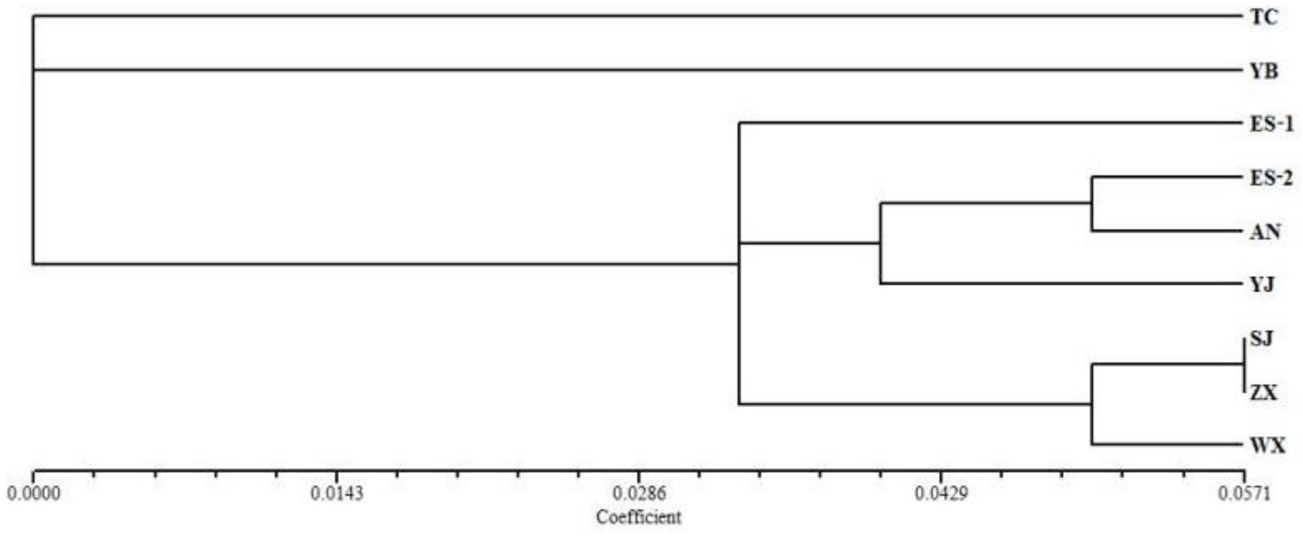

Fig. 7. Clustering analysis of *Citrus cavaleriei* from nine 9 populations in Yunnan Province (UPGMA).

Table 5. Correlation coefficient between leaf characters and geological factors of *Citrus cavaleriei*.

Leaf character	Longitude	Latitude	Altitude (m)
TLL (cm)	0.348**	0.291**	-0.422**
LWL (cm)	0.182**	0.046	-0.156**
WWL (cm)	0.298**	0.114*	-0.210**
LL (cm)	0.431**	0.442**	-0.572**
WL (cm)	0.468**	0.312**	-0.458**
LWL/WWL	-0.246**	-0.128**	0.132**
LL/WL	-0.129**	0.190**	-0.142**
LWL/TLL	-0.384**	-0.545**	0.577**
LWL/LL	-0.373**	-0.531**	0.558**
WWL/LW	-0.361**	-0.315**	0.402**

Discussion

The higher the genetic diversity of a species is, the stronger the adaptable ability is, and the easier for the species to expand its distribution and new environments (Gu, 2004). For example, the variation of leaf shape is closely related to the development degree of *Sagittaria* plants (Huang & Liu, 2014). As a wild *Papeda* endemic to China, *Citrus cavaleriei* is distributed widely and adapts to different kinds of environments, which results in the abundant diversity in many organs, such as the wing leaves, the flowers, and the fruits. For example, the leaves are the ulifoliate, sometimes the simple leaf during the seedling stage or bi-ulifoliate (Huang, 1997); the type along the banks of steams in areas at the north of the middle Guizhou Province has the shortest, thinner leaves with the obvious thin teeth, smaller fruit with thinner peel while the type from the northern areas of Guangxi Province has the biggest and thicker leaves; the type from Yangbi and Baoshan in west Yunnan has been described as a variety of this species (*Citrus ichangensis* var. *microcarpu*), based on these characters, such as the wing leaves that are longer and wider than the leaflets, the thinner leaves with obvious thin teeth, the smaller fruits with thinner peels (Bao *et al.*, 1991). Malik *et al.* (2006) studied the leaflet variation of *C. macroptera* var. *kerrii*, but lacked the wing leaf, which is very important morphology in *Papeda*. Based on the leaf characters of *C. cavaleriei*, it is concluded that the morphological diversity from nine populations in Yunnan Province is very high, which mainly results from the its genetic diversity, besides the ecological conditions, that the width of wing leaf showed the variation and influenced mainly by the environments while the ratio of the length of wing leaf and the total length of leaf is very significant to the taxonomy of the species.

Different individuals in the same population will show the differentiation in the different habitats, which allows it to survive in this environment (Zhong, 1992). In plant population ecology, the variation of morphology among the populations caused by the geographic difference is considered as the ecotypic differentiation, and the genetic characters of a species and its environmental factors can adapt, influence and interact one another (Jiang, 1984). The species *Citrus cavaleriei* originated from the southwest areas in China during Miocene and evolved from its ancestor during adapting the environmental change from the wet and hot habitats to the moist weather of the Central Asia; it is a species in the north of tropical Central Asia (Gou, 1985). The humidity played an important role in the distribution of the species and in the semi-humid areas of subtropical zone in Central Asia, the number of seeds decreases recognizably and the undeveloped seeds can be found usually, which means that it is at the margin of the distribution (Gou, 1985). In this study, clustering results of the leaf characters keep high accordance with the geographical distribution, specially the longitude, which illustrate that the variation rules of leaf characters can reflect the geographic variation. In Yunnan Province, the different longitude means different level of rainfall (namely humidity), which supports the opinion of Gou (1985).

Conclusion

Diversity of leaf characters: For most leaf characters of *Citrus cavaleriei*, the maximums are in the Weixin Population while the minimums in the Yangbi Population; the variation of most leaf characters in the Yangbi Population is highest and variations in Suijiang Population Eshan-1 Population lowest; the variations of WWL in all populations are higher, while that of LWL/TLL is lower which can be used as an important characters to identify the species; the variance of every leaf character among populations is very significant; the correlation between the most leaf characters are very positively or negatively significant.

Geographic differentiation: The longitude has a very significant correlation with all leaf characters of *Citrus cavaleriei*; the latitude with LL, WL, TLL, and LL/WL has a very significant positive correlation, that with WWL has a significant positive correlation, that with LWL/TLL, LWL/LL, WWL/WL, and LWL/WWL has a very significant negative correlation; the correlation of between the altitude and LWL/TLL, LWL/LL, WWL/WL, and LWL/WWL are positively significant and that between the altitude and LL, WL, TLL, WWL, LWL, and LL/WL are extremely negatively significant. The clustering of leaf characters from nine populations is in accordance with the geographical distribution, especially longitude. Longitude plays a key role in the variation of many leaf morphological characters in *C. cavaleriei*.

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