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RESEARCH PAPER

Genetic variation among wild and cultivated populations of the Chinese medicinal plant *Coptis chinensis* (Ranunculaceae)

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ABSTRACT

To examine if the cultivation process has reduced the genetic variation of modern cultivars of the traditional Chinese medicinal plant, *Coptis chinensis*, the levels and distribution of genetic variation was investigated using ISSR markers. A total of 214 *C. chinensis* individuals from seven wild and three cultivated populations were included in the study. Seven ISSR primers were used and a total of 91 DNA fragments were scored. The levels of genetic diversity in cultivated populations were similar as those in wild populations (mean PPL = 65.2% *versus* PPL = 52.4%, mean H = 0.159 *versus* H = 0.153 and mean I = 0.255 *versus* I = 0.237), suggesting that cultivation did not seriously influence genetic variation of present-day cultivated populations. Neighbour-joining cluster analysis showed that wild populations and cultivated populations were not separated into two groups. The coefficient of genetic differentiation between a cultivar and its wild progenitor was 0.066 (G_{st}), which was in good accordance with the result by amova analysis (10.9% of total genetic variation resided on the two groups), indicating that cultivated populations were not genetically differentiated from wild progenitors. For the seven wild populations, a significant genetic differentiation among populations was found using amova analysis (45.9% of total genetic variation resided among populations). A number of causes, including genetic drift and inbreeding in the small and isolated wild populations, the relative limited gene flow between wild populations (N_m = 0.590), and high gene flow between cultivars and their wild progenitors (N_m = 7.116), might have led to the observed genetic profiles of *C. chinensis*.

INTRODUCTION

During cultivation, populations of cultivated plants are subject to strong selective pressures, *e.g.* human determination of morphological, physiological and behavioural changes in the organisms (Otero-Arnaiz *et al.* 2005a). This process has greatly modified the natural mating systems and dispersal mechanisms of plant species (Pickersgill 1969), as well as their morphology, physiology and genetic structures (Doebley 1989; Buckler *et al.* 2001; Hernandez-Verdugo *et al.* 2001; Zeder *et al.* 2006).

The genetic structures of cultivated plant populations have been shaped by many factors in the course of cultivation (Hernandez-Verdugo *et al.* 2001; Oyama *et al.* 2006). Of importance is the way in which genetic material is passed from one cultivated generation to the next (Miller & Schaal 2006). Genetic drift and genetic bottlenecks caused by collection of seeds from a limited number of wild plants and used to found cultivated populations has resulted in cultivated populations that may have diverged significantly from their wild progenitor gene pools (Zohary 2004). Under such conditions, genetic diversity levels may be relatively low in cultivated populations. However, for wild populations in cultivated areas, the possibility of gene flow from wild to cultivated populations was raised, in such cases gene flow

can reduce genetic isolation and will maintain the identity of wild and cultivated populations (Otero-Arnaiz *et al.* 2005b).

China is a global centre of plant cultivation. Apart from many common crop and fruit species cultivated widely in China, traditional Chinese medicinal plants are also economically important and are cultivated widely by farmers. About 300 medical plants species are cultivated in China and this domestication process has been ongoing for approximately 5000 years (Tang 2005). Although several studies have examined the effects of cultivation on the genetic structure of crop species and fruit tree populations in China (Cai 2006; Zhu *et al.* 2007), little is known about how the cultivation process has impacted the extent and distribution of genetic variation in populations of the Chinese medicinal plant species (Wu *et al.* 2006).

Coptis chinensis Franch is an endangered herbaceous perennial in the Ranunculaceae. This species is mainly distributed in central China and can be found in cooler areas at altitudes of 500–2000 m in moist woodlands (Zhang & Wang 2006). The species flowers from early March to late May, with a peak in March. It has a generalist pollination system, with small insects and mammals probably the main seed dispersers (C. F. Yang, unpublished results). The rhizomes of *C. chinensis* are highly valued for their high berberine content and it is a well-known anti-microbial herb with a long history of use in traditional Chinese medicine for treating gastrointestinal problems, gall bladder inflammation, abdominal cramping and to control excessive bleeding (Yu *et al.* 2006). In addition, rhizomes have been used in preparations to relieve high fevers, sore throats, for cankers, pink eye, gingivitis and skin eruptions (Yu *et al.* 2006). This species is extensively cultivated in several provinces of China for use in traditional Chinese formulae. Hubei Province is a centre of cultivation for the species and cultivated populations are mainly established seeds and by direct transplanting wild *C. chinensis* individuals.

Inter-simple sequence repeats (ISSR) have been widely employed to reveal levels and patterns of genetic variation in plants (Qian *et al.* 2001), to determine evolutionary relationships (Zhou *et al.* 2005; Chen *et al.* 2006) and levels of genetic variation among wild and cultivated populations (Qiu *et al.* 2003; Wu *et al.* 2006). In this study, ISSR molecular markers were used to estimate: (i) levels and distribution of genetic variation in wild and cultivated populations of *C. chinensis* and (ii) the amount of gene flow among wild and cultivated populations. Such comparative genetic information on *C. chinensis* may clarify if the cultivation has eroded genetic variation in cultivated populations.

MATERIALS AND METHODS

Plant materials

During March and May 2006 seven wild (CW1–7) and three cultivated (CC1–3) populations of *C. chinensis* were sampled in Hubei Province. A total of 214 individuals with a range of 16–30 individuals per population were included in this study. Details on materials are given in Table 1. About 5 g of fresh leaves of individual plants were collected and preserved in silica gel until required for DNA isolation.

Total DNA extraction and ISSR PCR amplification

Total genomic DNA was isolated from 0.5 g of silica-dried leaf tissue following the procedure described by Fu *et al.* (2003). ISSR PCR reactions were done in a volume of 25 μl containing 0.25 mM of each dNTP, 2.5 μl 10× Taq buffer [10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$ and 50 mM KCl], 1 mM primer, 1 U Taq polymerase (Tian Yuan Biotech) and 40 ng of DNA template. Amplification of genomic DNA was done on a PTC-100^{TM} thermocycler (MJ Research, Inc.), and commenced with 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min annealing at 55 °C and 2 min extension at 72 °C, and a final extension cycle of 7 min at 72 °C. Amplification products were resolved electrophoretically on 1.5% (w/v) agarose gels run at 100 V in 0.5× TBE (Tris–boric

Table 1. Locations and sample size (number of individuals) of the 10 studied *Coptis chinensis* populations in Central China.

population	location	latitude/longitude	sample size
cultivated population			72
CC1	Fubaoshan, Lichuan	30°11′ N/108°42′ E	18
CC2	Xiagu, Shennongjia	31°23′ N/110°12′ E	30
CC3	Houhe, Wufeng	30°05′ N/110°33′ E	24
wild population			142
CW1	Houhe, Wufeng	30°04′ N/110°32′ E	16
CW2	Yangjiawan, Shennongjia	31°27′ N/110°25′ E	19
CW3	Xiangtongzai, Shennongjia	31°28′ N/110°26′ E	24
CW4	Fubaoshan, Lichuan	30°12′ N/108°40′ E	19
CW5	Jiugongshan, Tongshan	29°23′ N/114°36′ E	19
CW6	Xiagu, Shennongjia	31°24′ N/110°13′ E	25
CW7	Jiugongshan, Tongshan	29°24′ N/114°35′ E	20

Table 2. Primers and sequences of seven effective primers used in this study.

primers	sequences (5′–3′)
P808	$(AT)_8G$
P817	$(AT)_8G$
P826	$(GA)_8T$
P827	$(GA)_8C$
P834	$(AT)_8(CG)C$
P835	$(AG)_8(CG)T$
P862	$(AGC)_5$

acid–EDTA), visualised by staining with ethidium bromide and photographed under ultraviolet light. Sixty-five ISSR primers (SBS Genetech Co. Ltd, Shanghai, China) were screened on eight randomly selected individuals. The eight samples were amplified twice with the same primer. Seven primers that produced clear and 100% reproducible fragments were selected for further analysis (Table 2).

Data analysis

The ISSR amplified fragments were manually scored as present (1) or absent (0) for each sample. The resulting presence/absence data matrix of the ISSR was analysed to estimate genetic diversity parameters: percentage of polymorphic bands (PPL), Nei's (1973) gene diversity (H) and Shannon's information index (I). The estimations of genetic diversity were conducted not only at the total species level, but also within and among populations of each group (wild and cultivated). Population differentiation was analysed for polymorphism between wild populations, cultivated populations, and also the entire wild and cultivated population by G_{st} (Nei 1987). All calculations were performed using the POPGENE program version 1.31 (Yeh *et al.* 1997).

Analysis of molecular variance (AMOVA) was performed using squared Euclidean distances (Excoffier *et al.* 1992) among all samples to partition the variation into hierarchical components (among individuals within populations, among populations of each group, and between groups). The genetic analyses were performed with the WINAMOVA program 1.55 (Excoffier 1993). Input files for this program were generated using AMOVA-PREP (Miller 1998). Significance tests were performed using 1000 permutations.

Pairwise genetic distances (Nei & Li 1979) were calculated between populations or between sampled individuals. Based on these genetic distances, neighbour-joining trees were computed using MEGA 3.1 (Kumar *et al.* 2004).

Indirect estimation of the amount of gene flow between wild populations and between the two groups (wild populations and cultivated populations) were made from G_{st} values (Nei 1987) using the formula: $N_m = 0.5(1-G_{st})/G_{st}$ (McDermott & McDonald 1993), where N_m is the number of migrants per generation.

RESULTS

ISSR polymorphism

Seven ISSR primers that produced clear and reproducible fragments were selected (Table 2). The selected primers generated a total of 91 loci (average 13 loci per primer). A total of 89 loci were polymorphic among 214 individuals (Table 3). Among the seven wild populations, the mean PPL, H and I values were 52.4%, 0.153 and 0.237, respectively. Population CW6 had the highest level of variability (PPL, H and I values: 69.2%, 0.212 and 0.322, respectively), whereas population CW7 had the lowest level of variability (PPL, H and I value: 34.1%, 0.097 and 0.150, respectively) (Table 3). Among the three cultivated populations, mean PPL, H and I values were 65.2%, 0.160 and 0.255, respectively. Within the cultivated populations, PPL values ranged from 62.7% (CC1) to 68.1% (CC3), H and I values ranged from 0.133 (CC3) to 0.184 (CC1) and from 0.234 (CC3) to 0.282 (CC1), respectively (Table 3).

Genetic structure of populations

The coefficient of genetic differentiation (G_{st}) between wild populations was 0.459 (45.9% of total genetic variation resided among, and 54.1% within populations). Among the cultivated populations, G_{st} was 0.239. The level of gene flow (N_m) between wild populations was 0.590. G_{st} between wild and cultivated populations was 0.066; indicating only about 6.6% genetic variation

Table 3. Statistical analysis of genetic diversity in studied populations of *Coptis chinensis*.

population	PPL	H	I
cultivated population			
CC1	62.64	0.182 (0.184)	0.282 (0.263)
CC2	64.84	0.165 (0.183)	0.259 (0.258)
CC3	68.13	0.133 (0.143)	0.224 (0.209)
mean at population level	65.2	0.160	0.255
total of cultivated populations	89.01	0.204 (0.168)	0.327 (0.230)
wild population			
CW1	47.25	0.149 (0.188)	0.227 (0.271)
CW2	56.04	0.158 (0.176)	0.247 (0.256)
CW3	63.74	0.175 (0.177)	0.275 (0.254)
CW4	60.44	0.171 (0.177)	0.267 (0.256)
CW5	36.26	0.107 (0.164)	0.167 (0.244)
CW6	69.23	0.212 (0.200)	0.322 (0.277)
CW7	34.07	0.097 (0.162)	0.150 (0.237)
mean at population level	52.43	0.153	0.237
total of wild populations	95.60	0.279 (0.165)	0.430 (0.214)

Numbers in parentheses are standard deviations. PPL = Percentage of polymorphic loci; H = Nei's gene diversity; I = Shannon's information index; total number of loci surveyed = 91.

resided between the two groups (wild and cultivated). The level of gene flow (N_m) between cultivars and their wild progenitors was 7.116 individuals per generation. AMOVA analysis further revealed a similar pattern of genetic differentiation among and within the seven wild populations. Of the total genetic diversity, 42.0% was attributable to among-population diversity and the rest (58.0%) to differences within populations ($P < 0.001$) (Table 4). AMOVA analysis also revealed a low level of genetic differentiation but this was highly significant ($P < 0.001$) between the wild and cultivated populations. Of the total genetic diversity, 10.9% was attributable to between-group diversity and the rest (89.1%) to differences within groups (Table 4).

Neighbour-joining cluster analyses of the 10 study populations revealed that the wild populations were not clearly separated from the cultivated populations (Fig. 1). A neighbour-joining cluster analysis of 214 individuals indicated that the samples from each of the cultivated populations did not form a distinct group. A number of samples from the cultivated populations were shown to be closely related to the wild samples (data not presented).

Table 4. Analysis of molecular variance (AMOVA) for 214 individuals of *Coptis chinensis* using inter-simple sequence repeat markers, significance tests after 1000 random permutations.

source of variance	d.f.	SSD	variance components	total (%)	P-value
variance among groups	1	147.77	1.424	10.9	<0.001
variance within groups	212	2474.62	11.673	89.1	<0.001
among wild populations	6	773.56	5.309	42.0	<0.001
within wild populations	154	1130.32	7.340	58.0	<0.001
among cultivated populations	2	193.65	5.047	39.1	<0.001
within cultivated populations	50	393.86	7.877	61.0	<0.001

d.f. = degrees of freedom; SSD = sum of squares.

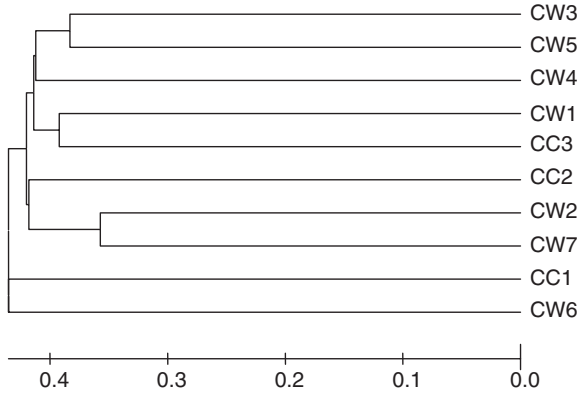

Fig. 1. Cluster analysis of populations from cultivated and wild *Coptis chinensis* using neighbour-joining from a matrix of pairwise genetic distances.

DISCUSSION

The present survey examined seven wild populations and three cultivated populations of *C. chinensis* from Central China. The level of genetic diversity obtained for *C. chinensis* can be compared to studies on other plant species by using dominant markers (*e.g.* RAPD, ISSR and AFLP markers). The level of genetic diversity (mean H = 0.15) in wild *C. chinensis* populations was lower than that of several other plant species (mean H = 0.22) (Nybom 2004). Genetic diversity in the wild *C. chinensis* populations was shown to be similar as that in studies based on RAPD markers for annual (mean H = 0.13) or selfing plant species (mean H = 0.12) (Nybom & Bartish 2000). The genetic diversity was also similar to that in studies on some other endangered Chinese medicinal plants, which were considered to maintain a low level of within-population genetic diversity (Wu *et al.* 2006; Yan *et al.* 2006). For example, using AFLP markers on 10 populations of *Phellodendron amurense*, a rare and endangered medicinal plant from China, Yan *et al.* (2006) found that H values within populations ranged from 0.12 to 0.17 (mean H = 0.15). Wu *et al.* (2006) found a low level of within-population genetic diversity (mean H = 0.18, range 0.14–0.20) for *Gastrodia elata*, another endangered Chinese medicinal plant, using ISSR markers.

Genetic diversity of plant populations is largely influenced by factors such as mating system, genetic drift, evolutionary history and life history (Loveless & Hamrick 1984). In general, outcrossing species have higher levels of genetic diversity than selfing and clonal plants (Rossetto *et al.* 1995). *Coptis chinensis* is a self-compatible species and can reproduce through both selfed and out-crossed seeds. The pollinators of this species are small insects (C. F. Yang, unpublished results). The transfer of pollen by insects between the different individuals may increase the possibility of sexual recombination and subsequently increase within-population genetic diversity. Although *C. chinensis* produces many flowers and seeds, herbivorous animals often disrupted a large proportion of the inflorescences before seed maturation (W. Shi and C.F. Yang, unpublished data). The failure of seeds of *C. chinensis* to mature would result in rare seedling recruitment in most *C. chinensis* populations, which may have contributed the relative low level of within-population genetic diversity found in this study.

In addition, the geographic range of *C. chinensis* in Central China has been reduced and its populations have also been greatly reduced in size. Human activities, such as digging up the rhizomes for traditional Chinese medicine, might have resulted in degradation of *C. chinensis* populations. Two genetic consequences of small population size are increased genetic drift and inbreeding

(Barrett & Kohn 1991). Low variability within a population is ascribed to small population size, which was constrained largely by habitat disturbance. In a small population, inbreeding and genetic drift will have greater effects, leading to loss of genetic variation within populations in Central China. However, no evidence of inbreeding was found in this study. In order to understand the population genetics and evolution of this species, more detailed studies on the reproductive biology and mating system are necessary.

Our survey of seven wild populations of *C. chinensis* revealed a high level of genetic variation at the species level, with 95.6% of loci being polymorphic. AMOVA analysis showed that a slightly higher proportion of the genetic variation resided within populations (58.0%), and a relatively lower degree of genetic variation resulted from differentiation among populations (42.0%). The coefficient of genetic differentiation (G_{st} = 0.420) of the wild *C. chinensis* populations in the present study was much higher than that in studies on outbreeding species using RAPD markers (average G_{st} = 0.220) and also using ISSR markers (average G_{st} = 0.340) (Nybom 2004). The G_{st} value was also higher than estimated values for several other medicinal plants. For example, Yan *et al.* (2006) found that a G_{st} value among *P. amurense* populations of 0.342, while Wu *et al.* (2006) found G_{st} values among *G. elata* population of 0.256.

Limited gene flow (N_m = 0.590) between wild populations of *C. chinensis* was indicated in the present study. As all the wild *C. chinensis* populations surveyed were in the same region of southwest Hubei Province, it is possible that gene flow occurs between these populations. However, our recent field investigations suggest that many populations of *C. chinensis* in Hubei Province have become fairly small and very isolated because of human activities. The very small isolated populations could reduce the chances of gene exchange among populations, which will enlarge genetic differentiation among populations. In addition, genetic drift caused by small population size and/or reduced gene flow or dispersal among patchy populations may also shape the current genetic structure of *C. chinensis* populations.

Unlike many other studied plant species where cultivars have lower genetic diversity than their wild relatives (Doebley 1989; Gepts 2004; Zhou *et al.* 2005; Miller & Schaal 2006; Wu *et al.* 2006), the three cultivated *C. chinensis* populations maintain similar levels of genetic diversity as their wild relatives (mean H value for cultivated populations was 0.16). Cultivation has not eroded the genetic diversity of the studied cultivated *C. chinensis* populations. Our findings are in accordance with several other cultivated plants where genetic diversity is as high as wild relatives (Hernandez-Verdugo *et al.* 2001; Cutsem *et al.* 2003; Khlestkina *et al.* 2004). The neighbour-joining cluster and AMOVA analysis similarly indicated low genetic differentiation between wild and cultivated populations.

Several factors should be included in attempts to account for the observed genetic structure (similar within-population genetic diversity and little genetic differentiation between cultivars and wild progenitors). The present-day cultivated *C. chinensis* may have been introduced to cultivation from the seeds of a large number of wild progenitors and also from the wild progenitors themselves. Seeds from the initial gene pool may contain most of the genetic diversity of the wild populations, and cultivated *C. chinensis* populations founded using these seeds may thus maintain the total initial genetic diversity. In southwest Hubei Province, the cultivation area of *C. chinensis* overlaps with the natural distribution of this species; for example, cultivated population CC1 and wild population CW4 were found in the same region of Fubaoshan Mountain in Lichuan County; cultivated population CC2 and wild population CW6 were both in Xiagu Mountain in Shenglongjia; cultivated population CC3 and wild population CW1 were both in Houhe County. Because of the sympatric distribution pattern of the wild and cultivated populations, subsequent gene flow from wild ancestors into cultivated populations may also increase or maintain genetic variation in *C. chinensis*. The high gene flow (N_m = 7.116) between the wild and cultivated populations is evident in this study from ISSR marker values. The high gene flow between the cultivars and their wild progenitors might have resulted from combine effects, including exchange of pollen by pollinators, transplantation of wild individuals to cultivated populations, and using the seeds from wild progenitors to found new cultivated populations. The high gene flow may have prevented genetic differentiation between cultivated *C. chinensis* populations and their wild progenitors.

Wild relatives of cultivated plants are important reservoirs of genes for improving commercial varieties (Oyama *et al.* 2006). Although the Chinese traditional medicinal plant *C. chinensis* currently maintains a high degree of genetic diversity at species level, a relative low level of within-population genetic diversity was found in the present study area. Human activity has led to the extant *C. chinensis* population facing a decline in number of individuals. There is a real danger that if the trend towards a decline in numbers is left unchecked, genetic consequences associated with small isolated populations, including genetic drift and inbreeding, will reduce most of the genetic diversity in wild *C. chinensis* populations. Thus, there is an urgent need to assess the diminishing population size and to adopt appropriate conservation strategies for the long-time survival of this species.

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