Genetic diversity and genetic structure of *Camellia nitidissima* in China by SSR[1]

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Abstract
To provide the scientific basis for protection and utilization of *C.nitidissima* resources, the genetic diversity and genetic structure of four *C.nitidissima* populations were studied by simple sequence repeats (SSR). There was significant positive correlation between the genetic distance and geographic distance of *C.nitidissima* populations. Genetic diversity and genetic differentiation of *C.nitidissima* was low. *In situ* conservation of *C.nitidissima* populations should be strengthened, and *ex situ* conservation by introduction also should be carried out.
Key words： *Camellia nitidissima*; genetic diversity; genetic structure; SSR; germplasm conservation

INTRODUCTION

Camellia nitidissima, a rare and endangered plants of *Camellia*, is distributed in a narrow region of Guangxi province, China. With golden yellow, waxy petals, *C.nitidissima* has been called "the queen of camellias family" and "the giant panda in plant kingdoms". The flowers and leaves of *C. nitidissima* contain many active components including flavonoids, tea polyphenols, saponins and polysaccharides, which have been proved to have hypoglycemic, hypolipidemic, hypotensive and antitumor action.

Moreover, in contrast to the flower colors of most *Camellia* cultivars which are red, pink, white and their combinations, *C.nitidissima* with yellow petals couyld provide a possible effective pathway to the breeding of yellow *Camellia* cultivars.

Because *C.nitidissima* has a higher economic value in terms of exploitation and utilization, this has resulted in the wild resources being almost destroyed by plants excavation, and flowers and seeds harvest. Deterioration of the ecological environment las led to further serious degradation of populations of *C.nitidissima*. The range of distribution has been

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shrinking and the number of populations has decreased dramatically. Comprehensive systematic investigation of *C. nitidissima* resources shows that natural populations mainly exist in Fangchenggang city and Nanning city, in Guangxi province, southern China, and the habitat of the species has been severely fragmented. So *C.nitidissima* was listed as the first class national protective keystone plant of rare endangered plants. Therefore, it is not only important but also urgent to carry out conservation biology research of *C.nitidissima*.

Plant conservation biology should not only protect its population, but also protect its genetic diversity. For rare and endangered natural populations seriously damaged by human disturbance, the genetic diversity and genetic structure of the populations should be first clarified, and the genetic variation of the populations should be mastered.

Genetic diversity and genetic structure of the *C.nitidissima* populations were studied using RAPD, ISSR, and AFLP, and that of *C.pubipetala* were studied using ISSR technology. Simple sequence repeat (SSR) markers are widely applied in researches of genetic diversity and genetic structure because of its many advantages: great quantity, extensive dispersion around the genome, high polyomorphism, mendelian codominant inheritance, rapid and convenient detection, among others. So it is very important to study genetic diversity and genetic structure of *C.nitidissima* using SSR technology, and evaluate the effects of ecological environment and human disturbance on population quantity and genetic diversity.

For SSR study on genetic diversity of C.*nitidissima*, ten pairs of SSR primers for the species were developed by cloning and sequencing technology, and they were tested and verified in 25 individuals of a *C.nitidissima* population. So up to now, SSR study on genetic diversity of *C.nitidissima* has mainly focused on the SSR primer development, and actual study on genetic diversity and genetic structure of *C.nitidissima* has not been carried out by SSR markers. We concluded there are two primary reasons to cause the current situation: on one hand, the lack of genetic information of *C.nitidissima*; on the other hand, the lack of wild individuals of natural populations.

In view of this, the paper intends to develop the SSR primers of *C.nitidissima* based on RNA-seq, and test and verify them in *C.nitidissima*. Then SSR primers were applied in the study on genetic diversity and genetic structure of *C.nitidissima* population, so as to provide scientific basis for the protection and utilization of the species.

1 Materials and methods

1.1 Plant materials

Four *C. nitidissima* populations in Guangxi province of China were selected as study objects bsaed on their natural distribution. The sampling locations of *C.nitidissima*

populations are shown in Table 1. Acording to interval distance sampling method, a total of 90 sample leaves of *C.nitidissima* wild individuals, from plants more than 50 m apart, were randomly collected and preserved in silica gel.

Table 1 Sampling location of *C.nitidissima* populations

Population	Provenance	Longitude (E)	Latitude (N)	Altitude (m)	Sample size
CD	Dalu town, Fangchenggang city	108°10′24″	21°18′16″	173.72	25
CH	Huashi town, Fangchenggang city	108°19′25″	21°19′32″	148.52	25
CF	Fushu town, Nanning city	107°47′15″	22°54′12″	144.45	20
CT	Tanluo town, Nanning city	107°45′24″	22°55′25″	215.38	20

1.2 Development of SSR primers from *Camellia*

RNA was extracted from leaf, and then was reverse transcribed to cDNA. The cDNA library was constructed according to Illumina TruseqTM RNA sample prep Kit method, and then was sequenced using Hiseq2500. The data were assembled, and Unigene were established. SSR primers were screened and designed acording to MISA method based on Unigene.

1.3 SSR amplification

DNA was extracted from dry leaves by using the CTAB method. SSR amplifications were peformed in 25 μL reaction mixtures, including template DNA 1.5 μL containing 100 ng, 2×TSINGKE Master Mix 12.5 μL，10 mmol·L^{-1} primers 2 μL，ultra pure water 9 μL。The reaction mixture was subjected to PCR amplification in Veriti 96-Well Thermal Cycler（Thermo Fisher Scientific）using a PCR program, Pre-denaturation at 94 ℃ for 5 min, followed by 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s and extending at 72 ℃ for 30 s, followed by extending at 72 ℃ for 9 min. PCR products were separated by electrophoresis and detected using Qsep100 automatic nucleic acid protein analysis system(Bioptic, Inc.), and peaks and alleles were analysed using Qsep10 software.

1.4 Data analysis

Genetic diversity parameters were analysed using GenAlEx 6.41, including observed number of alleles(*Na*), effective number of alleles(*Ne*), Shannon's information index(*I*), observed heterozygosity(*Ho*), expected heterozygosity(*He*) and percentage of polymorphic loci(*PPL*), and genetic differentiation coefficient, gene flow, genetic distance and genetic identity were also studied. Using NTSYSpc 2.0, cluster analysis was carried out according to UPGMA method based on the genetic distance, and clustering diagram was constructed. The relationship between genetic distance and geographical distance among populations was

analysed using TFPGA according to Mantel test method.

2 Results

2.1 The polymorphism of SSR primers of *C.nitidissima* populations

Some 237 pairs of primers were randomly extracted, and the DNA of three wild individuals of the *C.nitidissima* population were used as template for PCR amplification. The result showed that 176 primers generated bands, and the amplification rate was 74.26%. And 155 primers showed object bands that accorded with expected fragment size, and the effective amplification rate was 65.40%. Then the DNA of six wild individuals of the *C.nitidissima* population was amplified using the 155 primers, of which 78 were polymorphic with a polymorphism rate was 50.32%. Some 20 primers with higher polymorphism were used for PCR amplification of the *C.nitidissima* population. The information of 20 primers is shown in Table 2: two primers our of 20 were dinucleotide, and 18 were trinucleotide. Fragment size of amplification products ranged from 120 bp to 333 bp, which accorded with expected fragment size.

Table 2 Twenty primers used for SSR amplification of *C.nitidissima* populations

Primer name	Primer sequence 5'	Primer sequence 3'	Repeatitive units	Fragment Size/bp
SSR14	F: CATTGCACAATGTTTTTGGC	R: AAACAAGAATTGGCACCAGC	(TGC)7	162～198
SSR31	F: CAAGGCAGAGTAGGCTGAGG	R: AAAAGGGAAGGGGAAAGTCA	(AGC)7	123～273
SSR48	F: GTTGGAGGTTTTTCAGCCAC	R: ATTGAAAGTCGCTGCTTCGT	(CCG)7	255～306
SSR53	F: AAGTCTAAGGACGCAGCCAA	R: TGGAAGAAGGAGACGAAGGA	(AAC)6	258～306
SSR69	F: TTGTTGGGTATGCCAAGTCA	R: TCTGTAAGGAAGGCAAGGGA	(GAG)6	270～333
SSR75	F: TGATTGAGTTCAAGGGGAGG	R: CCAATGGAGGCATTGAAGTT	(ATC)6	174～213
SSR82	F: GGGGGAAAAACAAAACCCTA	R: TGACGCCATAGCCATAAACA	(AAG)6	120～150
SSR208	F: CGGAAGAAGAGACGGTGAAG	R: AATGGCGTCTCCAATTTGTC	(CAA)5	186～216
SSR212	F: GGCCGTGGATTAAAACTCAA	R: ATGGCAGGCTGTTCTGATTT	(TCA)5	240～273
SSR223	F: AGAGGTGGACTTGCAGGAGA	R: ATCACTTTCATCGGTTTCGC	(TGG)5	210～249
SSR224	F: ATGGTGGCAAGGAATCAAAG	R: TGTAAGCTCCTGTGCTGTGG	(TGG)5	177～228
SSR227	F: CAGCGACTGGAACAAAGAGA	R: GAAGAAGAGCAAATGGTGGC	(CGG)7	222～255
SSR229	F: GTCGAACCCACCATCATACC	R: CACAGGGTTATGGGTATCCG	(GCT)5	156～225
SSR239	F: AGATTTGCAAGGTTGGGTTG	R: TCTACCACACTCCCACTCCC	(GCA)7	183～258
SSR243	F: GACGACTCTGGTTCTGCCTC	R: AATCACCCTCTGCGAATCAC	(GTG)5	120～158
SSR250	F: ACCTCCAGCTTCCATCAGAA	R: ATCTTGACGGGCGTAACATC	(CCG)5	252～333
SSR265	F: GTAGCCCCAATCAGTGAGGA	R: GGAATCATCAAGCGCAATTT	(GGT)5	207～252
SSR288	F: ACCCGTCGCTAATAAAATGC	R: AAGGAATGTTTGGTTGACACTG	(AT)9	206～279
SSR297	F: CGGTACTTTCTGAGCTTCCG	R: TCACCCCTTTAAAAACCCATC	(CT)8	172～204
SSR326	F: GTCGAGTGGGAGTAGCTTCG	R: CTGGATCCAAGAAATTGGGA	(TGG)5	270～318

The genetic diversity parameters of 20 SSR primers in *C.nitidissima* populations is shown in Table 3. A total of 130 observed alleles (*Na*) and 74 effective alleles (*Ne*) were detected using the 20 SSR primers. *Na* and *Ne* were higher in SSR69 (12.2500 and 7.6326) and SSR31(10.000 and 5.7924), and minumum of *Na* and *Ne* was found in SSR223 (4.2500 and 2.0999). Observed heterozygosity(*Ho*) ranged between 0.1175 and 1.000, and the mean was 0.5625. Percentages of polymorphic loci (*PPL*) were higher in SSR69 (48.89%) and SSR31 (42.22%), and was lowest in SSR223 (23.72%). Minumum and maximum of Shannon's information index (*I*) were found in SSR223 (0.9053) and SSR69 (2.2305), and that of expected heterozygosity (*He*) were found in SSR223 (0.4907) and SSR69 (0.8637).

Table 3 The genetic diversity parameters of 20 SSR primers in *C.nitidissima* populations

Primer name	*Na*	*Ne*	*I*	*Ho*	*He*	*PPL*(%)
SSR14	5.5000	2.9567	1.2965	0.3600	0.6475	21.11
SSR31	10.0000	5.7924	1.8915	0.4075	0.7927	42.22
SSR48	4.2500	2.1278	0.9701	0.1175	0.5258	12.22
SSR53	9.5000	5.5568	1.9114	0.6800	0.8154	40.00
SSR69	12.2500	7.6326	2.2305	0.5800	0.8637	48.89
SSR75	6.2500	3.0283	1.3638	0.5125	0.6586	26.67
SSR82	5.2500	3.5656	1.3817	0.9250	0.7140	16.67
SSR208	4.7500	2.4712	1.0955	0.3875	0.5641	14.44
SSR212	5.7500	2.7888	1.2383	0.2725	0.6315	15.56
SSR223	4.2500	2.0999	0.9053	0.1250	0.4907	10.00
SSR224	5.2500	3.3545	1.2659	1.0000	0.6590	14.44
SSR227	4.7500	2.5629	1.0050	0.2075	0.4984	13.33
SSR229	4.5000	2.4965	1.0666	0.3275	0.5641	15.56
SSR239	5.0000	3.1024	1.1503	0.3750	0.5599	18.89
SSR243	5.0000	2.9259	1.1849	0.4875	0.6168	20.00
SSR250	6.7500	3.7674	1.4226	0.5475	0.6829	25.56
SSR265	6.0000	3.8518	1.4642	1.0000	0.7359	20.00
SSR288	7.7500	3.4863	1.4846	0.9875	0.7085	26.67
SSR297	8.7500	5.6672	1.8972	0.9800	0.8190	38.89
SSR326	8.7500	4.7667	1.7538	0.9700	0.7730	33.33

Note: *Na*, Observed number of alleles; *Ne*, Effective number of alleles; *I*, Shannon's information index; *Ho*,Observed heterozygosity; *He*, Expected heterozygosity; *PPL*, Percentage of polymorphic loci. The same below, in Table 4.

2.2 Genetic diversity of *C. nitidissima* populations based on SSR analysis

The related genetic data of *C.nitidissima* populations based on the SSR analysis are shown in table 4. The mean of observed alleles (*Na*) and effective alleles (*Ne*) were 6.5125

and 3.7001, and the *Ne* of population CD were less than that of the other three populations. The average observed heterozygosity (*Ho*) were 0.5625, and *Ho* of population CD were higer than that of the other three populations. The percentage of polymorphic loci(*PPL*), Shannon's information index(*I*) and expected heterozygosity(*He*) at the population level were 29.65%, 1.3990 and 0.6661 respectively. The *PPL* of the four *C.nitidissima* populations ranged from 25.00% to 32.25%. *I* ranged from 1.2708 to 1.5425, and *He* ranged from 0.6080 to 0.7093. The change trends of *I* and *He* of four *C.nitidissima* populations were uniform, and ranged in the order as follows: CH > CF > CT > CD. The genetic diversity of four populations was as follows: CH > CF > CT > CD, and that of CD was significantly lower than the other three populations.

Table 4 Genetic diversity of *C. nitidissima* populations

Population	*Na*	*Ne*	*I*	*Ho*	*He*	*PPL*(%)
CD	6.2500	3.4093	1.2708	0.6000	0.6080	25.00
CH	7.6000	4.1705	1.5425	0.5900	0.7093	30.60
CF	6.2500	3.6872	1.4034	0.5250	0.6771	32.25
CT	5.9500	3.5333	1.3793	0.5350	0.6700	30.75
Mean	6.5125	3.7001	1.3990	0.5625	0.6661	29.65

2.3 Genetic structure of *C. nitidissima* populations based on SSR analysis

For the genetic differentiation coefficient and gene flow of *C.nitidissima* populations, see table 5. The genetic differentiation coefficient of *C.nitidissima* populations was between 0.0376 and 0.1511, showing that the genetic variation was mainly within the populations. The genetic differentiation coefficient of *C.nitidissima* populations was at a minimum between CF and CT, and was at a maximum between CF and CD. Gene flow of *C.nitidissima* populations was between 1.4049 and 6.3916 (>1), which showed the populations was a high gene flow. Gene flow was minimum between CF and CD, and maximum between CF and CT.

The analysis of molecular variance(AMOVA)of *C.nitidissima* populations showed that 69.3739% of the genetic variation was within the populations, and 16.2991% of it was among the populations (Table 6). The results of AMOVA were basically identical with the genetic differentiation coefficient, that showed the genetic variation was happening among and within *C.nitidissima* populations, and the main variation was within populations.

Table 5 Genetic differentiation coefficient and gene flow of *C.nitidissima* populations

Populations	CD	CH	CF	CT
CD	*****	3.8686	1.4049	1.5232
CH	0.0607	*****	1.9628	2.2085
CF	0.1511	0.1130	*****	6.3916
CT	0.1410	0.1017	0.0376	*****

Note: Genetic differentiation coefficient (below diagonal) and gene flow (above diagonal)

Table 6 Analysis of molecular variance(AMOVA) among and within *C.nitidissima* populations

Source of variance	Degree of freedom *df*	Sum of squares *SS*	Mean square *MS*	Variance Component	Percent of variance Component （%）
Among Populations	3	202.8161	67.6054	1.3301	16.2991%
Among Individual	86	687.9450	7.9994	1.1691	14.3270%
Within Individual	90	509.5000	5.6611	5.6611	69.3739%
Total	179	1400.2611		8.1603	100.0000%

2.4 Genetic relationship of *C.nitidissima* populations based on SSR analysis

Genetic distance and genetic identify of *C.nitidissima* populations are shown in Table 7. The genetic distance of *C.nitidissima* populations was between 0.1191 and 0.9370 – at a minimum between CF and CT, and at a maximum between CD and CF. Genetic identify of *C.nitidissima* was between 0.3918 and 0.8877, which howed the genetic similarity was higher. Genetic identify of the populations of the species was highest between CF and CT, and that was lowest between CD and CF.

Table 7 Genetic distance and genetic identity of *C.nitidissima* populations

Populations	CD	CH	CF	CT
CD	*****	0.8123	0.3918	0.4316
CH	0.2079	*****	0.4474	0.5115
CF	0.9370	0.8044	*****	0.8877
CT	0.8401	0.6705	0.1191	*****

Note: Genetic distance (below diagonal) and genetic identity (above diagonal).

The unweighted pair group method with arithmeticmean (UPGMA) dendrogram of *C.nitidissima* populations was drawn based on genetic distance (fig.1). The result indicated that populations of the species had similar genetic background and certain difference. The CD and CH were clustered, and CF and CT was clustered. The four *C.nitidissima* populations were cluetered according to geographical distance, indicating that there was a significant positive correlation between the genetic distance and geographic distance of *C.nitidissima* populations.

Principal coordinates analysis(PCoA) indicated that 90 individuals were divided into two groups (fig.2). CD and CH were divided into one groups, and there was almost no interpenetration between the two populations. CF and CT was divided into another group, and there was obvious interpenetration between the two populations. Principal coordinates

analysis(PCoA) was consistent with the clustering results of unweighted pair group method with arithmeticmean (UPGMA), showing that CD and CH in relationship were near each other, and CF and CT were the same.

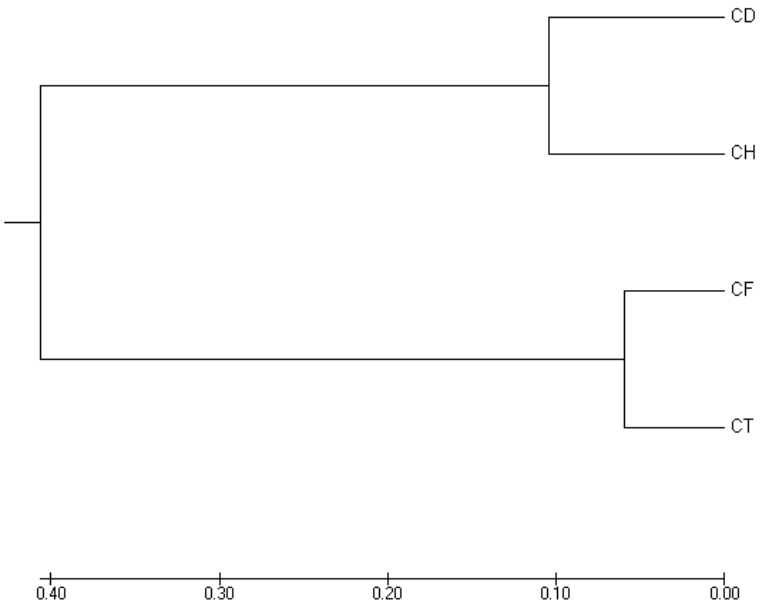

Fig. 1 UPGMA dendrogram of *C.nitidissima* populations based on genetic distance

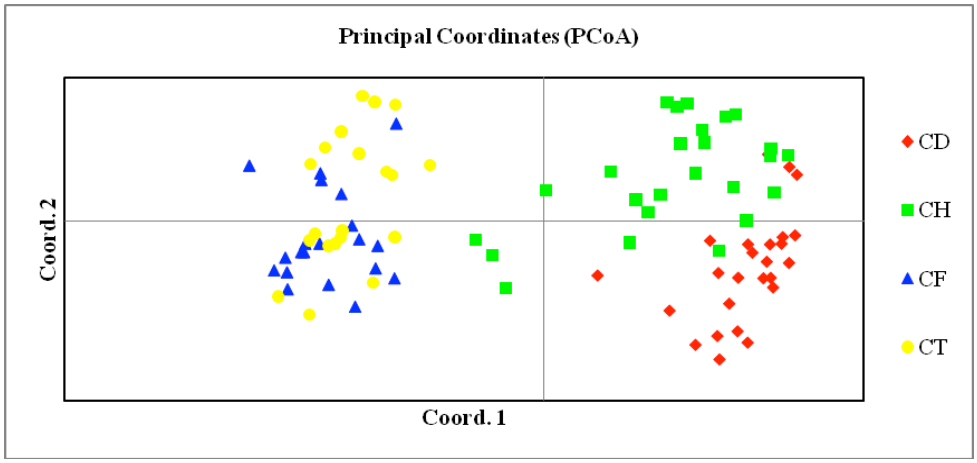

Fig. 2 Principal coordinates analysis (PCoA) of *C.nitidissima* populations

3 Discussion

Early molecular markers reseaching genetic diversity such as RAPD, AFLP and ISSR were dominant markers, and they are comparable in data analysis results. Statistical analysis of plant genetic diversity using different markers show the mean genetic diversity of perennial

plants was 0.25, and that of regionally distributed species was 0.21. For *Camellia japonica*, a widely distributed species of *Camellia*, the genetic diversity of ISSR analysis was 0.3414, higher than the mean of perennial plants (0.25). The genetic diversity of *C.pubipetala* using ISSR marker was 0.2451, higher than the mean of regionally distributed species (0.21). For *Camellia azalea*, only distributed in E'huangzhang provincial nature reserve of Guangdong, the genetic diversity of RAPD analysis was 0.1435, which was lower than the mean of regionally distributed species. RAPD analysis of three endangered plants of Theaceae shows that the genetic diversity of *Apterosperma oblata,* with wider distribution, was higher than that of *Euryodendron excelstan* and *C.azalea* with narrow distribution. So the genetic diversity of *Camellia* was closely related to its distribution range: the larger the distribution range, the higher the genetic diversity. And conversely, if the distribution range was more limited, the genetic diversity was lower.

Population genetic diversity is an important factor affecting species evolution and adaptability to the environment: the higher the genetic diversity, the greater the adaptation potential to the environment. ISSR analysis on 126 individuals of four *C.nitidissima* populations showed that the genetic diversity was 0.2302, and Shannon's information index was 0.3502. RAPD and AFLP analysis on 126 individuals of six *C.nitidissima* populations showed that the genetic diversity were 0.2698 and 0.2444, and Shannon's information index were 0.4043 and 0.3682. ISSR analysis on 12 *C.nitidissima* populations showed that the genetic diversity and Shannon's information index were 0.1561 and 0.2490 respectively, and there was no significant correlation between population size and genetic diversity. Compared with previous ISSR, RAPD and AFLP studies, the genetic diversity of *C.nitidissima* has been greatly reduced, and was much lower than the mean of regional distribution species (0.21).

Expected heterozygosity and Shannon's information index of *C.nitidissima* populations studied by SSR analysis were 0.6661 and 1.3990 respectively in this experiment, and related genetic diversity parameters were higher than that of RAPD, AFLP and ISSR analysis. This is mainly because RAPD, AFLP and ISSR are dominant markers, while SSR markers are co-dominant, which can detect dominant and recessive alleles and distinguish homozygous and heterozygous genotypes.

Related genetic diversity parameters of *C. nitidissima* using SSR markers in this experiment were lower than those of *C.sinensis* which were 0.73 and 0.61, and lower than that of *Schima superba* which were 0.725 and0.645, which indicates that the genetic diversity of *C.nitidissima* is lower. SSR analysis of *C.sinensis* showed that when the sample size reached 15 plants, the values of the genetic parameters tended to be stable. The samples of CH and CF were 25 each, and those of CT and CD were 20 each. But the genetic diversity ranged in the order as follows: CH > CF > CT > CD. The results indicated that when the sample

size reacheed a certain level, there were no obvious correlation between sample size and genetic diversity, which was consistent with previous studies.

The genetic variation of the three endangered plants in Theaceae mainly exist within the population, and the genetic differentiation among populations of *E.excelstan* was significantly higher than that of *A.oblata* and *C.azalea* because of severe fragmentation of habitats. The genetic variation of *C.japonica* mainly exists within the population, and island isolation played an important role in population genetic differentiation.

Analysis of molecular variance shows that the genetic variation of *C.pubipetala* populations, with 88.63% of the genetic variation within populations and 11.37% of that among populations, was mainly within populations, and there was a significant positive correlation between genetic distance and geographical distance. The genetic variation of *C.nitidissima* populations was mainly within populations, with 25.78% of the genetic variation among populations, and there was a significant positive correlation between genetic distance and geographical distance.

Wei et al（2008）studied the genetic variation of 12 *C.nitidissima* populations, and found that the variation within a population of *C.nitidissima* was the main genetic variation. In this experiment, analysis of molecular variance indicates that the genetic variation of *C.nitidissima* populations, with 69.3739% of the genetic variation within populations and 16.2991% of that among populations, was mainly within populations, and there was a significant positive correlation between genetic distance and geographical distance.

Distribution range, habitat fragmentation and breeding systems affect genetic diversity and genetic structure to various degrees. Habitat fragmentation affects population structure and population size, and affects the genetic characteristics of species, such as reducing heterozygosity and increasing genetic drift. Narrow distribution, habitat fragmentation, small population size and serious human disturbance results in the increase of inbreeding frequency and allelic loss of *C.nitidissima* populations, and then reduced genetic diversity and genetic variation of population. *C.nitidissima* is an outcrossing plant, and bees can effectively pollinate. But gene flow and genetic variation of populations are greatly limited because of a narrow distribution range and small population size. In addition, almost all of flowers and seeds are collected, so seedlings of population can not be effectively supplemented, thereby reducing genetic diversity and genetic variation of a population.

The resources of *C.nitidissima* have been seriously destroyed by human activities such as excavation of wild plants, collecting flowers and seeds, and the destruction of habitats, which has resulted in the narrow distribution range, small population size and low genetic diversity.

Genetic diversity and genetic differentiation of *C.nitidissima* populations was low, and genetic variation was mainly within populations.

So *in sit*u conservation of *C.nitidissima* populations should be strengthened, such as by establishment of protected area of natural populations, prohibition of digging up plants, picking flowers and collecting seeds. In addition, the growth environment and survival system of population were also strictly protected for promoting natural regeneration of population. ISSR markers were used to study the *ex situ* conservation population of *C.nitidissima*. The result showed that the genetic diversity of population was effectively protected by a collecting seed nursery, and it is feasible to carry out *ex situ* protection on the basis of *in situ* protection. The germplasm resource garden should be established for *ex situ* conservation,through seed collection and seedling raising of the *C.nitidissima* natural population.

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