Genetic diversity and relationships among populations of Camellia japonica, an endangered species in China

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ARTICLE

Genetic diversity and relationships among populations of *Camellia japonica*, an endangered species in China

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Abstract: *Camellia japonica*, an evergreen ornamental plant in the Theaceae family, has a natural range that is now shrinking. This is evident by the fact that the species is on the verge of extinction in Laoshan Mountain (Qingdao), the northernmost area of China known to have a natural population of *C. japonica*. Little is known about the genetic diversity and relationships among cultivated and wild *C. japonica* populations. One hundred and eighty samples of six *C. japonica* populations were tested for genetic diversity with simple sequence repeat (SSR) markers; these included three cultivated populations, two natural populations in Qingdao, and one natural population in Daqingshan. The average values of polymorphism information content (PIC), expected heterozygosity (He), and Shannon's information index (I) were 0.5849, 0.6385 and 1.3170, respectively, indicating that *C. japonica* has a high genetic diversity. The genetic diversities of the six populations in rank order were as follows: Daqingshan > Zhongshan Park > Changmenyan Island > Daguan Island > Botanical Garden > May Fourth Square. The geographical isolation of the islands had no significant influence on the genetic diversity of *C. japonica*. Clustering results showed that the six *C. japonica* populations could be grouped into three categories, and most populations were clustered according to their geographical origin and genetic background. These results also reconfirmed that the *C. japonica* (Naidong) population in Qingdao originated from Changmenyan Island. Genetic variation was highest within populations (89%), indicating that *C. japonica* can be protected at the population level. These findings will prove useful for the genetic analysis, protection, and horticultural use of *C. japonica*.

Key words: SSR markers, natural population, *Camellia japonica*, cultivated population, genetic structure.

Résumé : L'aire de répartition naturelle de *Camellia japonica*, conifère ornemental de la famille des Theaceae, rétrécit. La preuve? L'espèce a pratiquement disparu sur le mont Laoshan (Qingdao), territoire le plus au nord de la population naturelle, en Chine. On sait peu de choses sur la diversité génétique et les liens entre les populations sauvages et domestiques de *C. japonica*. Dans l'espoir d'y remédier, les auteurs ont examiné la diversité génétique de 180 échantillons de six peuplements du conifère avec des marqueurs SSR, en l'occurrence trois populations domestiques, deux naturelles dans le Qingdao et une naturelle dans le Daqingshan. La valeur moyenne du contenu polymorphe (PIC), le degré d'hétérozygotie prévu (He) et l'indice de Shannon (I) s'établissaient respectivement à 0,5849, à 0,6385 et à 1,3170, signe que *C. japonica* profite d'une très grande diversité génétique. Sur ce plan, les six populations peuvent se classer comme suit : Daqingshan > parc Zhongshan > île Changmenyan > île Daguan > jardin botanique > parc du Quatre-Mai. L'isolement géographique des îles n'a eu aucune influence déterminante sur la diversité génétique de l'espèce. L'analyse des résultats par grappes indique que les six populations de *C. japonica* pourraient être groupées en trois catégories et que la majorité des peuplements sont regroupés d'après leur origine géographique et leurs antécédents génétiques. Ces données confirment à nouveau que la population de *C. japonica* (Naidong) du Qingdao vient de l'île Changmenyan. Le plus important degré de variation génétique s'observe au sein de chaque peuplement (89 %), ce qui signifie qu'on pourrait protéger l'espèce à ce niveau. Ces constatations auront leur utilité pour l'analyse du génome, la protection de l'espèce et l'usage de cette dernière en horticulture. [Traduit par la Rédaction]

Mots-clés : marqueurs SSR, population naturelle, *Camellia japonica*, population domestique, structure génétique.

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Introduction

Camellia japonica belongs to the Theaceae family. As one of the top ten traditional famous flowers of China, it has high economic and ornamental value. It is a subcanopy tree (Yamamoto 1992) and its wild populations are widely distributed in Sichuan, Taiwan, Jiangxi, and Shandong provinces and on many islands along the eastern coast of China (Zhang and Ren 1998; Gao et al. 2005). Zhejiang province is the center for natural populations of *C. japonica* in southern China. The species is also found on the Korean Peninsula and on the southern islands of Japan (Kang and Huh 2014; King 2018). *C. japonica* is found mainly on hillsides facing the sea or in the forest understory of coastal islands. The Daqingshan scenic area of the Zhoushan Islands in Zhejiang is a representative area that contains some natural *C. japonica* populations. Laoshan Mountain in Qingdao and the adjacent island, due to their favorable mild oceanic climate, are the northernmost areas where *C. japonica* plants can be found. The wild *C. japonica* population in this area and the cultivated population derived from it are commonly known as *C. japonica* (Naidong). As a relict plant population, *C. japonica* (Naidong) was common in the Tertiary geologic period and is regarded as the original gene pool of northern *C. japonica* (Zhou et al. 1994; Wang et al. 1995). Therefore *C. japonica* (Naidong) has significant ecological value.

C. japonica (Naidong) was once a dominant species of the islands, naturally distributed on offshore islands in a longitude range of 120°37′–121°13′ and a latitude range of 36°07′–36°18′ (Wang et al. 2001). However, because of limited resources and habitat changes caused by human activities (e.g., building, harvesting, and excavation), the natural population is shrinking, and *C. japonica* (Naidong) has become an endangered plant (Wang and Zhang 1992). As the world population has grown and human activities have adversely affected the environment, the conservation of biodiversity has attracted widespread attention. Plant conservation includes the preservation of genetic diversity within and among populations and the maintenance of population numbers (Stinchcombe and Hoekstra 2008). Genetic diversity represents a particularly important component of conservation (Charles et al. 2018) because it affects species' adaptability, ecosystem stability, and sustainability (Hughes et al. 2008; Szczeciníska et al. 2016). To effectively protect *C. japonica*, its genetic diversity should first be characterized (Brian 2009).

The genetic diversity of natural *C. japonica* population has been examined by a few research, mainly using allozymes, amplified fragment length polymorphisms (AFLPs), and inter simple sequence repeats (ISSRs) (Oh et al. 1996; Chung and Chung 2000; Zhang 2008; Lin et al. 2012*a*, 2012*b*). All of these approaches are dominant marker systems and therefore have a limitated ability to precisely evaluate the diversity of *C. japonica*, an outcrossing species. Simple sequence repeats (SSRs) have the advantages of co-dominance, good polymorphism, and stable amplification conditions (Xie et al. 2010), and they are capable of distinguishing homozygous and heterozygous genotypes. Therefore, genetic diversity estimates may be higher when SSR markers are used (Li et al. 2020). SSR and expressed sequence tag (EST)-SSR markers based on transcriptome sequencing have recently been used to analyze the genetic relationships among *C. japonica* germplasms (Zhang et al. 2016; Zhao et al. 2017; Pan et al. 2019), but SSRs have not been used to analyze the genetic relationships among *C. japonica* populations. In addition, most AFLP and ISSR studies of *C. japonica* have focused on natural populations on islands. Genetic diversity and the relationships between cultivated and natural populations have not been studied to date.

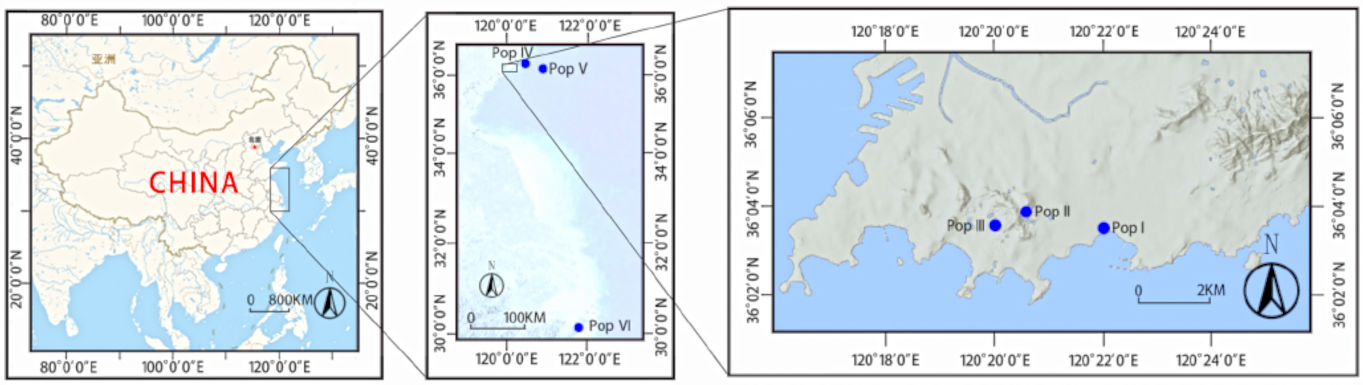
Humans have spontaneously cultivated wild *C. japonica* to protect it and ensure its reproduction, and these efforts have increased its population density. Natural populations of *C. japonica* (Naidong) are primarily found on Changmenyan Island and Daguan Island in Qingdao, whereas cultivated populations are found in three parks, the Botanical Garden, Zhongshan Park, and May Fourth Square in Qingdao (Wang 2006). In this study, we used SSR molecular markers to analyze the genetic diversity and relationships among five *C. japonica* (Naidong) populations in Qingdao in northern China and one *C. japonica* population in Daqingshan in southern China. The influences of geographic isolation and human factors on the genetic structure of the different populations were clarified. These results provide a scientific basis for the protection and utilization of *C. japonica* germplasm resources.

Materials and Methods

Sample collection

One hundred and eighty individuals of *C. japonica* were collected from six populations in northern and southern China (Fig. 1): May Fourth Square (Pop I) (Qingdao, Shandong, China), Botanical Garden (Pop II) (Qingdao), Zhongshan Park (Pop III) (Qingdao), Daguan Island (Pop IV) (Qingdao), Changmenyan Island (Pop V) (Qingdao), and Daqingshan (Pop VI) (Zhoushan, Zhejiang, China). Populations I–V are from the northern region where populations of *C. japonica* (Naidong) are concentrated, and Pop VI is a representative population from the southern range of its distribution. Populations I–III are cultivated populations, and Pop IV–VI are natural populations. From September to December 2017, an interval sampling method (Joshi et al. 2000) was used to collect samples from 30 individuals in each population: 2–4 young leaves were collected from the top of the stem of each individual, and sampled plants were located at least 50 m from a conspecific plant. The leaves were placed immediately into self-sealing plastic bags with dry color-indicating silica gel and transported back to the

Fig.1. Locations of the *C. japonica* populations sampled in this study. The left map shows the region in China from which six *C. japonica* populations were sampled (Map world, 2021), the middle map shows the locations of three natural *C. japonica* populations sampled in Qingdao, Shandong Province and Zhujiajian, Zhejiang Province (Map world, 2021), and the right map shows the locations of the three cultivated *C. japonica* populations sampled in Qingdao (Arcgis topographic map, 2021). [Colour online.]



laboratory within 2–3 h. After washing and drying, the samples were stored in a −80 °C freezer for future use.

DNA extraction

Genomic DNA was extracted from the samples using a modified cetyl trimethyl ammonium bromide (CTAB) method (Li et al. 2009). The concentration and purity of the DNA were measured using 2% agarose gel electrophoresis and UV spectroscopy, and the samples were dissolved in 1 × TE buffer and stored at −20 °C for future use.

PCR amplification and primer selection

The 20 μL SSR polymerase chain reaction (PCR) reaction system contained 7.2 μL ddH_2O, 10 μL Mix, 0.3 μL forward primer, 0.3 μL reverse primer, 2 μL DNA template, and 0.2 μL Taq enzyme. The amplification procedure was as follows: pre-denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 min, annealing at 54 °C for 35 s, and extension at 72 °C for 40 s; and final extension at 72 °C for 3 min. All PCR products were verified by capillary electrophoresis on an ABI 3730xl instrument (Applied Biosystems, Waltham, MA, USA). The electrophoresis results were photographed and analyzed using GeneMarker V2.2.0 software.

Four samples of DNA templates were randomly selected from 180 *C. japonica* individuals, and 64 pairs of primers were selected for primer screening based on previous SSR studies of *C. japonica* and related plants (Fang et al. 2012; Chen et al. 2016; Zhang et al. 2016). The PCR products were added to the upper sample buffer, denatured at 94 °C for 10 min, and then analyzed by vertical electrophoresis on a 6% denatured polyacrylamide gel. Twenty pairs of primer sequences were selected based on the results of this preliminary experiment (Table 1 and Supplementary Fig. S1[1]). Primer synthesis was performed at Huada Gene Company.

Statistical analysis

Capillary electrophoresis was performed using the ABI 3730xl gene sequencer, and the resulting raw data were analyzed with Fragment (plant) analysis software in GeneMarker. The position of each sample peak was compared with the molecular weight size markers in each lane to determine the fragment size. POPGENE version 1.32 software (Yeh et al. 1997) was used to calculate the effective number of alleles per loci (Ne), the expected heterozygosity (He), and the observed heterozygosity (Ho). The polymorphic information content (PIC) values of all loci were calculated using the online program PICcalc (Nagy et al. 2012). GenAlEx version 6.5 (Peakall and Smouse 2012) was used to estimate F-statistics, including the self-crossing rate within the population (F_{is}), the self-crossing rate among the populations (F_{it}), the population differentiation rate (F_{st}), the gene flow among different populations (N_m), and the principal coordinate analysis (PCoA). Analysis of molecular variance (AMOVA) in Arlequin 3.0 software (Excoffier et al. 2005) was used to estimate the genetic variation among and within populations and to conduct the significance test of genetic differentiation of the populations. The genetic distance was used to determine the genetic differences between various populations using the method described by Nei (1972). Based on the genetic distance, an unweighted pair group with arithmetic mean (UPGMA) dendrogram (Felsenstein 1984) constructed with NTSYS 2.1 (Rohlf 1988) was used to perform cluster analysis on various populations to construct a phylogenetic tree. The delta K criterion was analyzed with the

[1]Supplementary data are available with the article at https://doi.org/10.1139/CJPS-2021-0034.

Table 1. Twenty pairs of SSR primers.

Primer number	Forward primer sequence	Reverse primer sequence	Amplicon length (bp)	Fluorescent marker
P1	TATTGCCTACGACCATTTCCA	TTTGAGTTCGTTGCCTTCTCT	190–230	5′FAM
P2	AAGAAGAGCAGAGCAACAAGTG	CCACACACTTTCCACACTTTTG	180–230	5′HEX
P3	TGCCCCTAATTTTCTATCTTT	GGGAAATTGCTTACTCTCATT	160–200	5′TMRA
P17	GTGGGTTACGGGTTTA	TTTGAGTTCGTTGCCTTCTCT	210–260	5′FAM
P18	GCGTATGGAAAAGCTGAGAA	CCACACACTTTCCACACTTTTG	140–180	5′FAM
P19	TATTGCCTACGACCATTTCCA	GGGAAATTGCTTACTCTCATT	180–230	5′FAM
P21	CCATCATTGGCCATTACTACAA	CCATATGTGTGTGAATGATAAAACC	140–180	5′TMRA
P22	GGTGTGGTGTTTTGAAGAAA	TGTTAAGCCGCTTCAATGC	160–200	5′HEX
P23	GTGAAGTTAGTTGTTACTCTTTTTTGG	AGGGGAAGTGAGGAGGCAT	200–230	5′FAM
P27	GGCTTGGACACTTGGTTAGA	AAGATGGATTAGGGTAGGAT	160–200	5′TMRA
P28	TGGCCGATCGGGATTCAAT	TGACCAAACAGCTCATGCG	160–200	5′HEX
P37	ACGCTTCTGTTTGGTCTATT	GATCTTGCTCATCCCTTCAC	150–180	5′HEX
P39	ATAGGTCTTTGTCTGGTT	AAGATGGATTAGGGTAGGAT	210–250	5′FAM
P40	AATAAGAATCGGTGACCTCTG	CTTCATTAACCCCTAAACTAAAAC	130–160	5′HEX
P43	CCAAAACCCTAGTTTCACTCCA	ATCAAACGCTCTGTATCGGTG	240–289	5′FAM
P49	TTTTGGTTGCCTCGCCTCC	TGCTTCCCTCTAGGTCCCTCC	160–180	5′TMRA
P53	GCTAATGATAGACCATCTGCTCCT	GGCCATGCTCTCAATAGTAGAACT	140–170	5′FAM
P54	TTACATCTCTTTTGCAGCTGTCGG	CTTCGGGAACTTCTGCTTCATC	150–180	5′FAM
P55	CCTCTCCTTGCCTTTCATTTC	GCCACGGTTTTCTTCTCCTC	140–170	5′FAM
P58	AGGGGACGGATCTCATATCGT	GACTTCCTCACCGGAGTGCTT	190–230	5′HEX

program STRUCTURE HARVESTER (Earl and vonHoldt 2012). The genetic structure diagram of the six *C. japonica* populations was created using STRUCTURE 2.0 (Evanno et al. 2005). SRUCTURE analysis was performed with 200,000 replicates burn-in length and 1200,000 iterations of iterations Markov chain Monte Carlo (MCMC) from K = 2 to K = 10. Independent STRUCTURE runs were 6 conducted for each specialized K.

Results

Genetic diversity of *C. japonica* populations

Polymorphic alleles were amplified using 20 pairs of SSR primers (Table 2). The PIC values of these loci ranged from 0.3188 to 0.8177, with an average of 0.5849, revealing a high level of genetic diversity among *C. japonica* plants in China. Loci with low polymorphism (PIC < 0.25), moderate polymorphism (0.25 < PIC < 0.5), and high polymorphism (PIC > 0.5) accounted for 0%, 25%, and 75% of all loci, respectively, indicating that the SSR primers used in this study were highly reliable (Ashburner et al. 2000). The number of observed alleles (Na) at each locus ranged from 3 to 22; the average number of alleles was 9, and the total number of alleles was 180. The effective number of alleles per locus (Ne) ranged from 1.7620 to 6.1393, with a mean of 3.1842. The Shannon information index (I) ranged from 0.6253 to 1.9900, with a mean of 1.3170. Nei's gene diversity (GD) values indicated that there was a large amount of genetic diversity at all loci and ranged from 0.3817 to 0.8371. Observed heterozygosity (Ho) ranged from 0.2444 to 0.9722, and expected heterozygosity (He) ranged from 0.3827 to 0.8394. The average He (0.6385) was larger than the average Ho (0.5024), indicating low heterozygosity of *C. japonica*.

The genetic diversity among *C. japonica* populations based on SSR analysis is shown in Table 3. The mean values of Na and Ne were 4.8250 and 2.9538, respectively, and the Na and Ne of Pop VI were clearly higher than those of the other five populations. Values of I and GD at the population level were 1.1414 and 0.5978, respectively, and I and GD were also highest in Pop VI. The average value of He was 0.6086, and that of Pop VI was highest, but Ho of Pop VI showed the opposite pattern. The percentages of polymorphic loci (PPL) in the six *C. japonica* populations were all 100%. Among the *C. japonica* (Naidong) populations, Na, Ne, I, He, and GD were higher in Pop III than in the other four populations, although Ho had an intermediate value in Pop III. Pop IV and Pop V had similar genetic diversity values, as did Pop I and Pop II. Thus, the genetic diversities of the six populations in rank order were: Daqingshan (Pop VI) > Zhongshan Park (Pop III) > Changmenyan Island (Pop V) > Daguan Island (Pop IV) > Botanical Garden (Pop II) > May Fourth Square (Pop I). Ho was lower than He in all six *C. japonica* populations. These results were consistent with the geographic locations of the six populations (Fig. 1), indicating that environmental conditions may influence the genetic diversity of *C. japonica*.

Genetic structure of *C. japonica* populations

We analyzed the genetic differentiation index and gene flow of *C. japonica* populations based on the SSR

Table 2. Diversity statistics of 20 SSR loci across *C. japonica* populations.

Locus	PIC	Na	Ne	I	Ho	He	GD
P1	0.5249	10	2.3453	1.1851	0.4000	0.5752	0.5736
P2	0.7642	12	4.8474	1.7560	0.6056	0.7959	0.7937
P3	0.5096	6	2.4442	1.0460	0.9722	0.5925	0.5909
P17	0.7522	17	4.4588	1.9202	0.6000	0.7779	0.7757
P18	0.4576	6	2.1134	0.9452	0.4556	0.5283	0.5268
P19	0.5149	10	2.2877	1.1750	0.3611	0.5645	0.5629
P21	0.7815	22	4.9522	2.1663	0.4556	0.8003	0.7981
P22	0.6597	7	3.4703	1.3812	0.3820	0.7138	0.7118
P23	0.3504	4	1.7858	0.6704	0.2556	0.4412	0.4400
P27	0.5663	3	2.7682	1.0583	0.6556	0.6405	0.6387
P28	0.3188	4	1.6172	0.6253	0.4000	0.3827	0.3817
P37	0.4382	6	2.0456	0.9042	0.4056	0.5126	0.5111
P39	0.5891	3	2.9687	1.0934	0.7167	0.6650	0.6631
P40	0.7684	12	4.7894	1.8838	0.2889	0.7934	0.7912
P43	0.7212	11	4.0505	1.7151	0.6222	0.7552	0.7531
P49	0.6767	13	3.5602	1.5611	0.5111	0.7211	0.7191
P53	0.3413	3	1.7620	0.6381	0.2444	0.4337	0.4325
P54	0.6020	7	2.9297	1.2834	0.6111	0.6605	0.6587
P55	0.8177	11	6.1393	1.9900	0.6333	0.8394	0.8371
P58	0.5437	13	2.3477	1.3423	0.4722	0.5757	0.5741
Mean	0.3188	9	3.1842	1.3170	0.5024	0.6385	0.6367
St. Dev	0.4382	5.0576	1.2920	0.4685	0.1772	0.1361	0.1357

Note: Abbreviations: Na, number of observed alleles; Ne, number of effective alleles; I, Shannon information index; Ho, observed heterozygosity; He, expected heterozygosity; GD, Nei's gene diversity.

Table 3. Genetic diversity of *C. japonica* populations.

Populations	Na	Ne	I	Ho	He	GD	PPL (%)
Pop I	4.1000	2.5195	0.9906	0.4583	0.5442	0.5351	100.00
Pop II	4.1000	2.5447	1.0016	0.4667	0.5641	0.5547	100.00
Pop	5.5500	2.9983	1.2458	0.5067	0.6431	0.6324	100.00
Pop	4.2000	2.7906	1.0642	0.5152	0.5925	0.5826	100.00
Pop	4.8000	2.7594	1.0947	0.5537	0.5918	0.5846	100.00
Pop	6.2000	4.1100	1.4516	0.4895	0.7160	0.6972	100.00
Mean	4.8250	2.9538	1.1414	0.4984	0.6086	0.5978	100.00

Note: Abbreviations: Na, number of observed alleles; Ne, number of effective alleles; I, Shannon information index; Ho, observed heterozygosity; He, expected heterozygosity; GD, Nei's gene diversity; PPL, percentage of polymorphic loci.

markers (Table 4). F-statistics (F_{is}, F_{it}, and F_{st}) and gene flow (N_m) analysis of the six populations showed that only four loci had negative F-statistics. The remaining sixteen loci had positive values, indicating that the proportion of populations with heterozygotes was low and the degree of purity of the populations was high, consistent with the values of He and Ho in *C. japonica* individuals and populations (Table 2 and Table 3). The average F_{is}, F_{it}, and F_{st} were 0.1651, 0.2332, 0.0816, respectively (Table 4). The genetic differentiation of the different populations was analyzed based on the F_{st} and N_m values. The average F_{st} across all loci was 0.0816, indicating that there was clear genetic differentiation among the *C. japonica* populations. The average N_m was 2.8148, confirming that there was a moderate to high degree of genetic differentiation among the populations.

The AMOVA results showed that the highest genetic variation occurred within *C. japonica* populations (89%); the genetic variation among populations was only 11% (Table 5). Genetic variation among *C. japonica* populations was therefore low, and genetic variation arose mainly within members of individual C. *japonica* populations.

Relationships among *C. japonica* populations

The genetic similarity coefficients of the six *C. japonica* populations ranged from 0.6738 to 0.9874 (Table 6). The genetic similarity coefficient was highest between the May Fourth Square (I) and the Botanical Garden populations in Qingdao (II), and lowest between the five

Table 4. F-statistics and gene flow at 20 SSR loci of six *C. japonica* populations.

Locus	F_{is}	F_{it}	F_{st}	N_m
P1	0.1820	0.3092	0.1555	1.3575
P2	0.1951	0.2498	0.0680	3.4265
P3	−0.6545	−0.6185	0.0217	11.2630
P17	0.1911	0.2561	0.0804	2.8606
P18	0.0751	0.1195	0.0480	4.9591
P19	0.2718	0.3918	0.1647	1.2676
P21	0.3612	0.4395	0.1225	1.7911
P22	0.4721	0.4970	0.0473	5.0392
P23	0.4329	0.4642	0.0552	4.2764
P27	−0.0456	−0.0101	0.0340	7.1097
P28	−0.0598	−0.0183	0.0392	6.1353
P37	0.0846	0.2422	0.1722	1.2020
P39	−0.1205	−0.0663	0.0484	4.9150
P40	0.6064	0.6409	0.0875	2.6071
P43	0.1499	0.2044	0.0640	3.6543
P49	0.1889	0.3040	0.1419	1.5118
P53	0.4650	0.4897	0.0462	5.1666
P54	0.0542	0.0949	0.0430	5.5603
P55	0.2234	0.2669	0.0561	4.2096
P58	0.0996	0.2176	0.1310	1.6586
Mean	0.1651	0.2332	0.0816	2.8148

Note: Abbreviations: F_{is}, self-crossing rate within the population; F_{it}, self-crossing rate between the populations; F_{st}, population differentiation rate; N_m, gene flow estimated as $N_m = [(1/F_{st}) - 1]/4$.

C. japonica (Naidong) populations (I–V) and the southern *C. japonica* population (VI).

The unweighted pair group method with arithmetic mean (UPGMA) dendrogram of *C. japonica* was reconstructed based on genetic distance values (Fig. 2). UPGMA clustering of the six populations indicated that the genetic distance was 0.1818, and the populations could be grouped into three classes. Class I contained Pop I, Pop II, Pop III, and Pop V; class II contained Pop IV; and class III contained the southern *C. japonica* population, Pop VI.

Cluster analysis was performed based on differences in the allele frequencies of different individuals. The relationship between ΔK and K was determined using the method described by Evanno (2005) (Figs. 3*a* and 3*b*). The largest ΔK occurred at K = 3, and this value of K was therefore used to obtain a reasonable clustering division of the populations. The 180 individuals in the six populations could be divided into three genetically distinct groups (Fig. 3*c*). Most samples from Pop I, Pop II, Pop III and Pop V fell into one group, whereas Pop IV and Pop VI were placed in separate groups.

To further evaluate the genetic variation of different *C. japonica* populations, a principal coordinate analysis (PCoA) was performed. This PCoA indicated that the six populations divided into three clusters (Fig. 4): in one were Pop I, Pop II, Pop III and PopV, the other two groups consisted of Pop IV and Pop VI, respectively. The PCoA results were consistent with the clustering from the UPGMA (Fig. 2).

Discussion

Genetic diversity of *C. japonica*

Genetic diversity analysis of a species can provide guidance for its genetics and breeding, and the study of genetic structure and differentiation assists with the protection and utilization of germplasm resources (Wolf and Harrison 2001). Hamrick (1989) analyzed 449 plants in 165 genera and reported that the average genetic diversity index (He) was 0.11 within plant populations and 0.15 at the species level. Fang et al. (2012) used SSR markers to analyze the genetic diversity of *C. sinensis* cultivars and reported He values of 0.543. Expressed sequence tag-simple sequence repeat (EST-SSR) markers were developed for *C. reticulata*, a native plant of southwestern China, and analysis showed that the He values of *C. reticulata*, *C. saluenensis*, *C. pitardii*, and *C. yunnanensis* were 0.457, 0.342, 0.235, and 0.168, respectively (Tong and Gao 2020). Using EST-SSR markers, the average He of *C. japonica* cultivars was 0.638 (Pan et al. 2019), consistent with the He values reported here for 180 *C. japonica* samples (0.6385). Our findings also indicate that the genetic diversity of *C. japonica* is much higher than that of other *Camellia* species and woody plants. The Shannon information index (I) is also an important indicator that reflects population genetic diversity. Compared with the I value reported here (1.1414), the I values of *Camellia* populations from other species calculated from SSR markers were lower: 0.2689 in *C. japonica* (Zhao et al. 2017) and 0.988 in *C. nitidissima* (Li et al. 2020). Together, these results indicate that *C. japonica* exhibits high genetic diversity. In general, higher genetic diversity is thought to be associated with a greater ability to adapt to the environment (Reed and Frankham 2003). The high genetic diversity of *C. japonica* (Naidong) may explain why it has successfully survived from the third century.

Populations from the Daqingshan, Changmenyan Island, and Daguan Island generally showed higher genetic diversity, though they are natural island populations. Geographic isolation is a primary cause of differences in genetic diversity within the same species (Bellucci et al. 2013). Island populations exhibit genetic differences as a result of strong genetic drift acting on the population (Forsdick et al. 2017). It is a commonly held view that island species are characterized by geographical isolation, barriers to genetic flow, small population sizes, and possible historical bottleneck effects, which have reduced their population genetic diversity (Francisco-Ortega et al. 2000). Island populations have therefore become models for studying population genetic differentiation (Juan et al. 2000). However, numerous studies have demonstrated that not all island species have low genetic diversity. For example, marginal populations of *Cryptomeria japonica* on Yaku Island

Table 5. Analysis of molecular variance (AMOVA) in six *Camellia* populations.

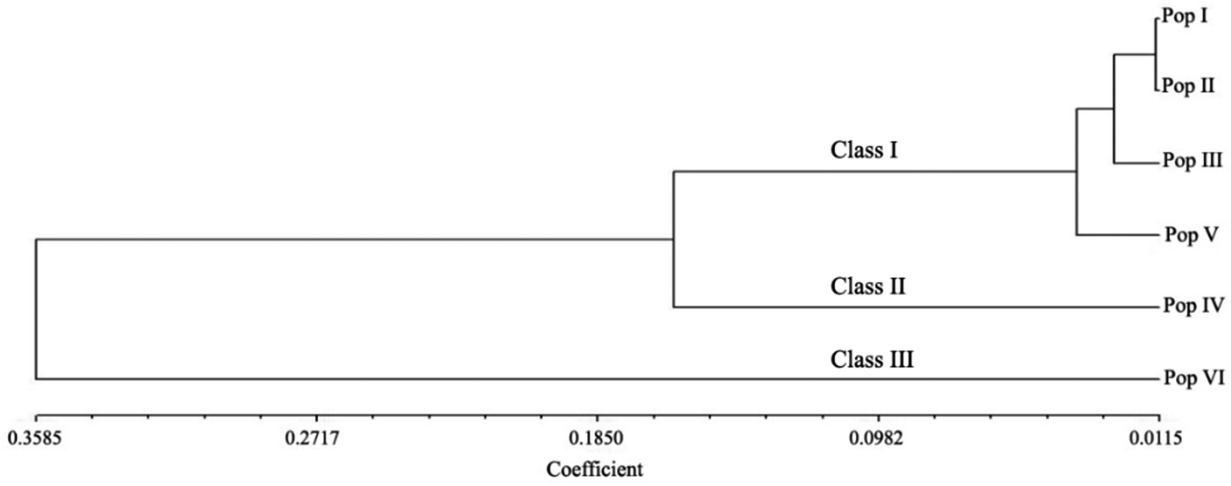
Source of variation	df	SS	MS	Est. variation	Percentage of variation (%)
Among populations	5	329.489	65.898	1.744	11
Within populations	174	2446.323	14.059	14.059	89
Total	179	2775.811		15.803	100

Note: df, degrees of freedom; SS, sum of squares; MS, Mean square.

Table 6. Genetic similarity coefficients of six *C. japonica* populations.

	I	II	III	IV	V	VI
Pop I	1.0000	0.9874	0.9721	0.8393	0.9606	0.6738
Pop II		1.0000	0.9776	0.8664	0.9671	0.6892
Pop III			1.0000	0.8646	0.9629	0.7357
Pop IV				1.0000	0.8338	0.6981
Pop V					1.0000	0.6987
Pop VI						1.0000

Fig. 2. Cluster analysis of six *C. japonica* populations based on genetic distance.



have maintained relatively high genetic variation compared with those of mainland Japan (Tsumura and Ohba 1993). Fineschi et al. (2004) analyzed the genetic diversity of *Zelkova schneideriana* on the islands of Crete and Sicily and found that the genetic diversity of the island species was high. Li et al. (1996) used allozyme electrophoresis to analyze the genetic diversity of *C. japonica* populations on the offshore islands of Qingdao and reported a high percentage of polymorphic loci (83.3%). Here, we found that island *C. japonica* populations (Pop VI, Pop V, and Pop IV) had higher genetic diversity than inland *C. japonica* populations (Pop II and Pop I), confirming that island organisms can maintain high levels of genetic diversity.

The island *C. japonica* population of Daqingshan (Pop VI) had the highest genetic diversity in our study, a finding that may be related to its rich plant species diversity and excellent geographical environment. Daqingshan on Zhujiajian is the main distribution area of wild *C. japonica*. It contains more than 1000 wild *C. japonica* plants with a rich variation in flower types, petal colors, and leaf shapes, which lead to a variety of breeding types and mating patterns and have caused the amount of genetic variation to increase. Why was higher genetic diversity also found in the two *C. japonica* (Naidong) populations from Daguan Island and Changmenyan Island? A study of *Argyroxiphium sandwicense* ssp. *sandwicense* on the island of Hawaii indicated that diversity was closely related to environmental differences; the pressure of natural selection was strong, and the genetic diversity was high (Friar et al. 2010; Zheng et al. 2010). Since the third century, climate

Fig. 3. STRUCTURE analysis results of 180 *C. japonica* individuals. (*a*) Population estimation using the mean of estimated log probability of data (lnP(D)) with the cluster number (K) varied from 2 to 10. (*b*) Population estimation using lnP(D)-derived delta K(ΔK) with the cluster number (K) varied from 2 to 10. (*c*) Genetic structure estimates of the six populations based on STRUCTURE analysis with cluster number K = 3. Each individual is represented by a vertical line, and different colors represent different genetic components. [Colour online.]

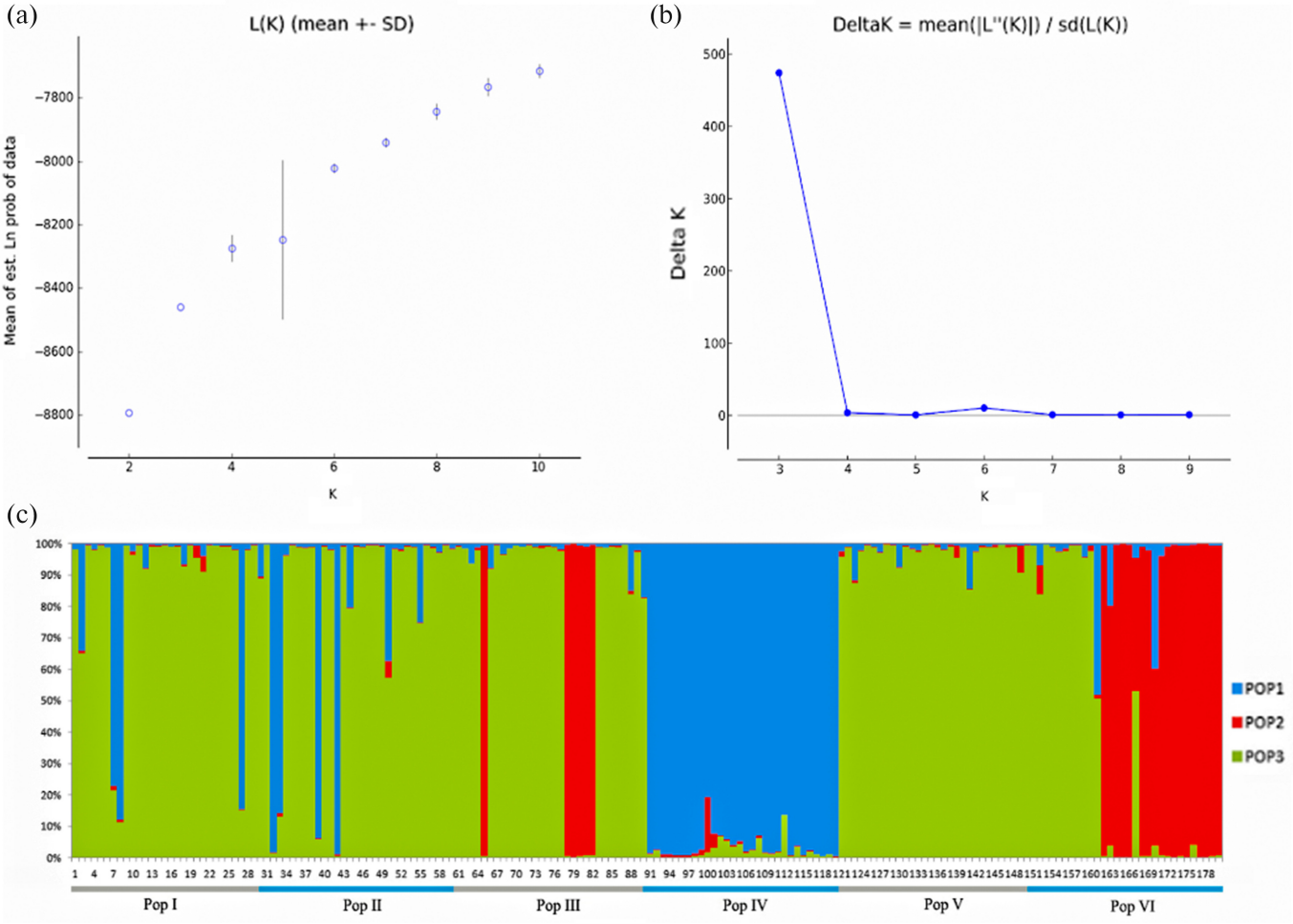
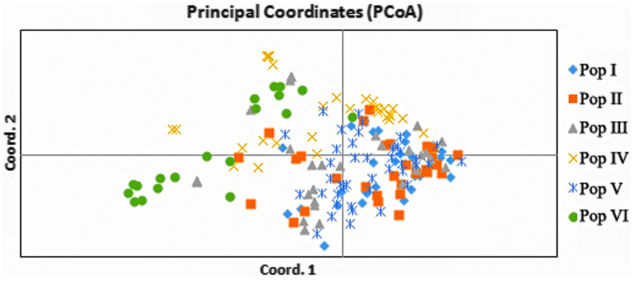


Fig. 4. Principal coordinates analysis (PCoA) of *C. japonica* populations. The colored dots indicated *C. japonica* individuals from different populations. [Colour online.]



change has produced variable and complex environmental conditions for *C. japonica* (Naidong). Its growth environment on Daguan and Changmenyan islands is relatively harsh, influenced by waves, exposed rocks, shallow soil (usually no more than 50 cm deep), and a shrinking distribution. Nonetheless, *C. japonica* (Naidong) has become the dominant tree species on the islands, perhaps because its genetic diversity has increased. Although its habitat is relatively small, human influence has also been relatively low (Zhou et al. 1994; Shi and Song 2005), a fact that may contribute to the high genetic diversity of *C. japonica* (Naidong).

Relationships among *C. japonica* populations

The clustering results of the six populations based on genetic distance showed that the *C. japonica* (Naidong) populations from May Fourth Square (Pop I), Botanical Garden (Pop II), and Zhongshan Park (Pop III) in Qingdao had the closest genetic relationship. These three populations were grouped with the *C. japonica* (Naidong) population from Changmenyan Island (Pop V) into class I. The clustering results indicated that the three cultivated populations and the natural population of *C. japonica* (Naidong) on Changmenyan Island were

closely related. Surveys have shown that the *C. japonica* (Naidong) population from the Botanical Garden in Qingdao was cultivated after the establishment of the garden in 1979. Many wild plants and seeds of *C. japonica* (Naidong) from Changmenyan Island and Daguan Island were transplanted or sown in the garden. Those which survived and multiplied gave rise to a *C. japonica* (Naidong) population in the Botanical Garden. *C. japonica* (Naidong) in Zhongshan Park and May Fourth Square in Qingdao were transplanted from the Qingdao Botanical Garden in 1988 and 1998, respectively (unpublished). Therefore, these four populations are very closely related. Li et al. (1996) conducted a genetic distance analysis of five *C. japonica* populations from Qingdao and Zhejiang and found that the genetic distance was shortest between the *C. japonica* populations of Changmenyan Island and the Botanical Garden in Qingdao, followed by Daguan Island. Their results agree with those of the present study, confirming that the three cultivated populations of *C. japonica* (Naidong) in Qingdao originated from Changmenyan island.

The relationships among the three island populations of Daqingshan (Pop VI), Daguan Island (Pop IV), and Changmenyan Island (Pop V) were consistent with the close genetic relationships reported for *C. japonica* populations on five geographically clustered islands (Lin et al. 2012*b*), indicating that the geographical isolation of the islands may have had an important effect on the genetic differentiation of *C. japonica* populations. At the same time, *C. japonica* (Naidong) exhibits morphological and physiological differences relative to southern *C. japonica*, and these may be the result of the long-term geographical separation of the species (Wang 2006; Hagenblad et al. 2019).

Genetic diversity conservation of *C. japonica* germplasm resources

C. japonica was the dominant species of the coastal woody plant communities on Changmenyan, Daguan, and Zhoushan Islands. Because of human disturbance, the species is currently found only on hillsides at 30–40 m altitude on Changmenyan and Daguan Islands in Qingdao and at 370 m altitude on islands in Daqingshan (Wang and Zhang 1992; Zhou et al. 1994; Lin et al. 2013). The AMOVA results showed that the within-population genetic variation of *C. japonica* (89%) was much higher than the among-population variation (11%), and similar results were found in a study of *C. nitidissima* (Li et al. 2020). This result indicates that there is abundant genetic variation within *C. japonica* populations and that genetic variation can therefore be protected at the population level. Hence, we propose that in situ conservation approaches for *C. japonica* populations should be carried out first. We suggest strengthening the protection of the natural *C. japonica* populations on islands. The rapid establishment of nature reserves may be an effective way to save rare and endangered *C. japonica*, avoid the destruction of existing island habitat, increase the environmental protection awareness of island residents, and prohibit seedling harvest and seed collection. These approaches can promote natural regeneration to conserve the genetic diversity of *C. japonica* populations (Wang and Hu 1996). For islands with human settlements (Daguan Island, Daqingshan Island), the government or the villagers' committee should publicize and organize the network of forest rangers. For islands without settlements (Changmenyan Island), the garrison could be responsible. To take full advantage of the protected areas, we should perform scientific research, breeding, and sales of *C. japonica* in an organized manner. For cultivated *C. japonica* populations in parks, we should strengthen the informational programs that educate tourists about *C. japonica*. In addition, a germplasm repository should be established to collect and protect the genetic resources present in *C. japonica* populations through seed collection and seedling cultivation from natural populations.

Conclusions

In this study, we assessed the genetic diversity and relationships among 180 *C. japonica* individuals from six populations. All analyses indicated that the genetic diversity of *C. japonica* was higher than that of other *Camellia* species, and island *C. japonica* populations generally had higher genetic diversity than inland populations. The 180 *C. japonica* individuals in the six populations were divided into three clusters that were consistent with their geographical distributions. We also confirmed that cultivated *C. japonica* (Naidong) from three parks were originally transplanted from Changmenyan Island. There was abundant genetic variation within *C. japonica* populations, and this variation can therefore be protected at the population level. This study represents the first systematic analysis of the genetic diversity and structure of natural and cultivated *C. japonica* populations. It provides guidance for protecting genetic resources and making horticultural use of *C. japonica*.

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