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ASSESSMENT OF GENETIC DIVERSITY AND POPULATION STRUCTURE OF ENDANGERED *CAMELLIA CHEKIANGOLEOSA* HU USING ISSR MARKERS

YUN XİE[1,2,3], JİYUAN Lİ[2*] AND DONGLİN ZHANG[3*]

[1]*Zhejiang A & F University, Jiyang College, Zhuji, Zhejiang, 311800, China*
[2]*Research Institute of Subtropical Forestry, Chinese Academy of Forestry, Hangzhou, Zhejiang, 311400, China*
[3]*University of Georgia, Department of Horticulture, Athens, GA 30602, USA*
**Corresponding author's email: jiyuan_li@caf.ac.cn or donglin@uga.edu*

Abstract

The genetic diversity of 210 individuals from seven *Camellia chekiangoleosa* Hu populations was analyzed using ISSR markers. Results revealed an extraordinarily high level of genetic diversity at the species level. The total percentage of polymorphic loci (*P*) was 96.88%. Shannon's information index (*I*) and Nei's gene diversity (*h*) were 0.4966 and 0.3331 in the species, respectively. At the population level, the genetic diversity of *C. chekiangoleosa* Hu was relatively low. The mean values of *P*, *I*, and *h* were 59.79%, 0.3106, and 0.2077, respectively. The level of genetic differentiation among populations was lower than that within populations, and a certain level of genetic differentiation (G_{ST} = 0.3758) was observed among the populations. Analysis of molecular variance revealed that among-population, among-region, and within-population components accounted for 28.0%, 12.0%, and 60.0% of the total variations, respectively. Genetic differentiation was high (0.6242), and the gene flow was low (0.8304). These results suggested that population isolation and inbreeding regression played major roles in genetic differentiation among the *C.chekiangoleosa* Hu populations. The pair-wise mean genetic distance among the populations was 0.1696.

Key words: *Camellia chekiangoleosa* Hu, Population, Genetic diversity, Genetic differentiation, ISSR.

Introduction

Camellia chekiangoleosa Hu (Zhejiang camellia), an evergreen broad-leaved woody species, is a rare ornamental plant (Liang, 2000) with a nice shape, large and bright red flowers, and strikingly bright red or yellow fruits. The plant usually blooms in winter, which is very valuable for ornamental plant world. Zhejiang camellia also offers economic and ornamental benefits because it could be utilized as oil, ornament, medicine, and wood. It provides high-quality edible seed oil with better content and fatty acid composition than those of the general *C. oleifera* (Hu, 1987). Wild *C. chekiangoleosa* Hu is endemic to China and narrowly distributed in Wuyi and Huaiyu mountain ridge within the altitude between 360 and 1600 m. The wild resource of *C.chekiangoleosa* Hu is being decreased rapidly due to deforestation. It was listed in Zhejiang as rare and endangered plants in 1994 (Zhang, 1994) and as a provincial key protected plant in Fujian (Liu & Xu, 1996).

Morpho-biochemical and molecular markers based evaluation is useful to identify new plant species/sub-species for further breeding programs (Jan *et al.,* 2017[a, b]; Hussain *et al.,* 2016; Jan *et al.,* 2016; Shinwari *et al.,* 2014; Shinwari *et al.,* 2013; Sultan *et al.,* 2013). Research on the genetic structure of *C. chekiangoleosa* Hu has not been reported yet although many studies were focused on its resource distribution, chemical components, seed germination, and cultivation. To protect and utilize the natural resources of *C. chekiangoleosa* Hu, seven populations of *C. chekiangoleosa* Hu in this work were analyzed in its whole distribution zone. The levels and patterns of genetic diversity and genetic differentiation were revealed using inter-simple sequence repeat (ISSR) markers.

Materials and Methods

Plant material: A total of 210 adult individuals from seven populations of *C. chekiangoleosa* Hu were collected from seven counties in Zhejiang, Anhui, Fujian, and Jiangxi provinces in China (113°54′36″-121°2′32″E, 26°49′19″-30°50′35″N) in the spring of 2010 (Table 1). In each population, 20 to 46 adult individuals were randomly sampled, with each individual being separated by a distance of at least 50 m. For molecular analysis, fresh leaves from each plant were collected, frozen immediately, and stored at −80°C until DNA extraction.

DNA extraction: Frozen leaves were grounded in liquid nitrogen and DNA was extracted from 0.3 g of powder by following the improved cetyl trimethylammonium bromide (CTAB) method, with 1% β-mercaptoethanol (v/v) and 1% PVP 40000 (w/v) added to the CTAB extraction buffer (Ni et al., 2009). The resulting DNA was air dried, dissolved in an appropriate amount of TE buffer, and preserved in a refrigerator at −20°C. DNA integrity was detected through 0.8% agarose gel electrophoresis. Then, 5 μL of the DNA solution mixed with 2 μL of 0.25% bromophenol blue was added as a sample to the 0.8% agarose gel in 1×TBE buffer for horizontal electrophoresis (electrophoretic apparatus type DYY-80, horizontal electrophoresis tank DYCP-33A). The known concentration of the DNA solution was used as the control. The electrophoresis was conducted at a voltage of 100 V for 30 min. After the electrophoresis, the gel was stained with ethidium bromide (EB) and observed and photographed with an automatic gel image analysis system (U.S. Alpha Innotech Corporation). The DNA solution was diluted to a concentration of 30 ng/μL and used for subsequent procedures.

Table 1. Sampling sites of *C. chekiangoleosa* Hu populations.

Population	Number of individuals	Locality	Geographical location	Mean altitude (m)	Ann mean temp (°C)	Ann precipitation (mm)
Anhui Yuexi (AYX)	24	Henghe village Laibang town	116°7′4″E30°50′35″N	700	12.7	1446
Fujian Xiapu (FXP)	20	Badouqiu village Shuimen town	120°1′26″E27°2′23″N	663	16.7	1720
Jiangxi Yongxin (JYX)	27	Jiulong village Sanwan town	113°54′36″E26°49′19″N	431	17.5	1530
Jiangxi Nanfeng (JNF)	29	Junfeng mountain	116°21′49″E27°12′59″N	1175	17.0	1685
Zhejiang Kaihua (ZKH)	46	Gutian Mountain Conservation Zone	118°9′18″E29°15′21″N	700	15.3	1843
Zhejiang Tiantai (ZTT)	40	Huading Mountain	121°2′32″E29°10′44″N	920	13.0	1900
Zhejiang Jinyun (HJC)	24	Dayang Mountain Huangjin village	120°16′52″E28°41′58″N	1060	13.5	1789

Table 2. Nucleotide sequences and annealing temperatures of the 21 selected ISSR primers and numbers of bands scored in *C. chekiangoleosa* Hu.

Primer	Sequence	Annealing temp /°C	Amplification band	Polymorphic band	Polymorphic loci	Primer	Sequence	Annealing temp /°C	Amplification band	Polymorphic band	Polymorphic loci
UBC-811	$(GA)_8C$	48.7	13	10	76.9	UBC-868	$(GAA)_6$	49.4	16	13	81.3
UBC-816	$(CA)_8T$	50	17	14	82.4	UBC-873	$(GACA)_4$	51	14	12	85.7
UBC-817	$(CA)_8A$	55	16	13	81.3	UBC-881	$(GGGTG)_3$	52	18	15	83.3
UBC-825	$(AC)_8T$	53.2	20	17	85.0	IR2	$(GGAGA)_3$	53.2	18	16	88.9
UBC-827	$(AC)_8G$	51.6	21	18	85.7	IR3	$(GA)_8CT$	50.2	20	17	85.0
UBC-834	$(AG)_8YT$	49.7	17	14	82.4	IR4	$(CAA)_6$	49.4	22	18	81.8
UBC-835	$(AG)_8YC$	53.2	15	13	86.7	IR8	$(AC)_8TG$	52	22	17	77.3
UBC-844	$(CT)_8RC$	50.2	13	11	84.6	IR9	(AC)8CG	49.4	15	12	80
UBC-845	(CT)8RG	51.6	14	12	85.7	IR12	(CAA)6G	52	13	11	84.6
UBC-846	(CA)8RT	49.4	15	12	80.0	IR13	(GACA)4T	52	16	14	87.5
UBC-855	$(AC)_8YT$	53.2	15	13	86.7						

Y = C or T R= A or G

Polymerase chain reaction amplification: All polymerase chain reaction (PCR) amplifications were performed with a GeneAmp® PCR System 9700 PCR instrument (Applied Biosystems, Perkin Elmer Corp., Norwalk, CT, USA). The electrophoresis results were observed, analyzed, and photographed with an Alpha automatic gel image system. The PCRs were performed in a volume of 20 μL containing 2.0 μL of 10 × reaction buffer (TaKaRa, Dalian), 30 ng of genomic DNA template, 2.0 mmol/L of Mg^{2+}, 300.0 μmol/L of dNTP (Promega), 0.8 μmol/L of primer, and 2.5 U of Taq DNA polymerase. Amplifications were carried out on 96-well plates through the following program: initial step of 5 min at 94°C followed by 40 cycles of denaturing 45 s at 94°C, annealing for 50 s at gradient temperatures (53-60°C) and then for 90 s at 72°C, and a final extension step of 7 min at 72°C. The resulting PCR product was preserved at 4°C. Thereafter, 10 μL of PCR products was separated through electrophoresis on 1.2 mg/L of agarose gels containing EB in 0.5 × TBE buffer at 100 V for 2 h (DYY-80 electrophoresis apparatus and DYCP-33A electrophoresis tank) by using 100 bp DNA Ladder as a control. The molecular weights were then estimated.

POPGENE version 1.32 software (Yeh *et al*., 1997) was used to analyze the polymorphic loci percentage (*P*(%)), number of effective alleles (*Ne*), expected heterozygosity (*He*), Shannon's polymorphic information index (*I*), Nei's gene diversity index (*h*), coefficient of gene differentiation (G_{ST}), population genome diversity (H_T), gene diversity within a population (*Hs*), and Nei's genetic uniformity and genetic distance (*D*). Gene flow (N_m) was calculated as $N_m = (1-G_{ST}) / 4G_{ST}$ (Xie *et al*., 2011; Barton, 1989) and UPGMA was used to perform a cluster analysis of every population. GenAlEx6.41 software (Genetic Analysis in Excel) (Li, 2008) was used in the analysis of molecular variance (AMOVA) for molecular variation (Li, 2008; Excoffier *et al*., 1992).

Results

Primer screening and ISSR amplification: A total of 14 plants (2 for each population) and 50 ISSR primers based on ISSR studies on other camellia plants (Ni *et al*., 2009; Luo *et al*., 2007; Bin *et al*., 2005; Yao *et al*., 2007; Wang *et al*., 2008) were used for ISSR primer selection. Twenty-one primers that could present abundant polymorphism and clear bands were screened out (Jin *et al*., 2006) for all sampling plants. The primer sequence, annealing temperature, and number of amplification bands were listed in Table 2. A total of 384 markers generated by 21 primers were scored, with sizes ranging between 150 and 2000 bp and with an average of 18.3 markers per primer. Among the 384 markers, 372 were polymorphic. The percentage of polymorphic loci was 96.88%. The amplification atlas of the JNF population with primer UBC844 is shown in Fig. 1.

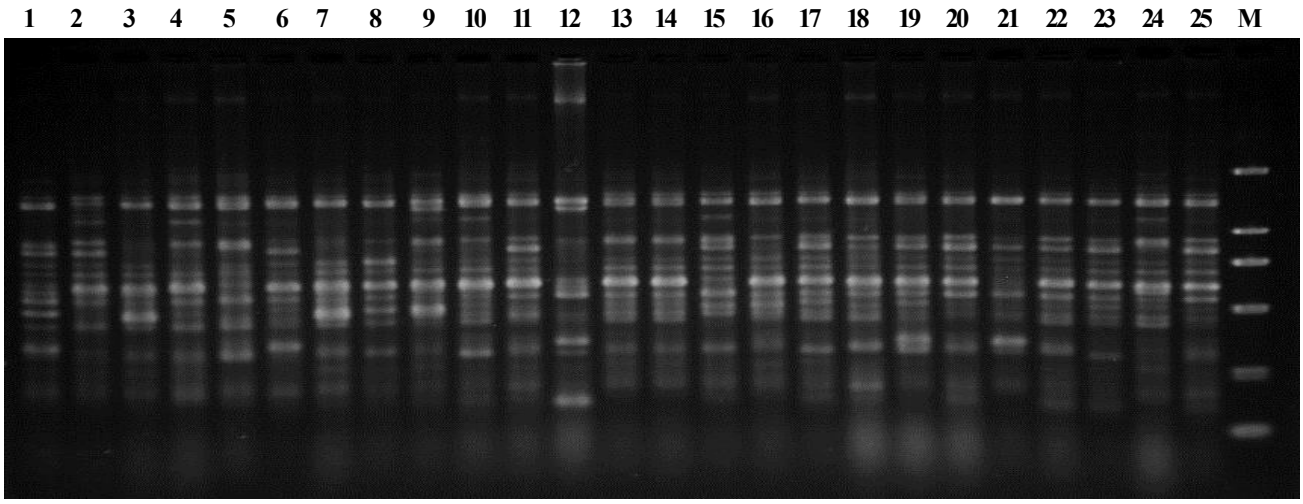

Fig. 1. ISSR fingerprinting in the JNF population of *C. chekiangoleosa* Hu by primer UBC844.
1-25: Individua; M: λDNA/EcoR Ⅰ+HindⅢ.

Table 3. Estimates of genetic diversity within *C. chekiangoleosa* Hu populations.

Populations	Polymorphic loci number	Percentage of polymorphic loci (*P*, %)	Observed number of alleles (N_a)	Effective number of alleles (*Ne*)	Nei's gene diversity (*h*)	Shannon's indices of diversity (*I*)
AYX	183	47.66	1.4766	1.2856	0.1662	0.2483
FXP	180	46.88	1.4688	1.3030	0.1745	0.2583
JYX	225	58.59	1.5859	1.3512	0.2020	0.3010
JNF	220	57.29	1.5729	1.3399	0.1993	0.2984
ZKH	282	73.44	1.7344	1.4217	0.2477	0.3719
ZTT	281	73.18	1.7318	1.4167	0.2477	0.3732
HJC	236	61.46	1.6146	1.3691	0.2164	0.3234
Multi population analysis	372	96.88	1.9688	1.5759	0.3331	0.4966
Mean	229.5714	59.78571	1.597857	1.355314	0.207686	0.310643

Table 4. Genetic differentiation among seven populations of *C. chekiangoleosa* Hu.

	Total gene diversity (H_T)	Gene diversity within populations (H_S)	Coefficient of genetic differentiation (G_{ST})	Gene flow (N_m)
Average	0.3328	0.2077	0.3758	0.8304
SD*	0.0248	0.0132		

*Standard deviation

Genetic variation: Within population, the percentages of the polymorphic loci of the seven populations (*P*, %) ranged from 46.88-73.44%, with an average of 59.79%. Nei's gene diversities (*h*) varied from 0.1662-0.2477, with an average of 0.2077. Shannon's indices (*I*) ranged from 0.2483-0.3732, with an average of 0.3106. A similar trend was observed in the values of *h* and *I*. The polymorphic loci percentage of each population showed a similar trend. The mean observed number of alleles (*Na*) ranged from 1.4688-1.7344, and the mean effective number of alleles (*Ne*) varied from 1.2856-1.4217. When calculated across populations (species level), the *h* and *I* values equaled 0.3331 and 0.4966, respectively, and the *Na* and *Ne* values equaled 1.9688 and 1.5759, respectively. Among the seven populations, the ZTT population exhibited the highest level of variability, whereas the AYX population showed the lowest level of variability (Table 3).

Genetic differentiation: Nei gene diversity at the species level was calculated with the POPGENE software. The total gene diversity (H_T) and gene diversity within populations (*Hs*) revealed in the mixed populations (at the species level) of *C.chekiangoleosa* Hu equaled 0.3328 and 0.2077, respectively. The coefficient of genetic differentiation (G_{ST}) was calculated as 0.3758 on the basis of H_T and H_S. This value indicated that 37.58% and 62.42% variation existed between populations and within populations. It also revealed the presence of a high degree of genetic differentiation among the mixed populations. The level of gene flow (*Nm*, number of migrating individuals among populations per generation) between populations based on *h* was estimated to be 0.8304 (Table 4), indicating a large genetic differentiation between populations and low gene exchange within population.

The AMOVA for the molecular differentiation of the seven populations showed that in terms of total gene diversity, 12.0% occurred between regions, 28.0% occurred between populations, and 60.0% occurred within populations. Differentiation within and between populations were both significant (p<0.001) (Table 5). Thus, AMOVA also supported the results of the Nei gene diversity statistic. Both analyses showed some variation between *C.chekiangoleosa* Hu populations and a high level of genetic differentiation within populations.

Table 5. Analysis of molecular variance (AMOVA) of 210 individuals from seven populations of *C.chekiangoleosa* Hu.

Source of variance	Degree of freedom	SSD*	MSD*	Variance component	Value	Percentage (%)	Probability
Among regions	4	2288.594	572.148	5.664	0.1222	12	<0.001
Among populations (AP)	2	977.826	488.913	12.996	0.3195	28	<0.001
Within populations (WP)	203	5619.286	27.681	27.681	0.4027	60	<0.001

*SSD, sum of squares; MSD, expected mean squares. Number of permutations=1000

Table 6. Nei's unbiased measures of genetic uniformity (above diagonal) and genetic distance (*D*) (below diagonal) of *C. chekiangoleosa* Hu.

	AYX	FXP	HJC	JNF	JYX	ZKH	ZTT
AYX	****	0.8127	0.8252	0.8128	0.7778	0.8517	0.8369
FXP	0.2074	****	0.8323	0.8000	0.7597	0.8322	0.8576
HJC	0.1921	0.1836	****	0.8130	0.7802	0.8303	0.8609
JNF	0.2072	0.2231	0.2070	****	0.7846	0.8490	0.8136
JYX	0.2513	0.2748	0.2482	0.2426	****	0.8110	0.8008
ZKH	0.1605	0.1837	0.1860	0.1636	0.2094	****	0.8861
ZTT	0.1780	0.1536	0.1497	0.2063	0.2221	0.1209	****

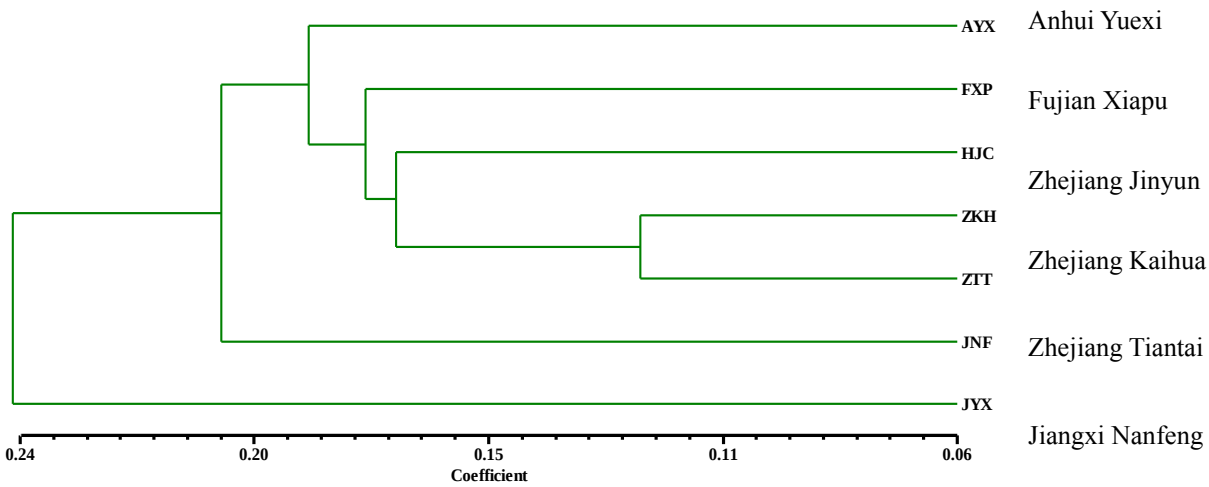

Fig. 2. Dendrogram of the 7 populations of *C. chekiangoleosa* Hu based on Nei's genetic distance.

Genetic relationships among populations: Nei's unbiased genetic distances were calculated with the POPGENE software and then used to estimate genetic differentiation between populations (Table 6). The mean genetic distance among the seven populations was 0.1986 (pairwise range of 0.1209-0.2748). The smallest genetic distance of 0.1209 was found between ZKH and ZTT, whereas the largest genetic distance of 0.2748 was detected between FXP and JYX. This result indicated that the genetic difference between FXP and JYX was the largest, and their similarity was the lowest. The genetic distance between ZKH and ZTT was the smallest, and their similarity was the highest. Nei's genetic uniformity for the seven populations was 0.7597-0.8861, and the average genetic similarity was 0.8204.

Cluster analyses: An UPGMA analysis was conducted on the basis of Nei's genetic distance for the seven populations (Fig. 2). The dendrogram indicated that the genetic distances of the seven populations were in line with their geographic distributions. JYX had the longest genetic distance from the other populations. It clustered with the other populations until the 0.24 threshold and was distributed at the west boundary of the areal area, farthest from the other populations. JNF clustered with the other populations until the 0.207 threshold and was isolated by Wuyi Mountain. AYX clustered with the other populations at the 0.19 threshold and was distributed at the northernmost boundary. FXP and HJC clustered with the other populations at 0.17-0.175, and the genetic distance between them was relatively small, similar to what a local old man said that FXP was a cultivated population introduced from Zhejiang 200 years ago. ZKH and ZTT clustered first, indicating that the genetic distance between the two populations was the smallest. Then, they clustered with HJC. The clusters of the three populations were consistent with their geographic distributions.

Discussion

The results showed that the genetic diversity of *C. chekiangoleosa* Hu at the species level was relatively high. Compared with *C. changii* Ye (Luo *et al*., 2007), *C. nitidissima* Chi (Bin *et al*., 2005), tea clones (Yao *et al.*, 2007), and *C. oleifera* Abel clones (Wang *et al*., 2008) (Table 7), *C. chekiangoleosa* Hu had a relatively high

genetic variation because of its distribution in mountainous regions. Its distribution area is wider than that of *C. changii* Ye and *C. nitidissima* Chi. In mountainous regions, varying climate, biotope heterogeneity, and geographic isolation result in genetic diversity. However, *C. chekiangoleosa* Hu showed a lower genetic diversity than other *C. japonica* L. varieties because many camellia varieties were derived from long-term artificial hybridization and thus showed high genetic diversity.

C. chekiangoleosa Hu also had a higher genetic diversity level than *Rhododendron fortunei* Lindl., which showed a similar biotope and small distribution range. At the species level, the *P* of *R. fortunei* Lindl. was 88.24%, its *I* was 0.4317, and its *h* was 0.2848 according to the ISSR analysis (Jin *et al*., 2006). This result indicated that *C. chekiangoleosa* Hu had a high genetic diversity level, and its distribution was abnormal, similar to an islet.

Table 7. Comparison of genetic diversity in Camellia plants.

Species	Bands size(bp)	*P* (%)	*h*	*I*	G_{ST}	*D*	Reference
Varieties of *C. japonica* L.	150-1500	95.4	0.40-0.48	0.57-0.67	0.5-0.7	—	Ni *et al*., 2009
C. oleifera Abel clone	200-2000	68.6	0.3132	0.4793	—	0.1542-0.6931	Wang *et al*., 2008
Tea clones	150-1500	99.7	0.23	0.38	—	0.337	Yao *et al*., 2007
C. changii Ye	—	55.29	0.2191	0.3215	0.0922	2.4619	Luo *et al*., 2007
C. nitidissima Chi	—	75.24	0.2302	0.3502	0.5752	—	Bin *et al*., 2005
C. chekiangoleosa Hu	200-2000	96.88	0.3331	0.4966	0.3758	0.1986	

Conclusions

Analysis of the genetic differentiation, cluster, and genetic distances of *C. chekiangoleosa* Hu showed that geographic isolation formed mutually independent populations and that a high degree of differentiation occurred between populations. *Nm* was only 0.8304, smaller than 1, indicating that the general level of gene flow was relatively low. Few gene exchanges and few individual confounders were observed, even between HJC and ZTT, which were less than 100 km apart. The genetic differentiation between populations was mainly related to the limit of gene exchange between populations. As a result of the separation of hilly terrains, short insect-pollinated distance, and nutrient-rich granule seeds (which were easily picked and damaged by people and swallowed by animals), only a few seeds fell under mother plants and spread in a limited distance by gravity. The random mating between populations was also limited. The difficulty of the genetic exchange between populations caused gene loss, resulting in the obvious genetic differentiation between populations. The limited seed flow and pollen flow between *C. chekiangoleosa* Hu populations could partly explain the significant genetic differentiation between different populations. Thus, the low gene flow caused by population isolation and inbreeding was the important reason for the genetic differentiation between populations of *C. chekiangoleosa* Hu. Limited genetic flow also caused genetic differentiation between *C. chekiangoleosa* Hu populations.

Among the *C. chekiangoleosa* Hu populations, three populations in Zhejiang had a higher genetic diversity level than the other populations. The Shannon's indices of diversity (*I*) of ZKH, ZTT, and HJC were 0.3719, 0.3732, and 0.3234, respectively. This result could be explained by the fact that Gutianshan Nature Reserve, Huading Mountain, and Huangjin Village without a driveway possessed a superior environment with minimal human interference, abundant ancient trees, and large effective populations. *I* (0.2483) of AYX was the lowest; this result is in line with the fact that the AYX population, which had the smallest area and the least individual number of less than 100, was distributed like an islet with a low diversity level. As the only cultivated population among the seven populations, FXP had a history of more than 200 years, but its genetic diversity was also relatively low, with the *I* value being only 0.2583. The dense plantation and inbreeding resulted in a low genetic diversity.

These results imply that one of the strategies to protect *C. chekiangoleosa* Hu is to expand its distribution area as much as possible and to establish a continuous distribution belt to promote gene exchange between populations. Another strategy is to establish a large natural conservation zone in Kaihua, Tiantai, and Jinyun while avoiding human interference to improve the genetic diversity of *C. chekiangoleosa* Hu.

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