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Identification of a Natural Hybrid between *Castanopsis sclerophylla* and *Castanopsis tibetana* (Fagaceae) Based on Chloroplast and Nuclear DNA Sequences

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Abstract: *Castanopsis* × *kuchugouzhui*Huang et Y. T. Chang was recorded in Flora Reipublicae Popularis Sinicae (FRPS) as a hybrid species on Yuelushan mountain, but it is treated as a hybrid between *Castanopsis sclerophylla* (Lindl.) Schott. and *Castanopsis tibetana* Hance in Flora of China. After a thorough investigation on Yuelushan mountain, we found a population of *C. sclerophylla* and one individual of *C.* × *kuchugouzhui*, but no living individual of *C. tibetana*. We collected *C.* × *kuchugouzhui*, and we sampled 42 individuals of *C. sclerophylla* from Yuelushan and Xiushui and 43 individuals of *C. tibetana* from Liangyeshan and Xiushui. We used chloroplast DNA sequences and 29 nuclear microsatellite markers to investigate if *C.* × *kuchugouzhui* is a natural hybrid between *C. sclerophylla* and *C. tibetana*. The chloroplast haplotype analysis showed that *C.* × *kuchugouzhui*shared haplotype H2 with *C. sclerophylla* on Yuelushan. The STRUCTURE analysis identified two distinct genetic pools that corresponded well to *C. sclerophylla* and *C. tibetana*, revealing the genetic admixture of *C.* × *kuchugouzhui*. Furthermore, the NewHybrids analysis suggested that *C.* × *kuchugouzhui*is an F2 hybrid between *C. sclerophylla* and *C. tibetana*. Our results confirm that *C.* × *kuchugouzhui*recorded in FRPS is a rare hybrid between *C. sclerophylla* and *C. tibetana*.

Keywords: *Castanopsis* × *kuchugouzhui*; natural hybrid; molecular identification; chloroplast DNA sequence; microsatellite

1. Introduction

Hybridization is a process through which there is interbreeding of individuals from two genetically distinct populations or species [1]. It was estimated that at least 25% of plant species, mostly the youngest species, were involved in hybridization and potential introgression with other species [2]. A large number of studies showed that natural hybridization is ubiquitous in different taxa [3–6], and it plays a significant role in generating genetic diversity, even the origin of new ecotypes or species [7–10]. Research on natural hybridization has become a hot spot in the field of plant systematics and evolution in recent years [11–13].

Hybrid identification is the first step in exploring the intricate evolutionary history of natural hybridization. The morphological characteristics of natural hybrids are usually intermediate or more similar to one of their parents, and they form a gradually morphological transition that often causes the blurred and indistinguishable boundary of the species [14]. Chloroplast DNA is uniparentally inherited (maternal inheritance in most angiosperm) and nuclear DNA is biparentally inherited; thus, a comparative analysis of nuclear DNA and chloroplast DNA would provide complementary and often

contrasting information on the genetic structure and phylogenies. Interspecific hybrids are commonly identified by cytonuclear discordance that may indicate different parental contribution to the hybrid genome [4,15–17].

Castanopsis sclerophylla (Lindl.) Schott. and *Castanopsis tibetana* Hance are dominant species in mid-subtropical evergreen broad-leaved forest in China. *Castanopsis sclerophylla* is mainly distributed in the north of Nanling mountain, south of the Yangtze River, and east of Sichuan and Guizhou provinces [18]. *Castanopsis tibetana* is widely distributed in subtropical China and overlaps with the range of *C. sclerophylla*. *Castanopsis tibetana* grows together with *C. sclerophylla* in a forest on Yuelushan mountain of Changsha City in Hunan Province, where a specimen of *Castanopsis* × *kuchugouzhui* Huang et Y. T. Chang was collected according to Flora Reipublicae Popularis Sinicae (FRPS) [18]. The leaves and cupules of *C.* × *kuchugouzhui* show intermediate morphologies between *C. sclerophylla* and *C. tibetana*; thus, it is recognized as a hybrid species in FRPS [18]. However, *C.* × *kuchugouzhui* is assumed to be a putative natural hybrid between *C. sclerophylla* and *C. tibetana* in Flora of China [19]. Up to now, there is no molecular evidence for this putative hybrid. The purpose of this study was to verify whether *C.* × *kuchugouzhui* is a natural hybrid between *C. sclerophylla* and *C. tibetana* by using chloroplast DNA sequences and nuclear microsatellite markers.

2. Materials and Methods

2.1. Plant Materials and DNA Extraction

After a thorough investigation of this forest on Yuelushan mountain in 2017–2019, we found a population of *C. sclerophylla* and one individual of *C.* × *kuchugouzhui*, but no living individual of *C. tibetana*. According to the information obtained from Yuelushan Mountain Scenic Area Administration Bureau and Hunan Normal University, this *C.* × *kuchugouzhui* is more than 100 years old and it is the same individual reported by FRPS. We sampled *C.* × *kuchugouzhui* and 20 individuals of *C. sclerophylla* on Yuelushan. We further sampled 22 individuals of *C. sclerophylla* and 19 individuals of *C. tibetana* from Xiushui, as well as 24 individuals of *C. tibetana* from Liangyeshan (Table 1). For each population, eight to 10 fresh leaves per tree were chosen and quickly dried with silica gel, and the individuals were sampled at least 20 m apart from each other. Voucher specimens were made for *C.* × *kuchugouzhui* and each population, and they were stored in the Dendrological Herbarium of South China Agricultural University (CANT).

Table 1. Location, sample size, and genetic diversity of the investigated populations.

Species	Location/Population Code	Latitude (N)	Longitude (E)	Sample Size	*A*	A_R	*H*	F_{IS}
Castanopsis sclerophylla	Xiushui/KZ-XS	28°55′23″	114°43′12″	22	5.6	5.426	0.629	0.103 *
	Yuelushan/KZ-YLS	28°10′49″	112°56′28″	20	5.5	5.430	0.580	0.052
	Average			21	5.6	5.428	0.605	0.078
Castanopsis × *kuchugouzhui*	Yuelushan/KZGK-YLS	28°10′49″	112°56′28″	1				
Castanopsis tibetana	Liangyeshan/GK-LYS	25°12′35″	116°10′48″	24	3.3	3.219	0.481	−0.057
	Xiushui/GK-XS	28°55′23″	114°43′12″	19	2.9	2.862	0.427	−0.068
	Average			22	3.1	3.041	0.454	−0.063

N: north, E: east, *A*: total number of alleles detected, A_R: allelic richness for 19 diploid individuals, *H*: gene diversity, F_{IS}: fixation index. * Deviated from Hardy–Weinberg equilibrium significantly ($p < 0.01$).

Total genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The quality and concentration of the genomic DNA were evaluated by electrophoresis on 1% agarose gels and NanoDropTM 2000 Spectrophotometers (Thermo Scientific, Waltham, MA, USA).

2.2. Chloroplast DNA Sequencing and Nuclear Microsatellite Genotyping

The chloroplast DNA of psbA-trnH and trnM-trnV intergenic spacers was amplified and sequenced with primers described in References [20,21]. The total volume of the polymerase chain reaction (PCR)

was 30 µL, which contained 1 × ES Tag Master Mix (Cwbiotech, Beijing, China), 0.2 µM each of forward and reverse primers, and 20 ng of DNA. PCR amplification was conducted for 33 cycles in a TaKaRa PCR Thermal Cycler Dice™ Gradient (TP600) (TAKARA, Kyoto, Japan). Each cycle included 45 s at 95 °C, 45 s at annealing temperature, and 45 s at 72 °C. An initial pre-denaturation for 5 min at 95 °C and a final extension for 7 min at 72 °C were added.

Polymorphic nuclear microsatellites were screened from 173 simple sequence repeat (SSR) markers originally developed in *C. sclerophylla*, *Castanopsis fargesii*, *Castanopsis sieboldii*, *Castanopsis tribuloides*, *Castanea sativa*, and *Castanea mollissima* [22–28]. One sample in each population and *C.* × *kuchugouzhui* were used in a preliminary experiment to test SSR amplification. PCR was performed in a total volume of 10 µL that consisted of 1 × ES Tag Master Mix, 0.5 µM each of forward and reverse primers, and 20 ng of DNA. The PCR program was the same as above apart from the annealing temperature. PCR products were separated by electrophoresis on 3% agarose gels.

A total of 75 pairs of primers that generated a clear electrophoretic band were applied to the multiple PCR, in which the forward primers were labeled with fluorochromes TAMRA, HEX, 6-FAM, and ROX. The primers with different fluorescence were combined, and we tried to keep their predicted products 30–50 bp apart. The Type-it Microsatellite PCR Kit (QIAGEN, Hilden, Germany) was used to prepare the multiple PCR with a total volume of 10 µL, which contained 1 × PCR Master Mix, 1 × Q-Solution, 0.2 µM each of forward and reverse primers, and 20 ng of DNA. Two samples in each population and *C.* × *kuchugouzhi* were used in this experiment. The PCR programs included an initial pre-denaturation of 5 min at 95 °C, followed by 28 cycles of 30 s at 95 °C, 90 s at 57 °C, 30 s at 72 °C, and a final extension of 30 min at 60 °C. PCR products were visualized on an ABI-3730XL fluorescence sequencer (Applied Biosystems, Foster City, CA, USA) using LIZ500 as an internal size standard. Alleles (Table S1) were scored using GeneMarker v 2.2.0 [29]. Finally, 32 pairs of primers with high polymorphism and stability were applied to genotype all samples.

2.3. Data Analyses

DNA sequences of psbA-trnH (GenBank accession numbers: MT635060-MT635092) and trnM-trnV (GenBank accession numbers: MT635093-MT635125) were manually checked using BioEdit [30]. Multiple alignments were carried out using MEGA v 7 [31] with *Castanopsis fabri* (GenBank accession numbers: MF592976, MF592882) as the outgroup. Haplotypes were retrieved using DnaSP v 6.12.03 [32], and a reduced median-joining network was constructed using NETWORK v 5.0 [33] to infer haplotype relationships.

Genetic diversity parameters including number of alleles detected (A), allelic richness (A_R), gene diversity (H), observed heterozygosity (H_O), gene diversity within populations (H_S), gene diversity in total population (H_T), fixation index (F_{IS}), and genetic differentiation among populations (F_{ST}) under an infinite allele model were calculated per locus using FSTAT v 2.9.4 [34]. The Hardy–Weinberg equilibrium was tested by permuting alleles and comparing the fixation index calculated from randomized datasets to that obtained from the observed dataset; p-values were subjected to Bonferroni correction for multiple comparisons. Three loci significantly deviated from the Hardy–Weinberg equilibrium both in *C. sclerophylla* and in *C. tibetana*; they were excluded from all subsequent analyses.

Genetic differentiation and exchange of *C. sclerophylla* and *C. tibetana* were assessed with a model-based Bayesian clustering method implemented in the program STRUCTURE v 2.3.4 [35] by choosing the admixture model and correlated allele frequencies between populations. Ten independent runs were conducted for each K value (from 1 to 5) with 100,000 MCMC (Markov chain Monte Carlo) iterations after 50,000 burn-in period. The optimal number of clusters (K) was determined through the statistic ΔK based on the second-order rate of change in the log probability of data between successive K values [36]. The average matrix of ancestry membership proportions was calculated over the 10 runs using CLUMPP v 1.1.2 [37].

Each individual was assigned to a genotype category with posterior probability by using the program NewHybrids v 1.1 beta [38]. We considered six genotype categories: Parent 1, Parent 2,

F1 hybrids, F2 hybrids, backcross generation to Parent 1, and backcross generation to Parent 2 [39]. The analysis was run for 100,000 rounds after a burn-in of 50,000 iterations.

3. Results

3.1. Chloroplatst DNA Variation

After alignment using *C. fabri* as outgroup, the total length of chloroplast sequences was 1158 bp, from which seven variable sites and six haplotypes were identified. The sequence variants and their positions are shown in Table 2. Within the lineage of *C. sclerophylla*/*C. tibetana*, five variable sites and five haplotypes were identified. Haplotypes H1 and H2 were detected in populations of *C. sclerophylla*, while haplotypes H3, H4, and H5 were found only in *C. tibetana*. *Castanopsis* × *kuchugouzhui* shared haplotype H2 with *C. sclerophylla* at the Yuelushan. Population GK-XS possessed two haplotypes H4 and H5, while the other populations were fixed for one unique haplotype. There was no shared common haplotype between *C. sclerophylla* and *C. tibetana*, nor between *C.* × *kuchugouzhui* and *C. tibetana*. Relationships among haplotypes are shown in Figure 1. Haplotype H2 was closely related to H1, and they diverged from haplotypes H3, H4, and H5, which constituted a haplogroup.

Table 2. DNA sequence variation and chloroplast haplotypes revealed in this study.

Species	Population Code	Haplotype	Frequency	Variable Site						
				psbA-trnH			trnM-trnV			
				100	258	284	478	491	672	892
Castanopsis sclerophylla	KZ-XS	H1	8	C	T	A	C	C	G	C
	KZ-YLS	H2	8	C	T	A	C	A	G	C
Castanopsis × *kuchugouzhui*	KZGK-YLS	H2	1	C	T	A	C	A	G	C
Castanopsis tibetana	GK-LYS	H3	8	C	T	A	C	C	T	A
	GK-XS	H4	7	C	T	A	A	C	T	A
		H5	1	C	C	A	C	C	T	A
Castanopsis fabri	outgroup	H6	1	T	T	T	C	C	G	C

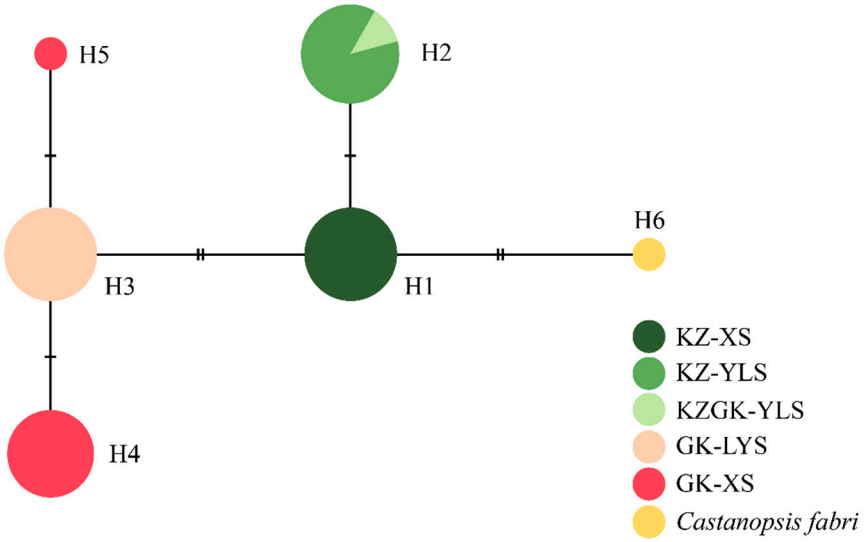

Figure 1. Haplotype relationships shown on median-joining network. The size of each circle is proportional to the haplotype frequency. Mutational steps between the haplotypes are indicated on the line. Population codes are the same as in Table 1.

3.2. Genetic Diversity at Nuclear Microsatellite Loci

In *C. sclerophylla*, a total of 195 alleles were revealed at 29 nuclear microsatellite loci (Table 3). Per locus, the number of alleles ranged from two to 15, and the observed heterozygosity (H_O) varied

from 0.143 to 0.932. The within-population gene diversity (H_S) and the overall gene diversity (H_T) ranged from 0.286–0.900 and from 0.312–0.907, respectively. Over the 29 loci, the values of F_{IS} and F_{ST} were 0.080 and 0.053, respectively. Five of 29 loci significantly deviated from the Hardy–Weinberg equilibrium ($p < 0.01$).

Table 3. Genetic diversity at 29 nuclear microsatellite loci in *Castanopsis sclerophylla*.

Locus	Allele Size	*A*	H_O	H_S	H_T	F_{IS}	F_{ST}
CC-30080	151–163	5	0.473	0.549	0.545	0.132	−0.013
CC-33079-1	189–209	7	0.259	0.468	0.546	0.455 *	0.244
CC-935	131–149	6	0.668	0.662	0.669	−0.011	0.022
CsCAT34	149–161	5	0.475	0.486	0.521	0.026	0.128
CC-14826	228–244	9	0.423	0.461	0.462	0.079	0.006
CC-2994	269–272	2	0.150	0.498	0.499	0.714 *	0.004
CC-3722	131–145	5	0.548	0.570	0.598	0.042	0.087
CC-6538	169–185	6	0.734	0.713	0.727	−0.029	0.037
CC-4950	247–283	15	0.693	0.900	0.907	0.233 *	0.016
CcC02022	351–375	11	0.764	0.795	0.807	0.040	0.029
CFA22	156–162	3	0.186	0.286	0.312	0.354	0.150
CC-25032	176–194	5	0.143	0.454	0.455	0.688 *	0.002
CC-41284	271–289	10	0.759	0.825	0.862	0.073	0.081
CS05	156–178	7	0.764	0.741	0.744	−0.025	0.008
CC-43042	186–210	6	0.464	0.582	0.622	0.215	0.120
CFA71	174–204	9	0.809	0.840	0.845	0.037	0.011
CC-42621	288–297	3	0.366	0.308	0.319	−0.183	0.064
CsCAT14	124–154	15	0.932	0.856	0.865	−0.087	0.020
CC-4562	144–156	3	0.782	0.657	0.661	−0.194	0.011
CC-26213	122–152	4	0.505	0.619	0.637	0.190	0.055
CFA61	184–212	11	0.761	0.601	0.601	−0.268	0.001
CS24	123–144	7	0.784	0.730	0.725	−0.077	−0.015
CS43	148–168	7	0.755	0.702	0.718	−0.079	0.042
CT161	148–157	4	0.395	0.362	0.388	−0.094	0.124
CT128	163–202	4	0.455	0.456	0.480	−0.003	0.099
CS20	249–273	8	0.741	0.728	0.780	−0.024	0.128
CS44	135–147	7	0.748	0.797	0.807	0.050	0.025
CC-33079	243–259	6	0.310	0.370	0.387	0.171	0.081
CC-4323	231–249	5	0.309	0.521	0.543	0.411 *	0.077
Mean		6.7	0.557	0.605	0.622	0.080	0.053

A: total number of alleles detected, H_O: observed heterozygosity, H_S: gene diversity within populations, H_T: gene diversity in total population, F_{IS}: fixation index (a coefficient based on the difference among observed and expected heterozygosity), F_{ST}: genetic differentiation among populations (a coefficient based on the difference among expected heterozygosity within populations and expected heterozygosity in the species). * Deviated from Hardy–Weinberg equilibrium significantly ($p < 0.01$).

In *C. tibetana*, a total of 115 alleles were revealed at 29 nuclear microsatellite markers (Table 4). Per locus, the number of alleles ranged from two to nine, and the observed heterozygosity (H_O) varied from 0.021 to 0.921. The within-population gene diversity (H_S) and the overall gene diversity (H_T) ranged from 0.155–0.746 and from 0.171–0.786, respectively. Over the 29 loci, the values of F_{IS} and F_{ST} were −0.061 and 0.204, respectively. Five of 29 loci significantly deviated from the Hardy–Weinberg equilibrium ($p < 0.01$).

Table 4. Genetic diversity at 29 nuclear microsatellite loci in *Castanopsis tibetana*.

Locus	Allele Size	A	H_O	H_S	H_T	F_{IS}	F_{ST}
CC-30080	148–166	7	0.465	0.680	0.722	0.268 *	0.113
CC-33079-1	197–205	3	0.644	0.495	0.547	−0.293	0.173
CC-935	131–133	2	0.372	0.356	0.356	−0.030	−0.002
CsCAT34	147–153	3	0.530	0.417	0.419	−0.246	0.008
CC-14826	226–244	6	0.702	0.614	0.734	−0.135	0.281
CC-2994	266–275	2	0.404	0.353	0.350	−0.126	−0.019
CC-3722	137–141	3	0.721	0.573	0.598	−0.275	0.079
CC-6538	161–187	7	0.627	0.547	0.711	−0.150	0.366
CC-4950	247–257	2	0.503	0.469	0.470	−0.049	0.003
CcC02022	353–361	5	0.753	0.697	0.731	−0.088	0.086
CFA22	156–158	2	0.342	0.228	0.285	−0.502	0.363
CC-25032	182–194	2	0.921	0.495	0.498	−0.876	0.010
CC-41284	271–273	2	0.419	0.361	0.503	−0.152	0.439
CS05	156–160	3	0.021	0.163	0.175	0.872 *	0.115
CC-43042	204–240	3	0.219	0.196	0.199	−0.125	0.032
CFA71	184–214	8	0.702	0.663	0.786	−0.045	0.269
CC-42621	291–294	2	0.354	0.231	0.439	−0.532	0.616
CsCAT14	130–138	2	0.167	0.194	0.198	0.089	0.037
CC-4562	144–168	6	0.659	0.575	0.772	−0.132 *	0.398
CC-26213	137–157	3	0.346	0.680	0.673	0.521 *	−0.021
CFA61	184–196	3	0.246	0.221	0.222	−0.113	0.004
CS24	117–144	9	0.602	0.640	0.683	0.042	0.119
CS43	144–164	7	0.791	0.746	0.765	−0.066	0.049
CT161	145–148	2	0.414	0.320	0.331	−0.279	0.066
CT128	181–199	6	0.451	0.468	0.722	0.027 *	0.528
CS20	239–253	7	0.555	0.672	0.707	0.171	0.095
CS44	137–141	3	0.325	0.508	0.513	0.363	0.020
CC-33079	247–257	3	0.539	0.448	0.591	−0.183	0.387
CC-4323	237–240	2	0.188	0.155	0.171	−0.208	0.156
Mean		4.0	0.482	0.454	0.513	−0.061	0.204

A: total number of alleles detected, H_O: observed heterozygosity, H_S: gene diversity within populations, H_T: gene diversity in total population, F_{IS}: fixation index (a coefficient based on the difference among observed and expected heterozygosity), F_{ST}: genetic differentiation among populations (a coefficient based on the difference among expected heterozygosity within populations and expected heterozygosity in the species). * Deviated from Hardy–Weinberg equilibrium significantly ($p < 0.01$).

Genetic diversity within populations is summarized in Table 1. *Castanopsis sclerophylla* exhibited a higher level of genetic diversity within population when compared to *C. tibetana*. At the population level, the average values of the number of alleles (A), allelic richness (A_R), and gene diversity (H) were 5.6, 5.428, and 0.605 in *C. sclerophylla*, but 3.1, 3.041, and 0.454 in *C. tibetana*. Population KZ-XS harbored the highest genetic diversity (A = 5.6, A_R = 5.426, and H = 0.629), while population GK-XS showed the lowest genetic diversity (A = 2.9, A_R = 2.862, and H = 0.427). The fixation index (F_{IS}) was 0.078 in *C. sclerophylla*, but −0.063 in *C. tibetana*. The population KZ-XS significantly ($p < 0.01$) deviated from the Hardy–Weinberg equilibrium across 29 loci.

3.3. STRUCTURE and NewHybrids Analyses Based on Nuclear Microsatellite Markers

In the STRUCTURE analysis (Figure 2A), the optimal K-value was found to be 2, indicating that all individuals sampled were assigned to two genetic clusters. One cluster corresponded to *C. sclerophylla* (42 individuals with cluster membership >0.993) and the other corresponded to *C. tibetana* (43 individuals with cluster membership >0.996). *Castanopsis* × *kuchugouzhui* showed genetic admixture with proportions of 0.606 and 0.394 for each cluster that appeared to be "heterozygous" with alleles inherited from *C. sclerophylla* and *C. tibetana*, suggesting that it could be a hybrid offspring of the two species. In the NewHybrids analysis (Figure 2B), 86 sampled individuals were clearly assigned to three genotype categories with high posterior probabilities (>0.999). Forty-two individuals of

C. sclerophylla and 43 individuals of *C. tibetana* were assigned to Parent 1 and Parent 2, respectively. *Castanopsis* × *kuchugouzhui* was classified as an F2 hybrid between *C. sclerophylla* and *C. tibetana*.

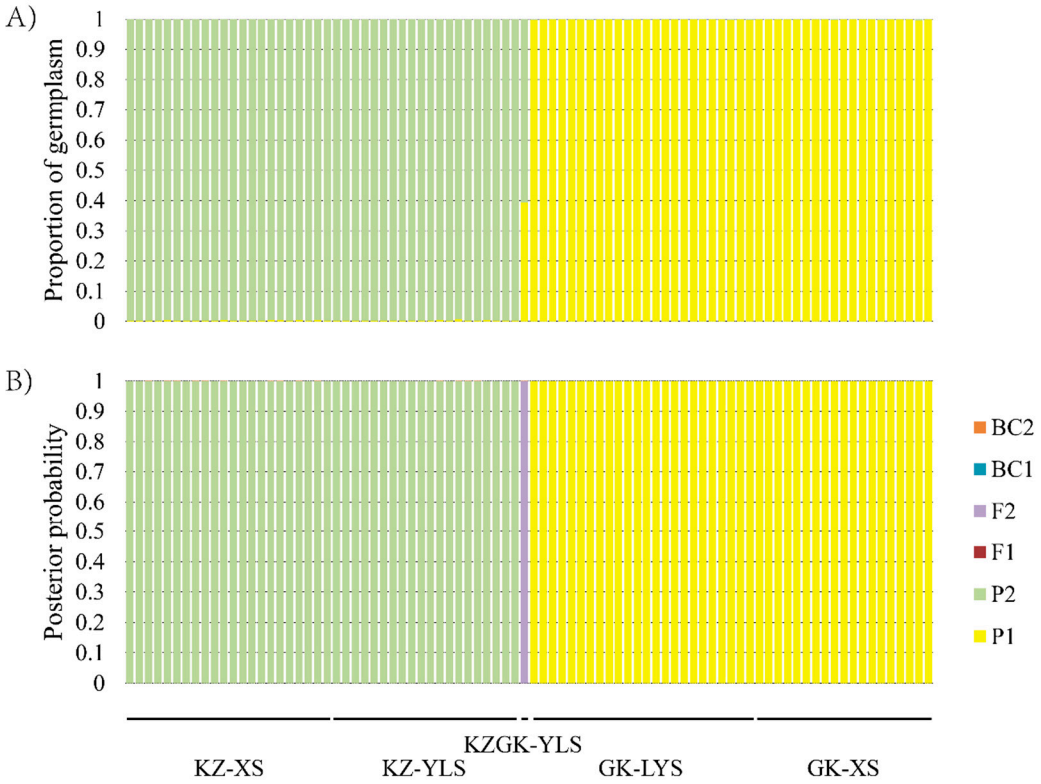

Figure 2. (**A**) Genetic admixture coefficient of the samples; (**B**) posterior probability of genotype category evaluated based on nuclear microsatellite markers. Population codes are the same as in Table 1.

4. Discussion

4.1. SSR Transferability among Castanopsis Species

The ability to transfer SSRs among species is related to the level of divergence between the species; a closer relationship, denotes higher transferability of the primers [40]. Ye et al. [23] screened 51 microsatellite markers originally developed from four *Castanopsis* species (*C. sclerophylla*, *Castanopsis chinensis*, *Castanopsis cuspidata*, and *C. sieboldii*) and found that 68.6% of SSR primer pairs successfully cross-amplified and 31.4% were polymorphic in *C. tibetana*. Li et al. [41] tested 124 EST(expressed sequence tags)-SSRs originally developed from *Castanea mollissima* and found that 42.7% of *C. mollissima* EST-SSR primers successfully cross-amplified and 56.6% showed polymorphism in *Castanopsis fargesii*. In this study, we screened 31 SSRs originally developed from *Castanopsis* species and 142 SSRs originally developed from *Castanea* species. We found that 80.6% of *Castanopsis* SSRs successfully cross-amplified in both *C. tibetana* and *C. sclerophylla*, and 52% showed polymorphism. In contrast, 35.2% of *Castanea* SSRs cross-amplified in both *C. tibetana* and *C. sclerophylla*, and 38% were polymorphic. These results were consistent with the expectation that successful cross-species amplification among closely related genera appears to be much lower than that within genera [40]. The moderate to very high cross-species transferability of SSRs among *Castanopsis* species implies that the species in this genus have more similar genetic makeup and may not be completely reproductively isolated; thus, there is a chance of natural hybridization between species in this genus.

4.2. Genetic Diversity of C. sclerophylla and C. tibetana

Genetic diversity is essential for populations to adapt to environmental change. Large populations of naturally outbreeding species usually have extensive genetic diversity, but it is generally reduced in small populations and endangered species. Habitat fragmentation caused by human interference would reduce the population size and increase the spatial isolation. Such changes will be accompanied by an erosion of genetic variation and an increase of inter-population genetic divergence due to increased genetic drift, elevated inbreeding, and reduced gene flow [42].

In the present study, moderate genetic variation was found in *C. sclerophylla*; the average number of alleles per locus was 6.7, and the mean observed heterozygosity was 0.557. The level of genetic diversity of *C. sclerophylla* was similar to that reported in other closely related species such as *C. fargesii* (A = 6.7, H_O = 0.690) [24], *Castanopsis acuminatissima* (A = 10.8, H_O = 0.517) [43], and *C. sieboldii* (A = 5.2, H_O = 0.563) [44]. Compared with the species above, a lower level of genetic diversity was observed in *C. tibetana* (A = 4.0, H_O = 0.482). The genetic diversity of *C. tibetana* may be greatly affected by habitat fragmentation and human interference given that *C. tibetana* was destroyed out on Yuelushan mountain and *C. tibetana* exhibited higher genetic differentiation than *C. sclerophylla*. Expanding the sampling of *C. tibetana* is required to examine how habitat fragmentation impacted the genetic diversity of this species.

4.3. Molecular Evidence of Natural Hybrid and Taxonomic Status for C. × kuchugouzhui

The genetic structure analyses based on nuclear microsatellite markers show a clear genetic differentiation between *C. sclerophylla* and *C. tibetana*, and two genetic clusters correspond well to the two species. The fact that *C.* × *kuchugouzhui* shows genetic admixture with proportions of 0.606 and 0.394 for each cluster indicated that it is a hybrid between *C. sclerophylla* and *C. tibetana*. Natural hybridization between *C. sclerophylla* and *C. tibetana* could be attributed to a full overlap in the flowering phenology of the two species that usually flower from April to May. *Castanopsis sclerophylla* is supposed to be the maternal parent of *C.* × *kuchugouzhui* since it shared with *C. sclerophylla* a common haplotype (H2) that is maternally inherited. *Castanopsis sclerophylla* inhabits lower elevation and would have a great chance of receiving pollen from *C. tibetana* that occupies higher elevation. *C.* × *kuchugouzhui* was assigned to an F2 hybrid between *C. sclerophylla* and *C. tibetana* with very high posterior probabilities in the NewHybrids analysis. However, the possible hybrid category that could occur after up to four generations of crossing between the two parent species was allowed [39]. The last specimen of *C. tibetana* on Yuelushan mountain was collected in 1977. Given that *C.* × *kuchugouzhui* is more than 100 years old, we can be sure that the hybridization event to form this hybrid occurred before *C. tibetana* disappeared from Yuelushan mountain. At the time of the presumable F1 hybrid formation, no other *Castanopsis* species was present in the region but *C. fargesii* and a preliminary test by four SSRs did not mark any gene flow from this species to *C.* × *kuchugouzhui* (data not shown). Because the dispersal distance of seeds and pollens of *Castanopsis* species is very limited [45–47], it is almost impossible that the formation of *C.* × *kuchugouzhui* was due to a hybrid seed dispersed from elsewhere or was contributed to by pollen of *C. tibetana* elsewhere.

The leaf and cupule morphologies of *C.* × *kuchugouzhui* were intermediate between *C. sclerophylla* and *C. tibetana* [18], and *C.* × *kuchugouzhui* showed mixed genetic characteristics between *C. sclerophylla* and *C. tibetana*. However, there is only one record of *C.* × *kuchugouzhui* up to now, and we did not identify natural hybridization elsewhere, such as in Xiushui, where the two species coexisted together. These facts suggest that natural hybridization between *C. sclerophylla* and *C. tibetana* is a very rare event. This is consistent with our expectation since the hybrid offspring between two species will suffer deleterious consequences termed outbreeding depression. The hybrid offspring in the F1 and subsequent generation will be rapidly eliminated by natural selection due to their minor fitness [48]. Therefore, instead of listing as a separate species, *C.* × *kuchugouzhui* should be comprehensively treated as a natural hybrid between *C. sclerophylla* and *C. tibetana*.

In recent years, *C. tibetana* on Yuelushan mountain disappeared due to serious disturbance from human beings such as tourism development. According to our investigation, *C. sclerophylla* inhabits lower elevations of approximately 200–1000 m and favors plenty sunshine, while *C. tibetana* generally prefers to grow in humid conditions at slightly higher elevations. These facts indicate that *C. sclerophylla* and *C. tibetana* have some differentiation in ecological niche occupation. The individuals of the two species seldom grow together just like they do on Yuelushan mountain and Xiushui county, although they could be found in the same forest communities. In this study, only one hybrid individual was corroborated. The very rare natural hybrid between *C. sclerophylla* and *C. tibetana* may imply that the two species have strong but not complete reproductive isolation, which may be caused by their ecological differentiation. Natural hybridization will generate new genotypes and increase genetic diversity, which is important for trees to adapt new environments in the face of rapid global change; thus, this new genotype of *C.* × *kuchugouzhui* is worth conserving as an important germplasm.

5. Conclusions

In this study, we provided compelling evidence for the natural hybrid of *C.* × *kuchugouzhui* using chloroplast DNA sequences and 29 nuclear microsatellite markers. *Castanopsis* × *kuchugouzhui* is a very rare event of natural hybridization, where introgression occurred between *C. sclerophylla* and *C. tibetana*. The genetic analysis of this rare natural hybrid is very helpful for us to understand the genetic differentiation and gene exchange between *Castanopsis* species.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/8/873/s1: Table S1. Alleles at 29 loci used for STRUCTURE and NewHybrids analysis.

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