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Heterozygosities and genetic relationship of tea cultivars revealed by simple sequence repeat markers and implications for breeding and genetic mapping programs

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ABSTRACT. Genetic maps are essential tools for quantitative trait locus analysis and marker-assisted selection breeding. In order to select parents that are highly heterozygous for genetic mapping, the heterozygosity (H_S) of 24 tea cultivars (*Camellia sinensis*) was analyzed with 72 simple sequence repeat markers. In total, 359 alleles were obtained with an average of 4.99 per marker. The H_S varied greatly from 37.5 to 71.0% with an average of 51.3%. On average, tea cultivars from Fujian Province showed a higher level of heterozygosity (59.8%) than those from Zhejiang (48.5%) and Yunnan (44.5%), and the 12 national tea cultivars were generally more heterozygous than the 12 provincial cultivars. Unweighted pair-group analysis using the arithmetic average grouping divided the 24 cultivars into 2 groups that are consistent with the morphological classification. All dual

combinations of the 24 cultivars were studied to calculate the percentage of mappable markers when using pseudo-testcross mapping strategy, and results showed that this value also varied greatly from 51.4 to 90.3%. The genetic relationships and H_S differences among different cultivars were discussed, and tea cultivars with high H_S were recommended as cross parents for genetic mapping programs.

Key words: Tea cultivars; Heterozygosity; Genetic mapping; Simple sequence repeat marker

INTRODUCTION

Tea is a ubiquitous commodity worldwide. In 2012, 3.28 million hectares of tea plant fields were harvested, producing 4.82 million tons of tea (FAO, http://faostat.fao.org/). China has a long tea-growing history; today, 20 of the 34 provinces of China cultivate the tea plant (Yao et al., 2012). In the past 30 years, 124 national tea cultivars (NTCs) have been released to famers in China, and a greater number of provincial tea cultivars (PTCs) have been registered by each tea-growing province (Chen et al., 2007). Investigations of these cultivars with DNA markers may provide useful information for future breeding programs.

Genetic maps are essential tools for implementing quantitative trait locus analysis and marker-assisted selection breeding in the 21st century (Collard and Mackill, 2008). The tea plant [*Camellia sinensis* (L.) O. Kuntze], is a perennial diploid (2n = 30) wood plant characterized by a large genome (~4 Gb) that is self-incompatible and highly heterozygous (Tanaka et al., 2006; Yao et al., 2012). The most used mapping strategy for this kind of plant is the pseudo-testcross strategy that is based on F_1 populations, and genetic markers should be heterozygous in at least one of the two cross parents (Grattapaglia and Sederoff, 1994). Theoretically, using a pair of parents with a high degree of heterozygosity could map a large number of markers in a single F_1 population and thus reduce the marker-screening cost.

Simple sequence repeat (SSR) or microsatellite markers are very attractive for genetic mapping and other studies because of the following features: codominant, multiple alleles, and high transferability among related species (Schlötterer, 2004). More than 1000 SSR markers have been developed for *C. sinensis* (Freeman et al., 2004; Zhao et al., 2008; Sharma et al., 2009, 2011; Fang et al., 2012; Ma et al., 2010, 2012; Taniguchi et al., 2012a; Yao et al., 2012), and a few SSR-based maps have been reported (Taniguchi et al., 2012b; Tan et al., 2013). In these studies, the average observed heterozygosity (H_O) of SSR markers varied greatly depending on the marker-developing strategies and plant materials assayed. However, the heterozygosities of different cultivars or germplasms, hereinafter denoted as H_S, were seldom discussed. Whether and to what extent the H_S changes in different tea cultivars and among different tea-growing areas and how its value affects the percentage of mappable markers in a certain F1 mapping population are also unknown. We will adress these questions in this paper.

MATERIAL AND METHODS

Plant materials

A collection of 24 tea cultivars that mainly originated from 3 tea-growing provinces

of China was used in this study (Table 1). Among them, 12 were NTCs and the other 12 were PTCs. All of them were landrace or field clone as described by Chen et al. (2007). The young shoots with two leaves and a bud of each cultivar were sampled at the China National Germplasm Hangzhou Tea Repository (Hangzhou, China). The samples were immediately snap frozen in liquid nitrogen and stored at -70°C prior to DNA extraction. Genomic DNA was extracted from the ground tissues using the cetyltrimethylammonium bromide method.

Table 1. Twenty-four tea cultivars used in this study.

No.	Name	Origin	Level	No.	Name	Origin	Level
1	Changyebaihao	Yunnan	PTC	13	Pingyangtezao	Zhejiang	PTC
2	Fudingdabaicha	Fujian	NTC	14	Fuandabaicha	Fujian	NTC
3	Baihaozao	Hunan	NTC	15	Meizhan	Fujian	NTC
4	Longjin43	Zhejiang	NTC	16	Fujianshuixian	Fujian	NTC
5	Wuniuzao	Zhejiang	PTC	17	Rougui	Fujian	PTC
6	Huangyan	Fujian	NTC	18	Foshou	Fujian	PTC
7	Tieguanyin	Fujian	NTC	19	Yunkang14	Yunnan	NTC
8	Mingshanbaihao	Sichuan	NTC	20	Yunkang43	Yunnan	PTC
9	Zhongcha102	Zhejiang	NTC	21	Yunkang27	Yunnan	PTC
10	Shuigucha	Zhejiang	PTC	22	Yunkang10	Yunnan	NTC
11	Huangyezao	Zhejiang,	PTC	23	Yunxuan9	Yunnan	PTC
12	Anjibaicha	Zhejiang	PTC	24	Yungui	Yunnan	PTC

NTC: national tea cultivar; PTC: provincial tea cultivar (also see Chen et al., 2007).

SSR markers and amplification

Seventy-two SSR markers developed by our group previously were used in this study (Table 2; Tan et al., 2013). Polymerase chain reaction (PCR) amplifications were performed in 10-µL reaction mixtures containing 1.0 µL 10X PCR buffer, 0.2 µL 10 mM dNTPs, 0.2 µL 10 µM of each primer, 0.5 U Taq polymerase (TaKaRa, Dalian, China) and 20 ng template DNA. Thermocycling conditions were as follows: 1) initial denaturation at 94°C for 4 min; 2) 35 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C; and 3) a final extension at 72°C for 10 min. PCR products were resolved on 10% polyacrylamide gels at 150 V for 90 min and visualized by silver staining.

Data analysis

Distinct alleles of the expected size of each marker were scored. The genetic parameters of these markers, including the number of alleles (N_A), H_O, Nei's gene diversity (H), and polymorphism information content (PIC) were estimated by PowerMarker (Liu and Muse, 2005). Nei's genetic identity and genetic distance between two cultivars were calculated by the POPGene program (Yeh and Boyle, 1997). The dendrogram based on unweighted pair-group method using the arithmetic average (UPGMA) was also drawn by POPGene and viewed by MEGA 4.0 (Tamura et al., 2007). The H_S of each cultivar was estimated as N_H / N x 100%, where N_H is the number of heterozygous markers and N is the total assayed marker number. The percentage of mappable markers (P_M) of each putative parental combination was estimated as (1 - N_O / N) x 100%, where N_O is the number of markers that are homozygous in both parents of that combination.

RESULTS

Marker polymorphism

A total of 359 alleles were detected by the 72 SSR markers (Figure 1). On average, there were 4.99 alleles per locus, and 2 (CsMF1176, CsMF1177, CsMF1256, and CsFM1268) to 12 (CsFM1131) alleles were scored for each marker. The PIC values ranged from 0.042 (CsFM1176) to 0.880 (CsFM1131) with an average of 0.584. The average H_O and H for all markers tested were 0.513 and 0.628, respectively. These values indicated that the 72 SSR markers had a high level of polymorphism, and detailed information for each marker is available in Table 2.

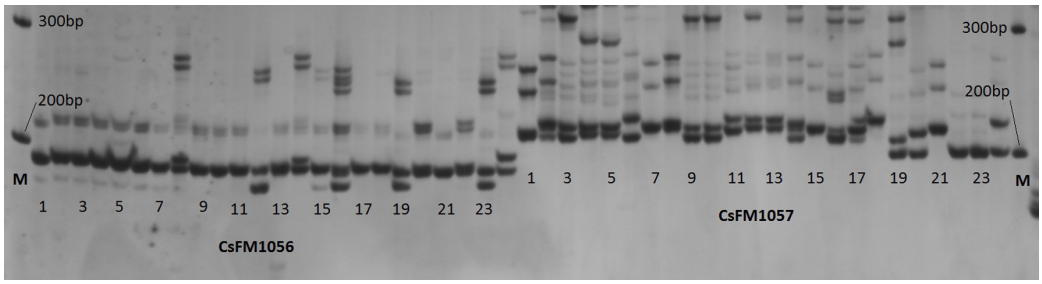

Figure 1. Simple sequence repeat (SSR) allele patterns amplified by CsFM1056 and CsFM1057 in 24 tea cultivars. Polymerase chain reaction (PCR) products were resolved on 10% polyacrylamide gels and visualized by silver staining. The cultivar names of *lanes 1-24* are shown in Table 1. *Lane M*: DNA ladder.

Genetic relationships and clustering analysis

The highest Nei's genetic identity (data not shown) was found between cultivars Yunkang27 and Yunxuan9 (0.674), while the lowest identity was found between Changyebaihao and Huangyezao (0.290). The 7 cultivars from Yunnan showed a high level of identity with each other, which ranged from 0.524 to 0.674. Among the cultivars that are not from Yunnan, the genetic identity between Fudingdabaicha from Fujian and Baihaozao from Hunan was the highest (0.655); this was followed by Meizhan and Rougui (0.612, both from Fujian) and Wuniuzao and Anjibaicha (0.594, both from Zhejiang).

The 24 cultivars were divided into two groups by the UPGMA based on Nei's genetic distances: the 7 cultivars from Yunnan were grouped together and the other 17 were assigned to another group (Figure 2). This result is consistent with the morphological classification. The cultivars from Yunnan belong to *C. sinensis* var. *assamica* that is characterized by semi-arbor trees and large leaves, while the other 17 cultivars belong to *C. sinensis* var. *sinensis* that is characterized by shrub trees and small- or moderate-sized leaves (Yao et al., 2012).

H_S of cultivars

The H_S of each cultivar was defined as the percentage of the heterozygous loci in all loci tested .In this study, the H_S ranged from 37.5 to 71.0%, with an average of 51.3% (Table 3). The lowest H_S was observed for Changyebaihao (37.5%) and was followed by Anjibaicha,

Yunkang27, Yunxuan9, and Yungui (all 40.3%). The highest H_S (71.0%) was scored by Fuandabaicha from Fujian Province. In fact, the average H_S of the 8 cultivars from Fujian Province (59.8%) was significantly higher than that from Zhejiang (48.5%, 7 cultivars) and Yunnan (44.5%, 7 cultivars).

Table 2. Number of alleles (N_A), Nei's gene diversity (H), observed heterozygosity (H_O), and polymorphism information content (PIC) for 72 SSR markers observed in the 24 tea cultivars.

Marker	N_A	H	H_O	PIC	Marker	N_A	H	H_O	PIC
CsFM1015	5	0.749	0.750	0.708	CsFM1155	6	0.657	0.348	0.616
CsFM1026	7	0.822	0.667	0.798	CsFM1156	7	0.801	0.773	0.773
CsFM1029	7	0.712	0.583	0.682	CsFM1157	4	0.554	0.542	0.471
CsFM1030	6	0.793	0.708	0.765	CsFM1158	5	0.629	0.565	0.570
CsFM1031	4	0.605	0.542	0.541	CsFM1159	6	0.733	1.000	0.691
CsFM1032	8	0.765	0.542	0.730	CsFM1162	5	0.622	0.500	0.581
CsFM1051	6	0.666	0.739	0.631	CsFM1165	5	0.720	0.500	0.677
CsFM1056	4	0.261	0.292	0.248	CsFM1166	3	0.477	0.208	0.427
CsFM1057	6	0.788	0.625	0.759	CsFM1167	7	0.826	0.792	0.804
CsFM1059	5	0.551	0.500	0.524	CsFM1169	7	0.799	0.696	0.770
CsFM1061	6	0.786	0.458	0.754	CsFM1170	5	0.264	0.292	0.254
CsFM1066	4	0.424	0.522	0.392	CsFM1173	6	0.687	0.375	0.652
CsFM1071	5	0.669	0.500	0.610	CsFM1175	4	0.678	0.292	0.614
CsFM1078	6	0.609	0.292	0.559	CsFM1176	2	0.043	0.043	0.042
CsFM1080	3	0.398	0.333	0.354	CsFM1177	2	0.325	0.136	0.272
CsFM1092	5	0.664	0.583	0.617	CsFM1182	5	0.624	0.833	0.584
CsFM1100	3	0.611	0.417	0.535	CsFM1186	4	0.529	0.348	0.490
CsFM1104	6	0.669	0.625	0.626	CsFM1255	4	0.730	0.458	0.680
CsFM1105	7	0.813	0.792	0.788	CsFM1256	2	0.499	0.261	0.375
CsFM1107	6	0.742	0.833	0.701	CsFM1261	6	0.764	0.500	0.734
CsFM1110	8	0.811	0.542	0.784	CsFM1262	3	0.559	0.542	0.488
CsFM1115	5	0.712	0.500	0.662	CsFM1263	3	0.414	0.542	0.356
CsFM1116	4	0.630	0.583	0.562	CsFM1267	3	0.284	0.333	0.254
CsFM1121	7	0.774	0.500	0.744	CsFM1268	2	0.194	0.217	0.175
CsFM1123	5	0.668	0.208	0.607	CsFM1283	3	0.448	0.333	0.397
CsFM1125	5	0.723	0.565	0.673	CsFM1286	4	0.740	0.458	0.693
CsFM1127	4	0.705	0.250	0.649	CsFM1290	6	0.658	0.833	0.617
CsFM1128	3	0.385	0.333	0.325	CsFM1295	4	0.690	0.583	0.632
CsFM1129	4	0.635	0.458	0.562	CsFM1307	4	0.576	0.522	0.534
CsFM1131	12	0.891	0.792	0.880	CsFM1316	4	0.676	0.667	0.617
CsFM1134	7	0.741	0.583	0.704	CsFM1317	5	0.702	0.609	0.648
CsFM1135	5	0.709	0.542	0.671	CsFM1322	4	0.633	0.500	0.586
CsFM1138	6	0.718	0.458	0.679	CsFM1324	4	0.666	0.552	0.613
CsFM1140	9	0.841	0.750	0.822	CsFM1348	5	0.712	0.439	0.667
CsFM1143	4	0.496	0.292	0.442	CsFM1351	3	0.448	0.381	0.404
CsFM1154	5	0.687	0.625	0.629	CsFM1357	5	0.631	0.650	0.589

The 24 cultivars can be classified into 3 groups based on their H_S values: 1) $H_S \geq 55\%$, including 7 NTCs and 2 PTCs; 2) $55\% > H_S \geq 45\%$, including 5 NTCs and 3 PTCs; and 3) $H_S < 45\%$, including 7 PTCs. The average H_S of the 12 NTCs (57.0%) was notably higher than that of the 12 PTCs (45.6%), and the Student *t*-test showed that difference had statistical significance ($P < 0.001$).

In order to choose the parental combinations that could make the best use of the available SSR marker resources for genetic mapping, we calculated the P_M for all the putative combinations of the 24 cultivars tested. The P_M values for the 276 dual combinations varied from 51.4 (Changyebaihao x Yunkang10) to 90.3% (Fuandabaicha x Fudingdabaicha or Rougui), with an average of 72.8% (Table 3). The average P_M values of each tea cultivar are shown on the diagonal line of Table 3 and Figure 3 and illustrate that they are positively related with the H_S values.

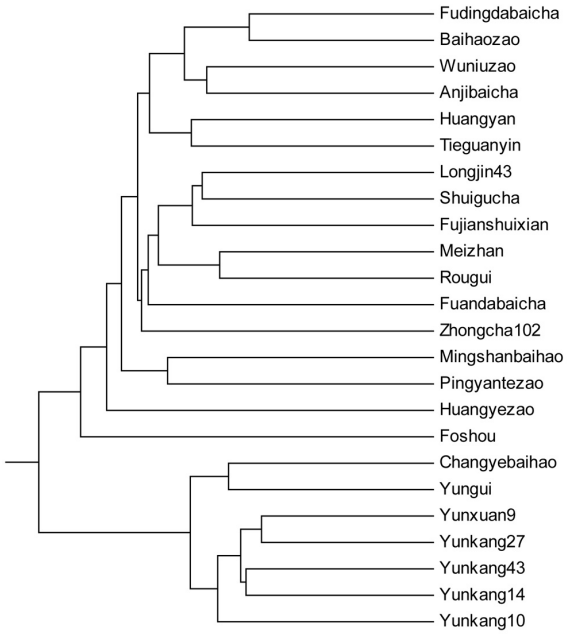

Figure 2. Dendrogram of 24 tea cultivars by the unweighted pair-group method using the arithmetic average (UPGMA) based on information of 72 SSR markers.

Table 3. Heterozygosities (H_S) of the 24 tea cultivars and percentages of mappable markers (P_M) of all putative combinations.

No. H_S	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1 **37.5**	**64.9**																							
2 **68.1**	77.8	**79.4**																						
3 **47.2**	59.7	76.4	**70.3**																					
4 **50.0**	63.9	79.2	69.4	**71.6**																				
5 **55.6**	69.4	84.7	69.4	70.8	**75.0**																			
6 **54.3**	69.4	79.2	69.4	73.6	75.0	**74.7**																		
7 **56.3**	70.8	76.4	70.8	69.4	76.4	76.4	**75.7**																	
8 **55.6**	69.4	81.9	73.6	73.6	77.8	77.8	75.0	**75.9**																
9 **46.5**	65.3	80.6	66.7	65.3	66.7	63.9	72.2	76.4	**70.4**															
10 **43.5**	62.5	75.0	68.1	63.9	70.8	65.3	70.8	75.0	61.1	**68.1**														
11 **50.0**	62.5	80.6	63.9	69.4	70.8	73.6	75.0	73.6	68.1	61.1	**70.4**													
12 **40.3**	61.1	76.4	69.4	69.4	70.8	66.7	75.0	73.6	62.5	56.9	63.9	**68.3**												
13 **53.5**	69.4	77.8	73.6	66.7	75.0	72.2	79.2	73.6	70.8	63.9	65.3	66.7	**73.0**											
14 **71.0**	66.7	***90.3***	79.2	80.6	83.3	79.2	84.7	83.3	77.8	76.4	83.3	79.2	81.9	**82.3**										
15 **63.9**	77.8	84.7	81.9	84.7	84.7	80.6	79.2	80.6	81.9	75.0	83.3	76.4	80.6	88.9	**81.0**									
16 **60.3**	66.7	88.9	75.0	79.2	80.6	87.5	81.9	84.7	80.6	76.4	70.8	73.6	77.8	86.1	81.9	**77.5**								
17 **56.9**	68.1	83.3	77.8	77.8	77.8	81.9	84.7	75.0	76.4	75.0	75.0	70.8	76.4	***90.3***	80.6	77.8	**76.2**							
18 **47.2**	63.9	79.2	72.2	73.6	76.4	76.4	73.6	72.2	70.8	68.1	66.7	66.7	72.2	83.3	77.8	73.6	69.4	**71.9**						
19 **60.6**	72.2	87.5	73.6	76.4	76.4	79.2	77.8	79.2	76.4	76.4	79.2	73.6	79.2	86.1	81.9	81.9	77.8	77.8	**77.0**					
20 **42.3**	56.9	79.2	66.7	66.7	72.2	69.4	73.6	70.8	63.9	62.5	65.3	59.7	69.4	80.6	77.8	70.8	69.4	66.7	75.0	**68.5**				
21 **40.3**	***51.4***	38.9	62.5	65.3	69.4	75.0	75.0	70.8	69.4	65.3	63.9	65.3	68.1	86.1	79.2	68.1	63.9	65.3	70.8	59.7	**65.8**			
22 **50.0**	59.7	84.7	68.1	75.0	77.8	77.8	76.4	81.9	68.1	68.1	72.2	66.7	75.0	87.5	81.9	76.4	76.4	73.6	72.2	70.8	61.1	**72.9**		
23 **40.3**	52.8	80.6	65.3	66.7	73.6	70.8	69.4	75.0	65.3	63.9	68.1	62.5	73.6	79.2	79.2	70.8	75.0	68.1	68.1	68.1	59.7	61.1	**68.5**	
24 40.3	55.6	81.9	63.9	65.3	75.0	77.8	76.4	70.8	68.1	65.3	62.5	63.9	69.4	79.2	81.9	70.8	70.8	65.3	72.2	59.7	59.7	65.3	59.7	**68.7**

The figures on the diagonal line are the average P_M values of each cultivar, e.g., the figure in the heave gray is the average of the 23 figures in the light gray.

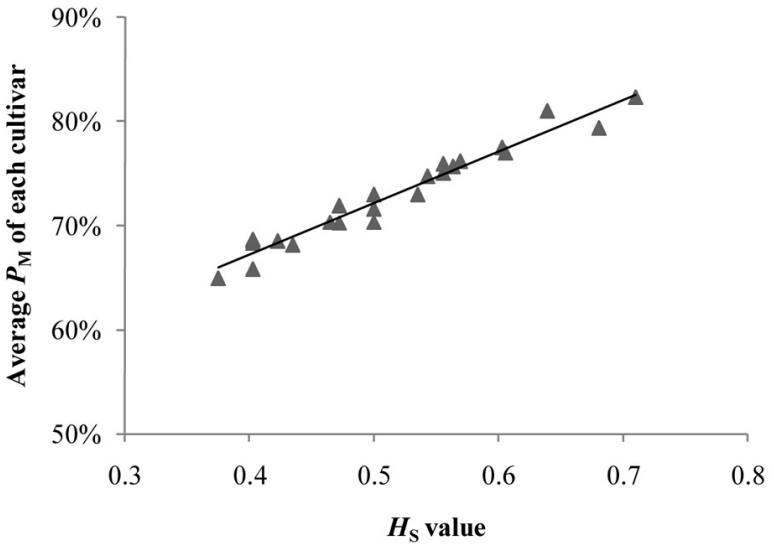

Figure 3. Relationship between the heterozygosity (H_S) of each cultivar and the average percentage of mappable markers (P_M).

DISCUSSION

In this study, we used 72 SSR markers to analyze the genetic relationships and heterozygosity of 24 tea cultivars in China. Overall, a high level of polymorphism was observed for the 72 markers, providing important genetic information as discussed below.

Yunnan is thought to be an original place for *C. sinensis* that has a high level of genetic diversity (Yao et al., 2012). However, the 7 cultivars from this province showed high levels of Nei's genetic identity, indicating close relationships among these cultivars. This is probably because all of these cultivars except Yungui were bred through field clones from the same county (Menghai). Although they originated from different provinces, Fudingdabaicha and Baihaozao also showed a high level of genetic identity (0.655). Another interesting observation is that these two cultivars shared at least one allele at most of the SSR loci tested (data not shown). It is likely that Baihaozao is an offspring of Fudingdabaicha, and this is practically possible because Fudingdabaicha, one of the most outstanding tea cultivars in China, was frequently introduced to other tea-growing areas from Fujian.

Clustering analysis failed to distinguish the cultivars tested geographically in this study except those from Yunnan. This result is consistent with other molecular marker studies of *C. sinensis* (Chen et al., 2005; Fang et al., 2012; Yao et al., 2012), and this result is probably because genetic exchanges occurred frequently among Fujian, Zhejiang, and Hunan because of human activities.

Genetic heterozygosity is the state of possessing different alleles at a given locus. Generally, *C. sinensis* is thought to be highly heterozygous because of long-term allogamy (Chen et al., 2007). With a set of 72 SSR markers, we showed that the H_S of different tea cultivars varied greatly from 37.5 to 71.0%. A high level of H_S (average 59.8%) was observed for the

cultivars from Fujian Province, suggesting frequent germplasm introductions and hybridizations in this region in history. However, the 7 cultivars from Yunnan showed a relatively low level of H_S (average 44.5%). The lowest H_S was scored by Changyebaihao from this province. Furthermore, in an investigation of 178 single nucleotide polymorphism loci, we also observed that Changyebaihao had a lower level of H_S (37.4%) compared to the other 6 tea cultivars that ranged from 39.9 to 48.9% (data not shown). Both the high levels of Nei's genetic identity and the low levels of H_S suggest some degree of inbreeding among the 7 cultivars from Yunnan Province.

Interestingly, the NTCs tended to have a higher H_S than the PTCs. According to the description in the Records of Tea Plant Cultivar in China (The Editorial Board, 2001), NTCs are the primary cultivars that could be spread in a large tea-growing area, while PTCs are supposed to spread mainly within that province. This means that NTCs generally have better fitness than PTCs. Because positive heterozygosity-fitness correlations have been reported for many other plants and animals (Zhang et al., 1996; Hansson and Westerberg, 2002; Chapman et al., 2009), it is possible that there is such a correlation in *C. sinensis* as well. Further studies with more markers and fitness traits of tea cultivars are needed to investigate this correlationship, which will be of great value in tea-breeding programs if validated.

The long period of juvenility of *C. sinensis* limits the possibility to work on a second generation of hybrids (F_2 or BC). Consequently, the genetic mapping of *C. sinensis* is normally established in F_1 populations using the pseudo-testcross strategy (Grattapaglia and Sederoff, 1994). For the sake of efficient genetic mapping by this strategy, the cross parents of the F_1 population are required to be highly heterozygous. As mentioned above, there are significant differences in terms of the H_S among the 24 tea cultivars tested. Therefore, it is important to consider the H_S of the cross parents to increase the mapping efficiency. In similar observations of another wood plant, *Citrus* species, the P_M varied from 21 to 80% (Luro et al., 2008). In *C. sinensis*, Ma et al. (2012) reported that 25 of 30 SSR markers (83%) are mappable between a cross of Yingshuan and Baiye Danzhu. Here, we showed that the P_M values for different tea cultivar combinations varied greatly from 51.4 to 90.3%. The cultivars from Fujian Province such as Fuandabaicha, Fudingdabaicha, and Meizhan were recommended as cross parents for genetic mapping, although there are other factors, like fruit-bearing rates and phenotype segregations, that need to be considered when making such choices.

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