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ASSESSMENT OF TEA PLANT (*CAMELLIA SINENSIS* L.) ACCESSIONS FOR POLLEN SOURCES IN NATURAL CROSSING BY USING MICROSATELLITES

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SUMMARY

Tea (*Camellia sinensis* L. [O.] Kuntze) is a highly cross-pollinated and self-incompatible plant. Seeds can be harvested from specific individual mother plants in polyclonal tea gardens. Whether the pollen donor plays an important role in seed formation remains unclear. This study aimed to identify the male parents of 72 natural hybridized progenies (F_1) from one female parent on the basis of a putative specific allele by using simple-sequence repeat (SSR) markers and the exclusion-likelihood method with Cervus 3.0 software. The genetic material, which comprised seven accessions of *C. sinensis* L., was acquired from Assamica planted in the Kayulandak polyclonal seed garden of the Pagilaran tea plantation in Batang District, Central Java, Indonesia, and was studied during 2019 and 2020. The genotype PGL-15 was used as the female parent, whereas the six candidate genotypes PGL-10, GMB-9, GMB-7, TPS-93, GMB-11, and TRI-2025 were used as the male parents. In this study, 13 SSR loci were used to identify the male parents of the F_1 progenies obtained through natural hybridization between one female and six male tea accessions. Results indicated that the exclusion-likelihood method, which correctly predicted 100% of the male parents, was more effective than the putative specific allele approach, which correctly predicted only 34.72% of the male parents in the 72 hybridized F_1 progenies of tea plants.

Keywords: *Camellia sinensis* L., natural pollination, SSR markers, paternity analysis, putative specific allele, exclusion-likelihood method

Key findings: The exclusion-likelihood approach was found to be more effective than putative specific allele analysis in the prediction of the male parents of F_1 tea plant progenies. The exclusion-likelihood method correctly predicted 100% of the male parents, whereas the putative specific allele method was able to predict only 34.72% of the male parents of 72 hybridized F_1 progenies.

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INTRODUCTION

Tea (*Camellia sinensis*), the oldest popular caffeine-containing beverage in the world, originated from China and the northeastern region of India (Akula and Akula, 1999; Chen *et al*., 2008). The global tea demand has increased with time due to population growth and improved life habits. The biochemical components of tea leaves include polyphenols, alkaloids, volatile compounds, polysaccharides, amino acids, lipids, and vitamins and demonstrate a variety of bioactivities.

Given that tea is a highly cross-pollinated and self-incompatible plant (Bandyopadhyay, 2011), controlled pollination can be performed by harvesting legitimate seeds from mother plants through natural hybridization and genetically identifying potential male parents through parentage analysis (Diaz *et al*., 2006). Although biclonal seed cultivars are currently rarely used to produce appropriate planting material, ex-situ field conservation banks still maintain their tea accessions by using polyclonal seed garden approaches. Such seed gardens can be utilized directly for breeding. Although controlled hybridization and individual selection have gradually become the predominant methods for tea breeding, tea breeding remains dependent on natural crossing for gene recombination due to the issues of self-incompatibility and inefficient artificial crossing (Muoki *et al*., 2007).

The prolonged cross-pollination of tea plants has produced considerable heritable variation, which has in turn resulted in a high level of genetic diversity (Fan *et al*., 2011; Kottawa-Arachchi *et al*., 2019). New high-yielding tea cultivars in the form of clonal plants can be exploited to increase yield. These tea cultivars can be selected individually from a population that originated from orchard seedlings or open-pollinated progenies.

The use of molecular techniques for parentage analysis has thrived over several decades. A highly polymorphic marker is required for successful parentage analysis, and microsatellite markers have been identified as the most reliable tool for the parentage analysis of peach (*Prunus persica* L.) (Yamamoto *et al*., 2002), oil palm (Thongthawae *et al*., 2010), and polyploid sweet potato (Buteler and LaBonte, 2002) due to their codominant inheritance and large number of alleles per loci.

The introduction of microsatellite markers into molecular ecology, accompanied by the proliferation and refinement of statistical techniques for the analysis of the parentage data of natural populations (Jones and Arden, 2003), is the most important technological innovation. As a result, parentage analysis can be performed as one of the most efficient and accurate analyses with simple-sequence repeat (SSR) markers.

ast studies have also classified different approaches for parentage analysis, i.e., exclusion, categorical allocation, fractional allocation, full probability parentage analysis, parental reconstruction, and sibship reconstruction (Jones *et al*., 2010). Mookerjee *et al*. (2005) proved that in olive plants, the chance of cumulative exclusion is very high because SSRs show a very low error probability in recognizing the male parent. Therefore, on the basis of the the above discussion, this study aimed to explore a similar approach for identifying the male parents of 72 seedling progenies of natural tea crosses. Male parent determination in open-pollinated progeny is useful for reconstructing the pedigree of outcrossed crops (Norman *et al*., 2018). Reliable pedigree information is useful for breeders in making decisions on existing divergence in progeny and hybrid vigor (Spanoghe *et al*., 2015) and determining genetic estimates, breeding value, and relationships (Gjedrem, 2010).

MATERIALS AND METHODS

Plant material

The genetic material, which comprised seven accessions and 72 hybridized F_1

progenies of *C. sinensis* L., was acquired from Assamica planted in the Kayulandak polyclonal seed garden of the Pagilaran tea plantation in Batang District, Central Java, Indonesia, and was studied during 2019 and 2020. The genotype PGL-15 was used as the female parent, whereas the six candidate genotypes PGL-10, GMB-9, GMB-7, TPS-93, GMB-11, and TRI-2025 were used as the male parents. The 72 F_1 progenies were obtained through natural hybridization.

DNA extraction and amplification

DNA was extracted from tea leaves by using the modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). A total of 200 mg of dry tea leaves was ground into smooth powder and added with 1500 µl of extraction buffer solution (2% CTAB, 0.1 M Tris-hydrochloric acid pH 8.0, 1.4 M sodium chloride, 0.02 M EDTA, 2% polyvinyl pyrrolidone, 2% β-mercaptoethanol, and aqua-bidest). The extracted solution was then transferred into a 1.5 ml microtube and incubated in a water bath at 65 °C for 60 min. The solution was added with 500 µl of chloroform isoamyl alcohol, then homogenized with a vortex and centrifuged at 12 000 rpm for 15 min. The last step was repeated three times. The supernatant was then transferred into a 1.5 ml microtube, then added with 60 µl of sodium acetate and 440 µl of isopropanol and stored in a refrigerator for 24 h. After 24 h, the sample was centrifuged at 12 000 rpm for 10 min. The supernatant was removed, and 500 µl of 70% ethanol was added. The mixture was then centrifuged at 12 000 rpm for 5 min. The supernatant was discarded to retain only the pellet. Subsequently, the supernatant was added with 500 µl of absolute ethanol and centrifuged at 12 000 rpm for 5 minutes. The supernatant was discarded, and the pellets were air-dried for 24 h and dissolved with 50 µl of aqua-bidest.

Thirteen SSR primers were used for DNA amplification via polymerase chain reaction (PCR). The SSR primers were selected because they were able to characterize the five accessions of tea that were exploited to develop plants with high-quality and high-quantity yield (Azka, 2019) in accordance with the aim of natural crossing. The PCR mixture was divided into 10 µl tubes. Each 10 µl reaction consisted of 0.25 µl of primer, 5 µl of GoTaq Green, 2.25 µl of nuclease-free water, and 2.5 µl of DNA (quantified by using GeneQuant spectrophotometer). The PCR mix was run on a Bio-Rad T100TM Thermal Cycler. The first heating cycle was carried out at 95 °C for 30 s then was followed by 54 cycles of touchdown. Denaturation was performed at 95 °C for 30 s. All primers were annealed at 60 °C, 58 °C, 56 °C, 54 °C, 52 °C, and 50 °C (Table 1) for 45 s at each temperature (touchdown). Elongation was performed at 72 °C for 1 min 30 s. The last cycle was followed by the final elongation cycle at 72 °C for 30 s. The amplified DNA was visualized by using 2% MetaPhor agarose gel electrophoresis (MAGE). The 2% MAGE gel consisted of 0.6 g of MetaPhor™ agarose, 30 ml of 1× Tris-borate-EDTA, and 5 µl of FluoroSafe DNA. The MAGE gel was then subjected to horizontal gel electrophoresis (Bio Rad PowerPack Basic™) for 75 min at 100 V and 400 mA.

Paternity analysis

Two approaches were used to identify the male parents in the natural hybridization progenies of Assamica. The first approach was based on the putative specific markers of the parents, whereas the second approach comprised the exclusion and likelihood approach. The putative SSR-specific markers in the parental genotypes were identified by looking at the presence and number of polymorphic alleles that were specific to each parent and that can be used to differentiate the parents from one another (Govindaraj *et*

Table 1. SSR primers used for the molecular characterization of the tea genotypes.

No.	Primers	Primer Sequences (5'–3')	Annealing Temperature (°C)
1.	CamsinM1	F: GAATCAGGACATTATAGGAATTAA	60 °C, 58 °C, 56 °C, 54
		R: GGCCGAATGTTGTCTTTTGT	°C , and 52 °C (five
2.	CamsinM2	F: CCTCTGGTGGTCCTACACCT	temperatures for all
		R: AAAGCCTTGATGCCTTTCG	primers - Touchdown)
3.	CamsinM3	F: GGTGTGGTGTTTTGAAGAAA	
		R: TGTTAAGCCGCTTCAATGC	
4.	CamsinM5	F: AAACTTCAACAACCAGCTCTGGTA	
		R: ATTATAGGATGCAAACAGGCATGA	
5.	CamsinM6	F: TGTTTTCTTAGGGTTGGATAAAGG	
		R: TTTTGTTGTAATGACGAAAATTC	
6.	CamsinM7	F: TGGTAAGGGTCCTAAGAGGTACAC	
		R: TTCCAATCTTTTTCTATAACATCTGC	
7.	CamsinM8	F: CCATCATTGGCCATTACTACAA	
		R: CCATATGTGTGTGAATGATAAAACC	
8.	CamsinM9	F: CTCATGGAGTCCAAGGAAGC	
		R: AAAGCAGTCTGGAACCTTGC	
9.	CamsinM10	F: TTACATCTCTTTTGCAGCTGTCGG	
		R: CTTCGGGAACTTCTGCTTCATC	
10.	CamsinM11	F: GCATCATTCCACCACTCACC	
		R: GTCATCAAACCAGTGGCTCA	
11.	CamsinM12	F: CATTATCGTCACTTGCAAAGAGGT	
		R: CGAGAAGAAGAGCTCTATTGGTT	
12.	CamsinM13	F: CACATTGTGGCGTGTTATTAATTT	
		R: ACATTGGCTATCTCTCATCATGG	
13.	M4	F: ACATTCAAGCAGTCCACATAT	
		R: CCTGATGCAGGACTGTCTATAGATGA	

Source: Freeman et al., (2004)

al., 2012). The putative specific alleles in the parental genotypes were used to identify every progeny. All the progeny genotypes were compared with the candidate male parent (PGL-10, GMB-9, GMB-7, TPS-93, GMB-11, and TRI-2025) and female parent (PGL-15) in reference to the Mendelian law for codominant inheritance. The male parent of the F_1 progeny was selected on the basis of the presence of identical putative specific alleles and the highest allelic similarity percentage.

Paternity analysis through the exclusion and likelihood approach was performed by using Cervus 3.0 software (Kalinowski *et al*., 2007). The six parental accessions (PGL-10, GMB-9, GMB-7, TPS-93, GMB-11, and TRI-2025) were used as the candidate male parents, whereas genotype PGL-15 was used as the female parent. The paternity analysis simulation was run with the likelihood method with the number of progenies, male candidates, the proportion of samples, the proportions of loci types, and mistyping set as 72, 6, 1.000 (100% of sample read), 0.7 (70% of the valid data given that data were missing [0]), and 0.01 (99% confidence interval), respectively. The logarithm of the odds (LOD) value of each possible parent–progeny pair was used to determine the true parent (Jones *et al*., 2010). A positive LOD score indicates that the parental candidate is the real parent. A zero LOD score (0) indicates that the probability of a candidate parent is the real parent and not the real parent is the same. A negative LOD score indicates that one or more loci of the candidate parent differ from those of the progeny (Kalinowski *et al*., 2007). The

critical LOD scores with 95% and 80% levels of confidence were calculated on the basis of the simulation analysis. In the paternity analysis, the level of confidence for each candidate male parent was determined by using the trio confident scores. If the trio confidence scores are followed by the symbol (*), then the confidence is 95%; if the values are positive but are not followed by the symbol (*), then the confidence is 80%; negative scores have less than 80% confidence (Kalinowski *et al*., 2007).

RESULTS

Parentage analysis based on putative specific alleles

In this study, 13 SSR loci (Table 1) were used to identify the male parents of the F_1 progenies obtained through natural hybridization between PGL-15 (female) and six male tea accessions of Assamica (PGL-10, GMB-9, GMB-7, TPS-93, GMB-11, and TRI-2025) in the Kayulandak second polyclonal seed garden of PT Pagilaran. The seven candidate parent accessions involved in natural hybridization had a fairly high percentage of allele similarities between accessions with an average of 67.85% (Table 2). Among individual pairs, the tea genotypes that exhibited the highest percentage of allele similarity (88.24%) were TRI-2025 and PGL-10. The highest level of similarity between these genotypes could be attributed to the fact that PGL-10 was obtained from a half-sibling progeny population selection with TRI-2025 as the female parent (Decree of the Agriculture Minister of the Republic of Indonesia No. 51/Kpts/KB.010/3/2020). The Decree of the Agriculture Minister of Republic of Indonesia No. 26/Kpts/KB.010/3/2020 regarding the release of tea accession PGL-15 as a superior cultivar stated that similar to PGL-10, PGL-15 was also obtained from half-sibling progeny population selection with TRI-2025 as the female parent. By using RAPD markers, Ramakrishnan *et al*. (2009) classified the tea accession TRI-2025 as the 'Cambod' cultivar (*C. assamica* subspecies lasiocalyx). The 'Cambod' cultivar is a hybrid of *sinensis* and *assamica* cultivars (Wambulwa *et al*., 2016). Accessions GMB-7 and GMB-11 were F_1 hybrids that were obtained from the crosses of the parental genotypes Mal-2 and PS-1, whereas the F_1 hybrid GMB-9 was obtained from the genotypes GP-3 and PS-1.

Furthermore, specific markers were estimated on the basis of the seven parental accessions by using 13 SSR loci. A specific marker or allele for each accession was identified by determining the unique bands that were found only in one accession and not in other accessions. A previous study revealed that among seven parental accessions, putative specific markers were recorded for only three accessions, namely, PGL-15, GBM-7, and GBM-9, (Nisa, 2020) (Table 3). The female tea parent PGL-15 was characterized by four putative specific loci,

Table 2. Allele similarity (above diagonal) and dissimilarity (below diagonal) percentage between the parental accessions of the tea genotypes.

Parental accessions	PGL15	TPS93	GMB7	GMB9	GMB11	PGL10	TRI2025
PGL15	-	66.67	66.67	61.11	57.89	64.71	64.71
TPS93	33.33	-	72.22	66.67	68.42	76.47	64.71
GMB7	33.33	27.78	-	61.11	68.42	58.82	64.71
GMB9	38.89	33.33	38.89	-	68.42	70.59	70.59
GMB11	42.11	31.58	31.58	31.58	-	76.47	88.24
PGL10	35.29	23.53	41.18	29.41	23.53	-	88.24
TRI2025	35.29	35.29	35.29	29.41	11.76	11.76	-
Average similarity	67.85%						

Table 3. Putative specific alleles in the parental accessions of tea.

Accessions	Loci	Putative Specific Allele (bp)
PGL-15	Camsin M3	230
	M4c	330
	Camsin M5	180
	Camsin M7	220
TPS 93	-	None
GMB 7	Camsin M8	140
GMB 9	Camsin M6	270
	Camsin M8	170
	Camsin M9	210
	Camsin M11	190
GMB 11	-	None
PGL-10	-	None
TRI 2025	-	None

Source: Nisa (2020)

i.e., the 230-bp Camsin M3 allele, the 330-bp M4c allele, the 180-bp Camsin M5 allele, and the 220-bp Camsin M7 allele. The male parental accession GMB-7 was characterized by one putative specific locus on the 140-bp Camsin M8 allele. The male parent accession GMB-9 was characterized by four specific loci on the 270-bp Camsin M6 allele, the 170-bp Camsin M8 allele, the 210-bp Camsin M9 allele, and the 190-bp Camsin M11 allele.

The presence of putative specific markers in the three parental accessions was then used to predict the male genotypes on the basis of the presence of that specific allele in each progeny by using the Mendelian law for codominant inheritance. In this case, the parental accessions that did not show a putative specific allele cannot be used to predict the male parents. Therefore, only two male parents were available for paternity analysis, i.e., GMB-7 and GMB-9, and PGL-15 was considered as the female parent.

Male parent determination was based on the presence of putative specific alleles in the progeny. If more than one putative specific allele in the progeny was obtained from different candidate male parents, the male parent prediction was based on the largest percentage of allelic similarities between the progeny and the candidate male parent. The largest percentage of allelic similarities between the progeny and candidate male parent implies a high probability of being the true pollen parent (Norman *et al*., 2018). On the basis of the results, the putative specific alleles can be used to predict the male parents of 25 progenies. The male parents were GMB-7 and GMB-9 (Table 4).

Parentage analysis by using the exclusion and likelihood approach

The second paternity analysis was performed through the exclusion and likelihood approach by using Cervus 3.0 software. Exclusion and likelihood analysis was used to compare the candidate parental genotypes with their F_1 progenies. Parental genotypes that had one or more different loci from their progeny were then excluded as candidate parents. The likelihood in this program was used to distinguish nonexcluded candidate parents (Kalinowski *et al*., 2007).

The exclusion and likelihood method was able to predict the male parents of all the 72 progenies. Ten male parent–progeny pairs were identified with a 95% level of confidence: GMB-7–O1.10, GMB-9–O2.7, GMB-7–O2.13-, TPS-93–O2.16, TRI-2025–O3.2, GMB-7–O3.20,

Table 4. Parentage analysis based on putative specific alleles in the tea genotypes.

Progeny ID	GMB7		GMB9		Expected Father	Progeny	GMB7		GMB9		Expected Father
	Allele Similarity %	Specific Allele	Allele Similarity %	Specific Allele			Allele Similarity %	Specific Allele	Allele Similarity %	Specific Allele	
O1.1	22.22	None	27.78	None	Unknown	O2.18	16.67	None	22.22	M11-190	GMB9
O1.2	11.11	None	16.67	None	Unknown	O2.19	16.67	None	22.22	M6-270	GMB9
O1.3	16.67	M7-190	16.67	M6-270	GMB7, GMB9	O3.1	5.56	None	5.56	None	Unknown
O1.4	11.11	None	16.67	None	Unknown	O3.2	11.11	None	27.78	M6-270	GMB9
O1.5	16.67	M8-140	11.11	None	Unknown	O3.3	0.00	None	5.56	None	Unknown
O1.6	5.56	None	11.11	None	Unknown	O3.6	11.11	None	11.11	None	Unknown
O1.7	16.67	None	22.22	None	Unknown	O3.7	16.67	None	5.56	None	Unknown
O1.8	11.11	M8-140	11.11	M8-170	GMB7, GMB9	O3.9	11.11	M7-190	11.11	None	GMB7
O1.9	5.56	None	16.67	M8-170	GMB9	O3.10	11.11	None	16.67	M11-190	GMB9
O1.10	27.78	M8-140	16.67	None	GMB7	O3.11	22.22	M7-190	11.11	None	GMB7
O1.11	11.11	None	5.56	None	Unknown	O3.12	11.11	None	11.11	None	Unknown
O1.12	11.11	M8-140	11.11	None	GMB7	O3.13	11.11	None	16.67	None	Unknown
O1.14	5.56	None	0.00	None	Unknown	O3.14	5.56	None	0.00	None	Unknown
O1.15	0.00	None	0.00	None	Unknown	O3.16	5.56	None	5.56	None	Unknown
O1.16	0.00	None	5.56	None	Unknown	O3.17	5.56	None	0.00	None	Unknown
O1.17	11.11	None	11.11	None	Unknown	O3.18	16.67	None	5.56	None	Unknown
O1.18	5.56	None	11.11	None	Unknown	O3.19	0.00	None	5.56	M6-270	GMB9
O1.19	22.22	M8-140	16.67	None	GMB7	O3.20	27.78	None	27.78	M9-210	GMB9
O1.20	0.00	None	5.56	None	Unknown	O4.1	11.11	None	11.11	None	Unknown
O2.1	11.11	None	11.11	None	Unknown	O4.2	22.22	None	16.67	None	Unknown
O2.2	11.11	None	0.00	None	Unknown	O4.3	22.22	None	11.11	None	Unknown
O2.3	16.67	None	11.11	None	Unknown	O4.4	5.56	None	22.22	M11-190	GMB9
O2.4	0.00	None	5.56	None	Unknown	O4.5	5.56	None	5.56	None	Unknown
O2.5	11.11	None	11.11	None	Unknown	O4.6	5.56	M8-140	11.11	M6-270	GMB7, GMB9
O2.6	11.11	None	16.67	None	Unknown	O4.7	11.11	M8-140	0.00	None	GMB7
O2.7	16.67	None	33.33	M8-170	GMB9	O4.8	11.11	M8-140	0.00	None	GMB7
O2.8	11.11	None	0.00	None	Unknown	O4.9	0.00	None	11.11	M8-170	GMB9
O2.9	11.11	None	5.56	None	Unknown	O4.10	5.56	None	16.67	M6-270	GMB9
O2.10	0.00	None	5.56	None	Unknown	O4.11	5.56	None	16.67	None	Unknown
O2.11	5.56	None	11.11	None	Unknown	O4.12	11.11	None	16.67	None	Unknown
O2.12	11.11	M7-190	11.11	None	GMB7	O4.13	11.11	None	11.11	None	Unknown
O2.13	22.22	M8-140, M7-190	11.11	M6-270	GMB7, GMB9	O4.14	5.56	None	11.11	None	Unknown
O2.14	5.56	None	5.56	None	Unknown	O4.15	11.11	None	5.56	M8-170	GMB9
O2.15	16.67	None	11.11	None	Unknown	O4.16	16.67	None	22.22	None	Unknown
O2.16	16.67	None	22.22	None	Unknown	O4.17	16.67	None	27.78	M8-170	GMB9
O2.17	5.56	None	5.56	None	Unknown	O4.18	5.56	None	16.67	None	Unknown

Table 5. Parentage analysis using the exclusion and likelihood method (progenies O1.1–O2.19) in the tea genotypes.

Progeny ID	Mother ID	Candidate father ID	Trio loci mis-matching	Trio LOD score	Trio confidence	Progeny ID	Mother ID	Candidate father ID	Trio loci mis-matching	Trio LOD score	Trio confidence
O4.1	PGL15	GMB7	9	−6.13E+00	-	O2.3	PGL15	GMB7	8	−2.24E+00	-
O4.2	PGL15	GMB7	8	1.19E+00	*	O2.4	PGL15	TRI2025	10	−4.00E+00	-
O4.3	PGL15	GMB11	10	5.20E−01	*	O2.5	PGL15	PGL10	10	−1.07E+01	-
O4.4	PGL15	GMB9	8	−1.61E−01	-	O2.5	PGL15	TRI2025	10	−1.07E+01	-
O4.5	PGL15	TPS93	9	−8.77E+00	-	O2.6	PGL15	PGL10	9	−1.30E+00	-
O4.6	PGL15	GMB9	11	−3.92E+00	-	O2.7	PGL15	GMB9	8	2.28E+00	*
O4.7	PGL15	GMB7	11	−4.39E+00	-	O2.8	PGL15	GMB7	9	−3.77E+00	-
O4.8	PGL15	GMB7	11	−4.39E+00	-	O2.9	PGL15	GMB7	9	−3.97E+00	-
O4.9	PGL15	TRI2025	11	−4.68E+00	-	O2.10	PGL15	PGL10	11	−6.26E+00	-
O4.10	PGL15	GMB9	12	−7.42E+00	-	O2.10	PGL15	TRI2025	11	−6.26E+00	-
O4.11	PGL15	PGL10	9	−1.08E+00	-	O2.11	PGL15	TPS93	10	−7.06E+00	-
O4.12	PGL15	GMB11	7	3.02E−01	*	O2.12	PGL15	PGL10	9	−3.56E+00	-
O4.13	PGL15	GMB11	10	−8.90E−01	-	O2.12	PGL15	TRI2025	9	−3.56E+00	-
O4.14	PGL15	TRI2025	8	−4.79E+00	-	O2.13	PGL15	GMB7	10	3.88E−01	*
O4.15	PGL15	GMB7	10	−8.16E+00	-	O2.14	PGL15	GMB11	10	−2.32E+00	-
O4.16	PGL15	PGL10	7	−4.18E+00	-	O2.15	PGL15	TPS93	9	−1.34E+00	-
O4.17	PGL15	GMB11	8	6.14E−01	*	O2.16	PGL15	TPS93	7	1.27E+00	*
O4.18	PGL15	TRI2025	9	−9.68E−01	-	O2.17	PGL15	TPS93	11	−4.49E+00	-
O2.1	PGL15	PGL10	9	−7.37E+00	-	O2.18	PGL15	GMB11	8	7.30E−01	-
O2.1	PGL15	TRI2025	9	−7.37E+00	-	O2.18	PGL15	PGL10	8	7.30E−01	-
O2.2	PGL15	GMB7	9	−3.77E+00	-	O2.19	PGL15	GMB11	11	−4.71E+00	-

*: 95% level of confidence

Table 6. Parentage analysis using the exclusion and likelihood method (progenies O3.1–O1.20) in the tea genotypes.

Progeny ID	Mother ID	Candidate father ID	Trio loci mismatching	Trio LOD score	Trio confidence	Progeny ID	Mother ID	Candidate father ID	Trio loci mis-matching	Trio LOD score	Trio confidence
O3.1	PGL15	TRI2025	11	−8.62E+00	-	O1.4	PGL15	GMB11	8	−5.73E+00	-
O3.2	PGL15	TRI2025	9	3.54E−01	*	O1.4	PGL15	PGL10	8	−5.73E+00	-
O3.3	PGL15	TPS93	11	−4.59E+00	-	O1.5	PGL15	GMB7	10	−6.61E+00	-
O3.6	PGL15	TPS93	9	−1.77E+00	-	O1.6	PGL15	PGL10	8	−3.23E+00	-
O3.7	PGL15	GMB7	11	−2.62E+00	-	O1.6	PGL15	TRI2025	8	−3.23E+00	-
O3.9	PGL15	PGL10	10	−4.88E+00	-	O1.7	PGL15	PGL10	7	−2.01E−01	-
O3.9	PGL15	TRI2025	10	−4.88E+00	-	O1.7	PGL15	TRI2025	7	−2.01E−01	-
O3.10	PGL15	TPS93	9	−1.77E+00	-	O1.8	PGL15	PGL10	8	−2.81E+00	-
O3.11	PGL15	GMB7	11	−9.77E−01	-	O1.8	PGL15	TRI2025	8	−2.81E+00	-
O3.12	PGL15	TPS93	9	−5.43E−01	-	O1.9	PGL15	PGL10	8	−1.82E+00	-
O3.13	PGL15	TPS93	9	−2.29E+00	-	O1.10	PGL15	GMB7	8	2.72E+00	*
O3.14	PGL15	GMB11	12	−5.03E+00	-	O1.11	PGL15	TPS93	10	−4.55E+00	-
O3.16	PGL15	GMB11	11	−8.05E+00	-	O1.12	PGL15	PGL10	8	−3.52E+00	-
O3.16	PGL15	TRI2025	11	−8.05E+00	-	O1.12	PGL15	TRI2025	8	−3.52E+00	-
O3.17	PGL15	GMB7	11	−1.04E+01	-	O1.14	PGL15	TPS93	10	−5.25E+00	-
O3.18	PGL15	GMB7	9	−6.22E+00	-	O1.14	PGL15	GMB7	10	−5.25E+00	-
O3.19	PGL15	GMB9	11	−5.42E+00	-	O1.14	PGL15	GMB11	10	−5.25E+00	-
O3.20	PGL15	GMB7	8	2.63E+00	*	O1.15	PGL15	TRI2025	11	−5.53E+00	-
O1.1	PGL15	TPS93	7	−1.07E+00	-	O1.16	PGL15	TRI2025	9	−2.97E+00	-
O1.2	PGL15	TPS93	10	1.31E−01	-	O1.17	PGL15	TPS93	9	−7.14E+00	-
O1.2	PGL15	PGL10	10	1.31E−01	-	O.18	PGL15	GMB11	10	−5.02E+00	-
O1.3	PGL15	GMB7	10	−6.72E+00	-	O1.19	PGL15	TRI2025	9	−3.48E+00	-
O1.4	PGL15	TPS93	8	−5.73E+00	-	O1.20	PGL15	GMB9	11	−9.25E+00	-
O1.4	PGL15	GMB9	8	−5.73E+00	-	O1.20	PGL15	GMB11	11	−9.25E+00	-

*: 95% level of confidence

Table 7. Matching results of the parentage analysis based on the specific putative allele and exclusion-likelihood approach in the tea genotypes.

Progeny	Parentage based on putative specific allele					Progeny ID	Parentage based on the exclude and likelihood method		
	GMB7		GMB9		Expected male		Candidate male ID	Trio LOD score	Trio confidence
	Allele similarity (%)	Specific allele	Allele similarity (%)	Specific allele					
Seedling 4.7	11.11	M8-140	0	-	GMB7	O 4.7	GMB7	−4.39E+00	-
Seedling 4.8	11.11	M8-140	0	-	GMB7	O4.8	GMB7	−4.39E+00	-
Seedling 4.10	5.56	-	16.67	M6-270	GMB9	O4.10	GMB9	−7.42E+00	-
Seedling 2.7	16.67	-	33.33	M8-170	GMB9	O2.7	GMB9	2.28E+00	*
Seedling 2.13	22.22	M8-140, M7-190	11.11	M6-270	GMB7, GMB9	O2.13	GMB7	3.88E−01	*
Seedling 3.11	22.22	M7-190	11.11	-	GMB7	O3.11	GMB7	−9.77E−01	-

GMB-7–O4.2, GMB-11–O4.3, GMB-11–O4.12, and GMB-11–O4.17 (Tables 5 and 6). The results also revealed several male parent–progeny pairs with positive LOD scores that were not followed by the symbol (*). This result indicated that the said pairs had an 80% level of confidence, whereas those with negative LOD scores had less than an 80% level of confidence (Kalinowski *et al*., 2007).

DISCUSSION

The transcriptomic analysis of the stylus after self and cross-pollination revealed that tea plants exhibit gametophytic self-incompatibility because they have gametophytic pollens (Zhang *et al*., 2016). Complete pollination can be successful only if the alleles in the pollen and pistil are different from each other (Chahal and Gosal, 2002). Cross-pollination results in generatively propagated tea with the highest heterogeneity, and natural hybridization can be exploited to develop high-yielding tea cultivars. Specific progenies produced through natural crosses at the Kayulandak second polyclonal seed garden of PT Pagilaran were used in this study.

Paternity analysis based on putative specific alleles predicted 25 male parent–progeny pairs because only three accessions exhibited putative specific alleles, i.e., PGL-15, GMB-7, and GMB-9, whereas the genotype PGL-15 was considered the female parent. Therefore, to increase the level of accuracy in paternity analysis, the data were also analyzed through the exclusion and likelihood method by using Cervus 3.0 software. The exclusion and likelihood approach was able to predict the male parents of 72 progenies with more than 95% level of confidence for 10 male parent–progeny pairs and 80% level of confidence or less for the remaining pairs. The comparison of paternity analyses based on the putative specific alleles and exclusion-likelihood approach revealed similarities in the estimation for six male parent–progeny pairs, namely, GMB-9-O2.7, GMB-7–O2.13, GMB-7–O3.11, GMB-7–O4.7, GMB-7–O4.8, and GMB-9–O4.10 (Table 7).

The paternity analysis using the putative specific allele method revealed that the male parent–progeny allele similarity was relatively low with an average of 11.38%, whereas the exclusion-likelihood method identified numerous mismatched loci likely as a result of several factors, including a limited number of parent accessions used in this study, and some progenies had alleles that could not be found in the female or male parent genotype. As observed by other researchers, the contribution of the shared and unshared parents cannot be unambiguously determined if the shared parent and one of its progeny have the same heterozygous genotype (Fiumera and Asmussen, 2001). Therefore, distinct parental candidates are required for accurate paternity analysis.

Pollinator involvement may also increase the occurrence of cross-pollination. Pollinators, such as flies (*Diptera* spp.) and bees, can carry pollen from relatively long distances for cross-pollination in tea plants (Mitra *et al*., 2017). The amount of pollen that is carried by the insect and deposited on the stigma may be influenced by the spatial isolation and population size that may reduce the pollinator visitation frequency in tristylous populations (Hodgins and Barret, 2006). A previous study showed that the fitness of *C. sinensis* pollen with respect to the germination ability varies due to many factors, such as pollen shedding duration, and environmental factors, such as temperature and rainfall (Muoki *et al*., 2007). Another factor that may play a significant role in low parent–progeny allelic similarity was the limited number of genetic markers used in this study because for successful paternity analysis, highly polymorphic markers are required (Jones *et al*., 2010). Therefore, the use of a very large number of markers with a high level of polymorphism is recommended for successful parentage analysis.

CONCLUSIONS

The results showed that the exclusion-likelihood approach was more effective than the putative specific allele method in the prediction of the male parents of F_1 tea progenies. The exclusion-likelihood method predicted 100% of the male parents, whereas the putative specific allele method predicted 34.72% of the male parents in 72 hybridized F_1 progenies.

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REFERENCES

Akula A, Akula C (1999) Somatic Embryogenesis in Tea (*Camellia sinensis* (L.) O. Kuntze). In: Jain SM, Gupta PK, Newton RJ (eds) Somatic Embryogenesis in Woody Plants. Forestry Sciences, vol. 59. Springer, Dordrecht. https://doi.org/10.1007/978-94-011-4774-3_15.

Azka NA, Widhianata H, Taryono (2019) Morphological and molecular characterization of 5 accessins of tea (*Camellia sinensis* (L.) O. Kuntze) exploited to develop high quality and quantity yield. 1st International Conference on Bioinformatics, Biotechnology, and Biomedical Engineering (Biomic 2018). *AIP Conf. Proc. 2099. https://doi.org/10.1063/1.5098408 .*

Bandyopadhyay T (2011). Molecular marker technology in genetic improvement of tea. *Int, J. Plant Breed. Genet.* 5: 23-33.

Buteler MI, LaBonte DR (2002) Microsatellite-based paternity analysis in polyploidy sweetpotato. *J. Amer. Soc. Hort. Sci.* 127(3): 392-396.

Chahal GS, Gosal SS (2002). Principles and Procedures of Plant Breeding: Biotechnological and Conventional Approaches. Alpha Sci. Int. Ltd., UK.

Chen D, Milacic V, Chen MS, Wan SB, Lam WH, Huo C, Landis-Piwowar KR, Cui QC, Wali A, Chan TH, Ping Q (2008). Tea polyphenols, their biological effects and potential molecular targets. *Histol. Histopathol*. 23(4): 487-96.

Diaz A, Martin A, Rallo P, Barranco D, De-la-Rosa R (2006). Self-incompatibility of Arbequina and Picual olive assessed by SSR markers. *J. Am. Soc. Hort. Sci.* 131: 250-255.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. *Focus* 12: 13-15.

Fan FY, Yan DH, Borthakur D, Liang YR, Luo XY, Wei J, Liu SC, Lu JL (2011). Application of molecular tools in parentage identification and gene diversity study in tea (*Camellia sinensis*) germplasm of Guizhou province, China. *Two and a Bud* 58: 39-43.

Fiumera AC, Asmussen MA (2001). Difficulties in parentage analysis: the probability that an offspring and parent have the same heterozygous genotype. *Genet. Res. Camb.* 78: 163-170.

Freeman S, West J, James C, Lea V, Mayes S (2004). Isolation and characterization of highly polymorphic microsatellites in tea (*Camellia sinensis*). *Mol. Ecol. Notes* 4: 324-326.

Gjedrem T (2010) The first family-based breeding program in aquaculture. *Rev. Aquac.* 2:2-15.

Govindaraj P, Balamurugan A, Natarajam US (2012). Identification of intergenic hybrid between *Erianthus arundinaceus* and *Saccharum spontaneum* through STMS markers. *Int. Sugar J.* 114: 350-356.

Hodgins KA, Barrett SCH (2006). Female reproductive success and the evolution of mating-type frequencies in tristylous populations. *New Phytol.* 171: 569–580.

Jones AG, Ardren WR (2003). Methods of parentage analysis in natural populations. *Mol. Ecol*. 12: 2511-2523.

Jones AG, Small CM, Paczolt KA, Ratterman NL (2010). A practical guide to methods of parentage analysis. *Mol. Ecol. Resour.* 10: 6-30.

Kalinowski ST, Taper ML, Marshall TC (2007). Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol. Ecol.* 16: 1099-1106.

Kottawa-Arachchi JD, Gunasekare MTK, Ranatunga MAB (2019). Biochemical diversity of global tea [*Camellia sinensis* (L.) O. Kuntze] germplasm and its exploitation: A Review. *Genet. Resour. Crop Evol.* 66: 259-273.

Mitra B, Roy S, Shah SK, Mishra P (2017). Inventorisation of true flies (Insecta: Diptera) and their association with tea plants in Dooars, West Bengal, India. *Int. J. Entomol. Res.* 2: 21-26.

Mookerjee S, Guerin J, Collins G, Ford C, Sedgley M (2005). Paternity analysis using microsatellite markers to identify pollen donors in an olive grove. *Theor. Appl. Genet.* 111: 1174-1182.

Muoki CR, Wachira FN, Pathak RS, Kamunya SM (2007) Assessment of the mating system of Camellia sinensis in biclonal seed orchards based on PCR markers. *J. Hort. Sci. Biotechnol.* 82(5):733-738.

Navascues M, Emerson BC (2005) Chloroplast microsatellites: Measures of genetic diversity and the effect of homoplasy. *Mol. Ecol.* 14(5):1333-13341.

Nisa, YS (2020) Morphological and SSR characterization of tea accessions (*Camellia Sinensis* (L.) O. Kuntze) in Kayulandak Polyclonal Garden of PT Pagilaran. *Manuscript in preparation.*

Norman PE, Asfaw A, Tongoona PB, Danquah A, Danquah EY, Koeyer DD, Asiedu R (2018) Can parentage analysis facilitate breeding activities in root and tuber crops? *MDPI J. Agric.* 95(8):1-24.

Ramakrishnan M, Rajanna L, Papanna N, Simon L (2009). Assessment of genetic relationship and hybrid evaluation studies in tea (*Camellia* sp.) by RAPD. *Int. J. Plant Breed.* 3: 144-148.

Spanoghe M, Marique T, Riviere J, Lanterbecq D, Gadenne M (2015). Investigation and development of potato parentage analysis methods using multiplexed SSR fingerprinting. *Potato Res.* 58:43-65.

Thongthawae S, Tittinutchanon P, Volkaert H (2010) Microsatellites for parentage analysis in an oil palm breeding population. *Thai Journal of Genetics* 3(2):172-181.

Vieira MLC, Santini L, Diniz AL, Munhoz CF (2016). Microsatellite markers: what they mean and why they are so useful. *Genet. Mol. Biol.* 39: 312-328.

Wambulwa MC, Meegahakumbura MK, Chalo R, Kamunya S, Muchugi A, Xu J (2016). Nuclear microsatellites reveal the genetic architecture and breeding history of tea germplasm of East Africa. *Tree Genet. Genomes* 12: 11. https://doi.org/10.1007/s11295-015-0963-x

Yamamoto T, Mochida K, Imai T, Shi YZ, Ogiwara I, Hayashi T (2002). Microsatellite markers in peach (*Prunus persica* (L.) Batsch) derived from an enriched genomic and cDNA library. *Mol. Ecol. Notes* 2: 298-301.

Zhang CC, Wang LY, Wei K, Wu LY, Li HL, Zhang F, Cheng H, Li DJ (2016). Transcriptome analysis reveals self-incompatibility in the tea plant (*Camellia sinensis*) might be under gametophytic control. *BMC Genomics* 17: 359. https://doi.org/10.1186/s12864-016-2703-5.