Journal of Oil Palm Research
DOI: https://doi.org/10.21894/jopr.2025.0042

SSR MARKERS FACILITATE TESTING OF GENETIC PURITY AND PREDICTION OF BIOLOGICAL PARENTS FOR BREEDING CROSSES OF OIL PALM

NORHALIDA MOHAMED SERDARI[1,3]*; TING NGOOT-CHIN[1]; RAJINDER SINGH[1]; KANDHA SRITHARAN[2]; MARYAM MOHAMED REHAN[3]; JALANI BIN SUKAIMI[3] and MAIZURA ITHNIN[1]

ABSTRACT

The genetic purity of a controlled cross is essential for achieving the objectives of a breeding program for any crop. This study evaluated SSR markers for assessing the legitimacy of two oil palm interspecific BC_1 *x* BC_1 *populations, namely, Populations 9 and 10. A total of 40 primer-pairs were screened, resulting in the identification of 19 and 20 informative markers for Populations 9 and 10, respectively. The markers revealed sufficient genetic variation in allele frequency, heterozygosity and polymorphic information content in both populations. The phylogenetic trees revealed three and 32 outliers in Populations 9 and 10, respectively. In addition, the palms originally recorded as parents to the crosses had numerous loci that were mismatched with the non-outlier offspring. We reconstructed the expected genotypes of the real parents based on the segregation profiles observed among the legitimate offspring in the two families, resulting in 100% parent-offspring assignment. This study discusses how the SSR genotypes of palms were examined to detect outliers and to predict the genotypes of the true parents in these interspecific hybrids, the first such report for oil palm. The information will be useful for various controlled crosses of oil palm interspecific hybrids and other outcrossing plant species.*

Keywords: BC_1 x BC_1, genetic purity, interspecific hybrid, parental genotypes reconstruction.

Received: 17 October 2024; **Accepted:** 26 May 2025;

INTRODUCTION

The genus *Elaeis* (Arecaceae) consists of *E. guineensis*, which originated from Central and West Africa, and *E. oleifera* from Latin America. Both species produce versatile oil that has significant economic importance (Corley & Tinker, 2015; Rajanaidu *et al.*, 2017). The two palm species exhibit contrasting growth characteristics where, *E. guineensis* grows 45-75 cm/yr (Barcelos *et al*., 2015) with an upright vascular stem that can reach over 12 m after 25 years, whereas *E. oleifera* is naturally shorter, as its trunk grows around 5-10 cm/yr (Corley & Tinker, 2015; Rajanaidu *et al.*, 2017; Rivera *et al.*, 2013). Their fruit morphology and oil composition are also very different. *E. guineensis* produces large fruitlets with thick mesocarp, coated with deep-violet/black colored skin when young and turns red when ripe. In contrast, *E. oleifera* produces much smaller fruitlets with a thinner layer of mesocarp, and the yellowish-green skin changes to ivory/orange when the fruits are ripe (Corley & Tinker, 2015). The distinctive change in color upon ripening is somewhat similar to virescens fruits observed in *E. guineensis* (Singh *et al.*, 2014). The thick fleshy mesocarp is rich in oil, making *E. guineensis* the oil palm of commerce. *E. oleifera* is well-known for its natural high unsaturated oil, with the composition of oleic and linoleic acids comparable to that of olive and canola oils (Corley & Tinker, 2015; Mendoza *et al.*, 2023).

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[1] Malaysian Palm Oil Board,
6, Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.

[2] United Plantation Berhad, Jendarata Estate,
36009 Teluk Intan, Perak, Malaysia.

[3] Universiti Sains Islam Malaysia, Bandar Baru Nilai,
71800 Nilai, Negeri Sembilan, Malaysia.

* *Corresponding author e-mail: norhalida@mpob.gov.my*

The two species of oil palm can crossbreed and generate fertile interspecific hybrids, which are tolerant to bud rot disease (also known as lethal decay), have a slower increment of height (Hormaza *et al.*, 2012; Madon *et al.*, 2018; Meléndez & Ponce, 2016) and produce oil with higher levels of unsaturated fatty acids than *E. guineensis* (Lieb *et al.*, 2017; Mozzon *et al.*, 2020). However, pollen viability, fruit sets and seed germination rates of the hybrids are relatively lower (Daza *et al.*, 2020; Escobar & Chauza, 2018; Hardon & Tan, 1969; Hormaza *et al.*, 2012; Mosquera *et al.*, 2021; Romero *et al.*, 2021; Socha *et al.*, 2019). These could be due to incomplete chromosome-pairing and an increasing number of univalent chromosomes (Hardon & Tan, 1969; Sunilkumar *et al.*, 2013). One way to overcome the constraints is to backcross the interspecific hybrids with a carefully selected *E. guineensis,* aiming to generate more viable and good-yielding backcross palms and yet possessing the desired traits of *E. oleifera* (Noh *et al.*, 2012). The backcross-breeding strategy has also been successfully adopted in rice, where several disease resistance traits were introgressed from the wild species into the cultivated varieties (Collard & Mackill, 2008; Kannan *et al.*, 2017; Sharma *et al.*, 2012) as well as, for the improvement of grain quality, amylose content, aroma and seed weight traits (Bao *et al.*, 2014; Cho *et al.*, 2020; Miura *et al.*, 2022). In *Elaeis*, repeating backcross to the recurrent parent (*E. guineensis*), aiming to eliminate undesirable traits of *E. oleifera*, could cumulatively reduce the genetic variability in subsequent generations. Nonetheless, genetic variability can be slightly increased by backcrossing to a different elite *E. guineensis*. Alternatively, intercrossing BC_1 individuals can help increase the genetic viability while stabilising the genetic background. This breeding strategy may also help combine desirable traits, particularly quantitative traits. Therefore, BC_1 x BC_1 crosses were initiated in this study with the long-term objectives of evaluating the levels of genetic heterozygosity in these crosses and the efficiency of trait introgression, particularly in comparison to the conventional interspecific backcross breeding programs of oil palm.

In oil palm breeding, potential parental palms are continuously evaluated through well-designed controlled crossing schemes and field experimental plots. The primary steps of this important activity include isolating pollen from selected male parents, bagging flowers on selected female palms and introducing the desired pollen onto the bagged flowers. Production of low number male inflorescences coupled with the small quantity and poor viability of the pollen has been proven a major challenge in utilising interspecific hybrids as the pollen donor (Daza *et al.*, 2020; Meléndez-Jácome *et al.*, 2024; Meléndez & Ponce, 2016; Mosquera *et al.*, 2021), making the interspecific breeding program a time-consuming process. The procedures involved in the breeding steps must be strictly complied with, to make sure that the offspring produced are free from illegitimates. In addition, great care is also undertaken during seed processing and germination, nursery development and field planting to prevent mix-up, which can significantly affect the quality and integrity of a breeding program (Budiman *et al.*, 2019; Josia *et al.*, 2021).

In recent years, DNA markers have been applied for genetic fingerprinting and identification of outliers and illegitimates in the controlled crosses involving pure *E. guineensis* (Corley *et al.*, 2005; Okoye *et al.*, 2020; Pomiès *et al.*, 2019; Teh *et al.*, 2019; Zolkafli *et al.*, 2021). However, the use of DNA markers for accessing the genetic purity and parentage analysis in interspecific crosses has not been commonly reported. In other crops *e.g.*, sunflower, maize, wild rye, cabbage and coconut, simple sequence repeat (SSR) panels ranging from 10-100 markers have been commonly used to screen for genetic purity in breeding trials and identification of true parental lines (Daniel *et al.*, 2012; Liu *et al.*, 2007; Pallavi *et al.*, 2011; Rajesh *et al.*, 2012; Saiful-Lazim *et al.*, 2024; Shinde *et al.*, 2021; Sudha *et al.*, 2022; Wang *et al.*, 2002). SSR markers have been applied in small-scale legitimacy assessment in oil palm due to their abundance in the genome, high reproducibility and polymorphism owing to their multi-allelic nature (Rosli *et al.*, 2022; Singh *et al.*, 2007). Furthermore, the genotype datasets are also useful for genetic diversity and lineage assessments, particularly, for the valuable materials owned by an organisation (Ahmad *et al.*, 2018; Saiful-Lazim *et al.*, 2024; Sudha *et al.*, 2022). By leveraging DNA markers, the overall efficiency and precision of a breeding program can be enhanced, which, ultimately, can lead to the precise development of improved planting materials.

This study evaluates the efficiency of SSR markers in assessing the genetic purity in two interspecific BC_1 x BC_1 crosses as the prerequisite to ensure the integrity of the established breeding populations. The two-level check was performed, first to identify outliers among the offspring and subsequently, to validate their biological parents. Using the multi-allelic SSR markers, we demonstrated that it is also possible to reconstruct the genotypes of the biological parents of a family.

MATERIALS AND METHODS

Palm Samples

Two interspecific backcrosses of one intercrossing (BC_1 x BC_1) populations, namely, Population 9 (trial 6.6-9.0) and 10 (trial 6.6-10.0), were included in the present study. Population 9 (332/5.2-5.0 x 19/5.2-6.0) and 10 (438/5.2-5.0 x 19/5.2-6.0) consisting of 95 and 110 progeny palms, respectively (*Figure 1*), were created by crossing two sibs of the same family to a common paternal palm (19/5.2-6.0).

DNA Extraction

Young leaves were collected and preserved in the -80°C freezer while waiting for DNA extraction. The extraction of DNA was carried out using the modified CTAB method (Suzana *et al.*, 2015). The quality of extracted DNA was checked by restriction enzymes (*Hae*III and *Eco*RI) digestion and gel electrophoresis (1.0% agarose). This was followed by measuring the optical density (OD) of DNA using the Multiskan Go spectrophotometer (Thermoscientific, USA) to determine their purity (A_{260}/A_{280} and A_{260}/A_{230}) and concentration (ng/µL). Good quality DNA samples with purity ≥1.8 were diluted to the 50 ng/µL working stocks for SSR analysis.

SSR Markers Screening and Genotyping

Screening was first carried out using 40 SSR primer-pairs (*Table 1*) in a subset of 28 samples (including the parents) to identify informative SSR markers. Subsequently, all samples in both populations were genotyped using the polymorphic SSR markers pre-selected from the screening experiment. The standard polymerase chain reaction (PCR) conditions as recommended by the manufacturer (Meridian Bioscience, USA), were applied in both screening and genotyping procedures. The PCR reaction for each sample contained 2.0 µL DNA (50 ng/µL), 0.03 µl M13-forward primer, 0.03 µL M13-flourescent dye, 0.03 µL reverse primer, 5.0 µL 2X myTaq™ master mix (Meridian Bioscience, USA) and 2.9 µL dH_2O. The Fluorescent Dye Set consisting of ROX, 6-FAM, VIC and NED was used for multiplexing and differentiating the SSR amplicons during fragment analysis (Applied Biosystems, USA). Sizing and calling the SSR alleles were carried out using GeneMapper™ v4.1 (Applied Biosystems, USA). Alleles observed were coded according to the bi-parental segregation configurations (Billotte *et al.*, 2005; Ting *et al.*, 2014).

SSR Data Analysis

The Neighbor-joining phylogenetic trees were constructed using DARwin 6.0 (Perrier & Jacquemoud-Collet, 2006). Allele frequency, simulation and parentage analyses were carried out using CERVUS 3.0.7 (Kalinowski *et al.*, 2007, 2010; Marshall *et al.*, 1998). For parentage assignments, the logarithm of the odds (LOD) at a 95.0% confidence level was determined through simulations performed on 100,000 samples, 1.0% typing error and 90.0% sampling proportion, executed separately for Populations 9 and 10. The first round of the parentage analysis was carried out to validate whether the candidate parents provided were the true parents. For comparison, a set of predicted genotype calls was also configured for each of the parents based on the genotypes observed among the legitimate progeny palms. The predicted parental genotypes were then included in the second round of parentage analysis (with parent sexes unknown).

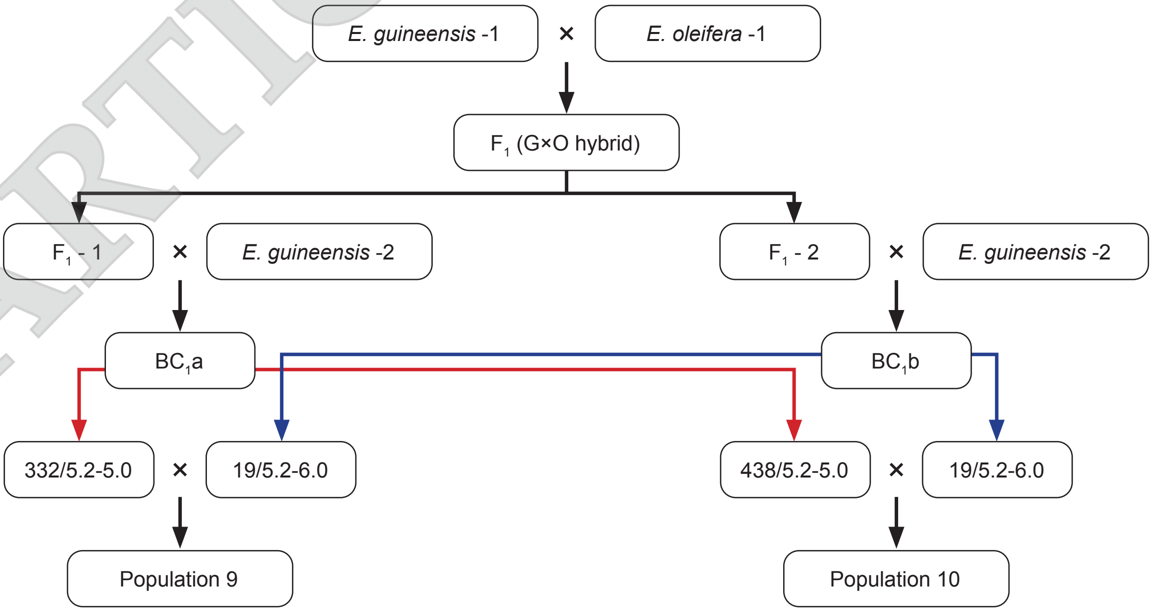

Figure 1. Pedigree of the interspecific BC_1 x BC_1 populations in this study.

TABLE 1. LIST OF 40 SSR PRIMER-PAIRS FOR SCREENING POPULATIONS 9 AND 10

No.	SSR marker	Forward sequence	TM (°C)	Reverse sequence	TM (°C)	TA (°C)	Expected fragment size (bp)	Observed fragment size (bp)	Reference
1	sEg00011*	GGAACCACGTTGGAGACTGT	60	TCCGACAAACTAGCAAAGCA	60	57	135	146-159	Low *et al.* (2008); Yaakub *et al.* (2020)
2	sEg00117*	GAGTGGTAGTGGCTATGGTCT	56	CCGTTGTATCTGTTCTCGTAGT	56	53	150	166-173	Low *et al.* (2008); Yaakub *et al.* (2020)
3	sMo00166*	CATAACATGCATTCACGGCT	60	AAGGGGCGGAAGGATTACTA	60	57	151	167-198	Yaakub *et al.* (2020); Ting *et al.* (2014)
4	Hght7_oSSR*	ATCCAACTTCCAACCAACCA	60	acgTGGGGATGTAGTTCCAA	60	58	203	176-204	Yaakub *et al.* (2020)
5	sMg00175*	TTTGTCTCCGTTTCCTTCTCT	58	CCCTAATCTCCTTCATCTCCTC	58	54	173	177-187	Ting *et al.* (2013); Yaakub *et al.* (2020)
6	sMo00132	ATAGCCAGAGGGCAAAACTGT	60	GCAACACACGGACTCAAAACTA	60	58	161	180-198	Yaakub *et al.* (2020); Zaki *et al.* (2010)
7	sEg00167*	AAAGAAAACACAACCACTGCC	59	AGGCTTATCGGAGATGGGAT	60	57	171	188-201	Low *et al.* (2008); Yaakub *et al.* (2020)
8	sMo00138*	AGGGTTGTCGCTCCAATTTAT	60	GGCATCTTTTTGACCTGTAGAAG	59	56	190	194-210	Yaakub *et al.* (2020); Zaki *et al.* (2010)
9	sEg00213*	TCCCCCAATACTCATCATGC	60	TGATCGACGGTTGTCACATT	60	57	178	200-209	Yaakub *et al.* (2020)
10	sMo00055*	GGCATTTCAGATAACGACAAA	58	GCACCCAAGTCTCTCTACCTC	58	54	202	205-216	Yaakub *et al.* (2020)
11	sMg00196*	CACAAACCAGGATAGCACACT	58	GTTGCTGGAGGTAGGGAAATA	58	56	200	208-230	Yaakub *et al.* (2020)
12	sEg00017	ATGGGTTGCTAATCTGTGGG	60	CATAAGAAGCAGAACCGTTGC	60	57	107	217-138	Low *et al.* (2008); Yaakub *et al.* (2020)
13	sPSc00488*	TTTCCTCGGAATGCTATTGG	60	TCCTCTCCCCAAAGGAAAAT	60	57	215	229-249	-
14	sEg00126*	CCGTCTCAAAAGCCCTAAAC	59	TTGTTGTCCCACTCCCTCTT	60	52	216	231-234	Yaakub *et al.* (2020); Ting *et al.* (2010)
15	sMo00027	TTACAGTTGAGGCAGTATGTCAAT	58	CTGTATGTCAAACCTTCTGCAC	57	50	209	235-252	Ting *et al.* (2013); Yaakub *et al.* (2020); Zaki *et al.* (2010)
16	sMg00051*	CCCTCCCTCTCCTTTTTATC	57	CCCTTCTCTCTCTCGTGTTAT	55	52	231	235-252	Ting *et al.* (2013); Yaakub *et al.* (2020)
17	sMg00011*	GTCCAAACCCTAACCCTAAC	55	GTCCTCGTAGTCCTCCTCTT	55	52	232	240-254	Yaakub *et al.* (2020)
18	sMo00246*	TGATGATGGAAAAGCTGCTG	56	TCCACCACAAATCAGGTCAG	60	53	220	241-249	Yaakub *et al.* (2020)
19	sMo00126*	AAGAGCAAGGGGAGAACTGACT	61	GTAAACACGCACACGGACAC	60	58	232	242-255	Yaakub *et al.* (2020)
20	sMg00235*	ATGGGAAGAGTACCAGTGCTT	58	CACCCTTTGCTTCAATTCAG	58	58	235	245-273	Ting *et al.* (2013); Yaakub *et al.* (2020)
21	sMg00168*	CGGGTAGGAACAAAAGAACC	59	GACGAGCAAGAGTCCAGAGAG	59	55	232	246-259	Ting *et al.* (2013); Zolkafli *et al.* (2021)

TABLE 1. LIST OF 40 SSR PRIMER-PAIRS FOR SCREENING POPULATIONS 9 AND 10 (continued)

No.	SSR marker	Forward sequence	TM (°C)	Reverse sequence	TM (°C)	TA (°C)	Expected fragment size (bp)	Observed fragment size (bp)	Reference
22	sMo00066	GTACAAGATGGAAAGGGAAAAA	57	TCAAGGGTCAGTTCATAAATCA	57	55	247	246-265	Yaakub *et al*. (2020)
23	sMo00222*	TCCACCCTTTGCTTCAATTC	60	AGAGTGGGGATGGGAAGAGT	60	57	236	255-285	Ting *et al*. (2013); Yaakub *et al*. (2020)
24	sEg00154*	TCCCCCAATACTCATCATGC	61	TGATCGACGGTTGTCACATT	60	57	238	257-268	Ting *et al*. (2013); Yaakub *et al*. (2020); Zolkafli *et al*. (2021)
25	sPSc00546*	GTCTAAATCCTATTCGATGGTGTT	58	CACAGCGTGGTTTCTCTGTT	59	56	241	258-264	Yaakub *et al*. (2020)
26	sMg00071*	CCAAATGTAAAAGGGGAAAATAA	58	GACGAGGAGAACGAAGGTAGA	59	54	272	270-284	Ting *et al*. (2013); Yaakub *et al*. (2020); Zolkafli *et al*. (2021)
27	sEg00019*	AGTGAAGCCCTTTCCCAAAT	60	CAGGCACCTGTAAGGGAAAA	60	57	261	271-281	Low *et al*. (2008); Yaakub *et al*. (2020)
28	sEg00146	TGTAGCAGCTGCAGAAGAGC	60	TCCTTTTTCTTGCTTTGCGT	60	57	210	245	Low *et al*. (2008); Yaakub *et al*. (2020)
29	sMg00030	AGCAAGGAATTTGAACTCTA	52	GATACCTCCCTTCTTGATGC	56	52	272	290	-
30	sMo00208	GCATGGCCAGTTTTCCTAGA	60	TAGCCCATATAGCACCCTGC	60	58	257	285	Ting *et al*. (2013); Yaakub *et al*. (2020)
31	sMg00093	CAATAACCTGAAACCCCTCTAATC	59	ATCTCCATCACCCTCTTCTTCTC	60	57	241	Not amplified	Yaakub *et al*. (2020)
32	sMg00066	TCTTGTTTCCGTTACTTGATGTT	58	AAGTTCTCGGCCTTCAAAAG	59	52	200	Not amplified	Ting *et al*. (2014); Yaakub *et al*. (2020)
33	sMo00023	TGACCCAATGATACCACCAC	59	GAGAACACAAACAGATGCACAC	58	53	243	Not amplified	Ting *et al*. (2013); Yaakub *et al*. (2020)
34	sPSc00481	TTTTCTGGATAAGTTGCTAAGATTT	57	CAACTCTCTTCCCTCCTCCTG	60	55	222	Not amplified	-
35	sMo00088	AGATACTGAGGGAAAGGTTAGG	56	TTCAAGACGAAACTGGTAAAA	55	54	204	Non-specific	Yaakub *et al*. (2020)
36	sMg00152	ATCGGACCAGCGCATATCTAA	62	CCTTGGGATTCTTATGGAACTTG	61	54	243	Non-specific	Ting *et al*. (2013); Yaakub *et al*. (2020)
37	sPSc00550	TAGATTCTTTCCGTGCGCTT	60	AAGAAGCTAAAAGGGCCCAC	60	57	185	Non-specific	-
38	sEg00072	TCTCTATGCAGCAGCCAAAT	59	ATAGACAACAGGCTTTTCATCA	57	54	136	Non-specific	Low *et al*. (2008); Yaakub *et al*. (2020)
39	sMg00016	GCGATTCCGGTTATCTTTAG	57	GAGTTTTTGCTGTGATGATTAG	54	52	274	Non-specific	Ting *et al*. (2014); Yaakub *et al*. (2020)
40	sMg00063	CACGAAAACGATAATAAAGTACA	54	ATGTGCCAGAACTTTTGTCTAT	56	52	209	Non-specific	Ting *et al*. (2014); Yaakub *et al*. (2020)

Note: * informative marker; TM - melting temperature; TA - annealing temperature.

RESULTS AND DISCUSSION

Informative SSR Markers

Of the 40 SSR markers screened, a total of 19 and 20 polymorphic markers were identified following the analysis of selected palms from Populations 9 and 10, respectively. The remaining markers were either monomorphic, not amplifiable, or amplified with unspecific and ambiguous bands. The shortlisted informative SSR markers were then used to genotype all palms of the two populations, and the observed allelic information is summarised in *Figure 2*. For Population 9, the number of alleles (k) identified by the SSR markers ranged from two to six, where two alleles were observed in 31.6% of the markers, three to four segregating alleles were observed in 52.6% of the markers whereas the remaining 15.8% of the markers revealed five to six segregating alleles. For Population 10, 25.0% of the markers showed two alleles, 50.0% detected three to four segregating alleles and 25.0% showed five to six segregating alleles.

When the SSR markers were ranked from low to high in terms of the number of alleles (*Figure 2*), a clear pattern was also observed, where increased levels of observed heterozygosity (H_{Obs}), expected heterozygosity (H_{Exp}) and polymorphic information content (PIC) were detected. This trend was consistent for both populations where markers with a lower number of alleles (*e.g.*, two and three) had on average lower H_{Obs} (0.41 and 0.48 for Population 9 and 0.33 and 0.46 for Population 10) and PIC (0.34 and 0.38 for Population 9 and 0.51 and 0.72 for Population 10) than markers with four to six segregating alleles. In this set of markers, the average of H_{Obs} and PIC ranged from 0.70-0.99 and 0.51-0.72 (for Population 9) and 0.67-0.73 and 0.52-0.60 (for Population 10). The present marker panel also included four to five markers that deviated from the Hardy-Weinberg equilibrium (HWE), and this is not uncommon for such a small population as Population 9 and 10. In general, the results indicated that the selected SSR markers are useful for assessing the genetic variation in the two interspecific backcross populations analysed.

Progeny Outliers

The Neighbor-joining phylogenetic trees revealed three and 32 outliers among the offspring in Population 9 and 10, respectively (*Figure 3*). These outliers formed a separate sub-cluster in Population 9, whereas in Population 10, the outliers formed two sub-clusters. The two sub-clusters in Population 10 are likely to be closely related, possibly with one common parent. Further examination of their genotypes confirmed that the outliers in both populations were indeed illegitimates. For the three outliers in Population 9, different genotypes were observed in 14 SSR markers. For instance, in sMg00196, the outliers were called *dd* and *ae*, whereas, genotypes for other progeny palms were either *ab* or *bb*. In marker sMg00051, genotypes *cc* and *bd* were only observed in the three outliers, but the likely legitimate sibs had genotype calls *ab, ac, bb* and *bc*.

For Population 10, distinct genotypes in the outliers were also observed in 14 SSR markers, including the markers described in the two examples above. The two markers (sMg00196 and sMg00051) revealed a mixture of *aa, ab, ac* and *bc* genotypes in most of the progenies in Population 10. However, for the 32 outliers, distinct genotypes such as *ad, af, bb, cc, cf* and *de* were observed. As a result, the outliers were removed from further analysis and the number of justifiable legitimate offspring for Populations 9 and 10 was 92 and 78, respectively (*Figure 3b*).

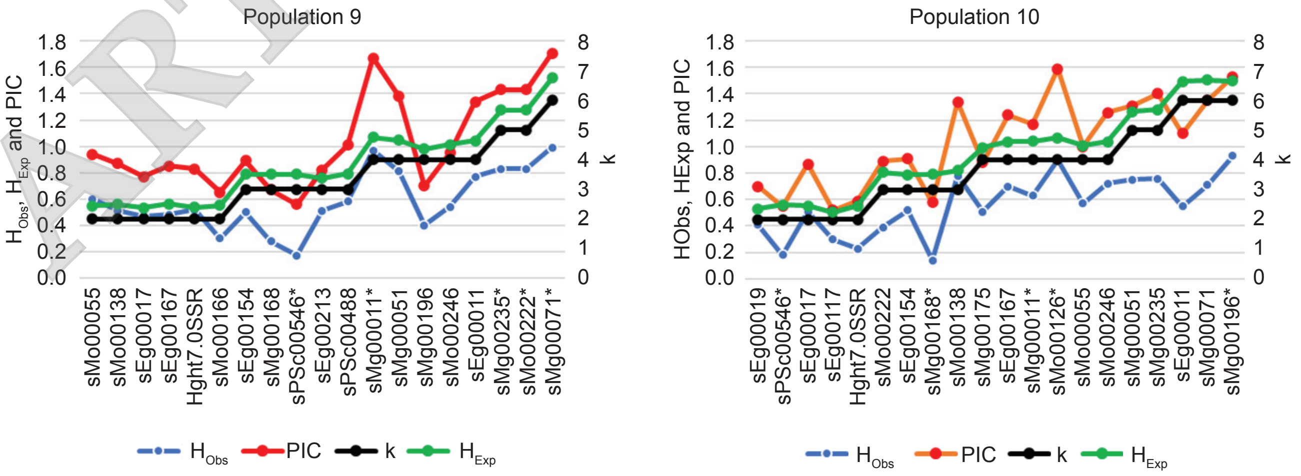

Figure 2. SSR allelic information including number of alleles (k), observed heterozygosity (H_{Obs}), expected heterozygosity (H_{Exp}) and polymorphic information content (PIC) for Populations 9 and 10. Markers (*) are those that deviated from the Hardy-Weinberg equilibrium at $p<0.001$.

Figure 3. The Neighbor-joining phylogenetic trees for Populations 9 and 10 show the original candidate parents in green (P1_ and P2_ori), the predicted parents in blue (P1_and P2_pred), outliers in red and the legitimate offspring in black.

Miss-assignment of Parents for the Selected Crosses

The parentage analysis was first performed by including palms 332/5.2-5.0 and 19/5.2-6.0 (for Population 9) and 438/5.2-5.0 and 19/5.2-6.0 (for Population 10) as the parental candidates, based on earlier records. This analysis was conducted for the legitimate offspring after the removal of outliers. Unfortunately, no significant assignment was obtained, indicating that the candidate parents are not the true parents (*Figure 4*).

To verify this further, we re-examined the genotype calls of selected SSR markers. Markers sEg00117 and sEg00019 revealed heterozygous genotypes (of two and three alleles) in the two parental palms of Population 9, showing monomorphic genotypes (of single allele) among the offspring. Following the Mendelian inheritance, crossing of the two heterozygous alleles, *e.g.*, *ab* x *ab* in the parents, was expected to recombine and produce the *aa*, *ab* and *bb* offspring. Similarly, crossing two parents with *ab* and *ac* heterozygous alleles (revealed by sEg00019) was expected to give rise to a mixture of *aa*, *ab*, *ac* and *bc* genotypes in their next generation instead of the monomorphic *cc* genotype that was observed throughout the offspring.

Unexpected genotypes in the parents of Population 9 were also observed for another six SSR markers (*Table 2*) - sEg00213, sMo00222, sMo00166, Hght7_oSSR, sMg00235 and sMo00132 where the maternal and paternal palms were genotyped as *aa* and *bb*, respectively. However, the progeny palms had *ac*, *cc*, *bb* and *bc* instead of the expected *ab* genotype. Among the six markers, sEg00213 revealed a similar scenario in Population 10, where the respective female and male parents were *aa* and *bb*, but the offspring exhibited up to four different genotypes.

Furthermore, the sMo00222, sMo00166 and Hght7_oSSR genotype data showed that both parental palms of Population 10 were homozygous (*aa*); however, their offspring were unexpectedly heterozygous, consisting of *ab*, *ac*, *bb* and *bc* genotypes (*Table 3*). In another example observed for Population 10, the parental genotypes for markers sMg00011, sMg00051, sMo00055, sMg00071, sEg00154 and sMg00235 were *ab*, which was expected to segregate and form three genotypes (*aa*, *ab* and *bb*) in their offspring. Instead, two to five different genotypes were observed among the progeny palms. These observations indicate that the candidate parents sampled for Population 9 and 10 may not be the true parents. Mislabeling or mix-up could have occurred, and these have been described as the potential causes of illegitimacy among oil palm progenies (Budiman *et al*., 2019; Corley, 2005; Hama-Ali *et al*., 2014; Thongtawee *et al*., 2010; Zolkafli *et al*., 2021).

Prediction of the Genotypes of the Biological Parents

To predict the genotypes of the actual biological parents, only genotype calls among legitimates were analysed while outliers were excluded. As an example, when genotypes *ac*, *bc*, *ad* and *bd* were observed in the offspring, the expected parental genotypes would be *ab* and *cd*. The predicted parental genotypes for all the informative markers (*Table 4*) were included as the new candidate parents for parentage analysis, resulting in 100% of the progeny palms in Populations 9 and 10 being successfully assigned to the predicted parent-pairs at the 95% confidence threshold (*Table 5*).

Although the sexes for the two predicted parents were not known, parent pairs comparison (offspring-first parent and offspring-second parent) showed a perfect match across all the loci analysed. Similarly, for the trio comparison, the trio logarithm of the odds (LOD) scores ranging from 0.05-0.48 were observed in 98.0% offspring of Population 9, and LOD scores of 0.06-5.90 in 80.8% offspring of Population 10.

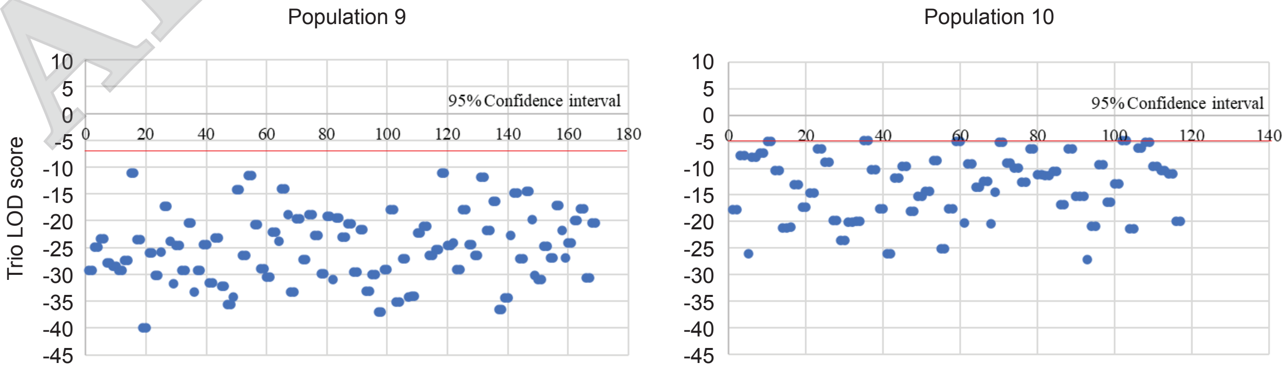

Figure 4. Distribution of trio LOD scores observed for Populations 9 and 10. No offspring were assigned to the sampled candidate parents.

TABLE 2. SUMMARY OF UNEXPECTED GENOTYPES OBSERVED IN POPULATION 9

SSR marker	**Parental genotype**		**92 offsprings genotype**						
	332/5.2-5.0	**19/5.2-6.0**	***aa***	***ab***	***bb***	***cc***	***ac***	***bc***	**missing**
sEg00117	*ab*	*ab*	92	-	-	-	-	-	-
sEg00019	*ab*	*ac*	-	-	-	92	-	-	-
sEg00213	*aa*	*bb*	46	46	-	-	-	-	-
sMo00222	*aa*	*bb*	-	25	15	-	31	20	1
Hght7_oSSR	*aa*	*bb*	-	49	43	-	-	-	-
sMg00235	*aa*	*bb*	-	25	15	-	28	22	2
sMo00132	*aa*	*bb*	50	-	38	-	-	-	4

TABLE 3. SUMMARY OF UNEXPECTED GENOTYPES OBSERVED IN POPULATION 10

SSR marker	**Parental genotype**		**78 offsprings genotype**						
	438/5.2-5.0	**19/5.2-6.0**	***aa***	***ab***	***bb***	***bc***	***ac***	***cc***	**missing**
sEg00213	*aa*	*bb*	1	24	12	-	16	12	15
sMo00222	*aa*	*aa*	43	21	14	-	-	-	2
sMo00166	*aa*	*aa*	58	-	-	-	-	-	22
Hght7_oSSR	*aa*	*aa*	25	18	20	-	-	-	17
sMg00011	*ab*	*ab*	11	50	19	-	-	-	-
sMg00051	*ab*	*ab*	18	26	1	8	17	-	10
sMo00055	*ab*	*ab*	39	40	-	-	-	-	1
sMg00071	*ab*	*ab*	-	25	31	15	9	-	-
sEg00154	*ab*	*ab*	9	38	33	-	-	-	-
sMg00235	*ab*	*ab*	20	23	-	15	22	-	-

TABLE 4. PREDICTED PARENTAL GENOTYPES BASED ON OBSERVED OFFSPRING GENOTYPES

Marker	**Population 9**		**Population 10**	
	Female parent	**Male parent**	**Female parent**	**Male parent**
sMg00011	*cd*	*ab*	*ab*	*ab*
sMg00051	*bc*	*ab*	*ac*	*ab*
sMo00055	*ac*	*aa*	*ab*	*aa*
sMo00246	*ab*	*ab*	*bd*	*ab*
sMg00168	*ab*	*ab*	*ab*	*ab*
sMg00071	*ae*	*bc*	*ab*	*bc*
sMg00196	*bb*	*ab*	*cc*	*ab*
sMo00138	*ab*	*ab*	*ac*	*ab*
sPSc00546	*ab*	*ab*	*ab*	*ab*
sEg00017	*ab*	*bb*	*ab*	*bb*
sEg00011	*ad*	*ab*	*ab*	*ab*
sEg00167	*ac*	*ac*	*ab*	*ac*
sEg00154	*ab*	*ab*	*ab*	*ab*
sMo00222	*bc*	*ab*	*ab*	*ab*
Hght7_oSSR	*bb*	*ab*	*ab*	*ab*
sMg00235	*bc*	*ab*	*ac*	*ab*
sPSc00488	*bc*	*bc*	-	*bc*
sMo00166	*ac*	*ac*	-	*ac*
sEg00213	*aa*	*ab*	-	*ab*
sMo00126	-	*cd*	*ab*	*cd*
sEg00117	-	*aa*	*ab*	*aa*
sMg00175	-	*bb*	*ab*	*bb*
sEg00019	-	*bb*	*ab*	*bb*

TABLE 5. ASSIGNMENTS OF THE PREDICTED PARENTS IN BC_1 x BC_1 POPULATIONS

Population	Number of legitimate offspring	Significance level (%)	Critical LOD	Parental assignment rate (%)
9	92	95.0	-1.84	100.0
10	78	95.0	-1.42	100.0

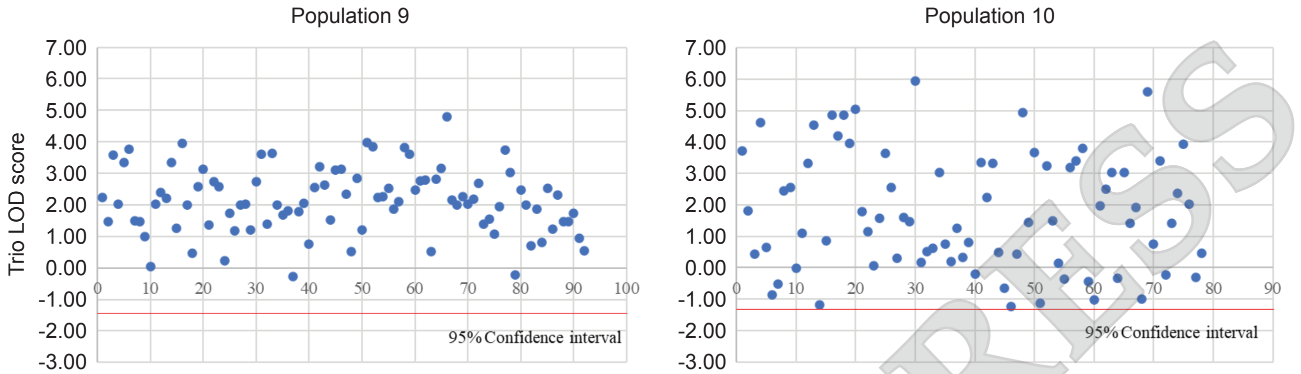

Figure 5. Distribution of trio LOD scores observed for Populations 9 and 10. All the progeny palms were successfully assigned to the predicted parental-pairs at 95.0% confidence level.

A small number of offspring - two palms in Population 9 and 15 palms in Population 10 had slightly lower trio LOD scores ranging from –0.23 to –0.28 and –0.02 to –1.22, respectively (*Figure 5*), indicating that more markers are required for a better analysis of these samples.

As demonstrated in this study, the reconstruction of parental genotypes is possible by evaluating the segregation of genotypes among the offspring in a family, which is being demonstrated here for the first time for an interspecific hybrid cross of oil palm. This is particularly useful when the true parental information is unavailable either due to a mix-up or the original parental palm being not available before DNA analysis of the cross. In case of a mix-up, the predicted genotypes can be used as the reference to determine the actual parents in the seed garden. In general, the same approach can be applied for any species where the offspring share at least one parent (Jones *et al.*, 2009) such as *Eucalyptus nitens* (Gea *et al.*, 2007; Telfer *et al.*, 2015). Reconstruction of parental genotypes has also been conducted for more complex breeding systems such as salamanders (Myers & Zamudio, 2004) and fish (Neff *et al.*, 2001) where multiple paternity is involved.

CONCLUSION

This study reveals a set of SSR markers that effectively assess the genetic purity of two oil palm families where outliers have been identified among the progeny palms. More importantly, analysis using the selected SSR markers revealed that the available records did not reflect the true parentage, raising concerns about the legitimacy of the two families being true interspecific BC_1 x BC_1 hybrids. Nevertheless, the reconstruction of actual parental genotypes was successfully carried out. Although the number of informative markers reported in this study has allowed for an accurate assignment of offspring to the true parents, the inclusion of additional markers would further enhance the resolution of the parentage analysis.

ACKNOWLEDGEMENT

The authors would like to thank the Director-General of the Malaysian Palm Oil Board (MPOB) for permission to publish this article. This work was funded by MPOB (MOA/3(i)/ABBC/GENOMIK).

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