

IMPROVING OIL PALM BREEDING EFFICIENCY VIA MIXED POLLINATION AND PATERNITY DETERMINATION USING SINGLE NUCLEOTIDE POLYMORPHISM (SNP) PANEL

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ABSTRACT

This study introduces a novel approach in oil palm breeding, where pollen from different sources is mixed and utilised in a single controlled cross, and the seedlings generated are subsequently separated based on their paternal source. Mixes of up to four pollen sources were created and crossed each time with a single dura maternal palm, generating seedlings for fingerprinting analysis. A total of 12 crosses were generated to produce 1,811 progenies (43–415 progeny/cross) from a pool of 16 candidate parental palms. DNA fingerprinting analysis was carried out using 24 and 108 SNP panels. The assignment found that the 108 SNP panel is more effective in tracking genetic lineage and assigning seedlings to their true parental combination. The dominant contribution of certain pollen sources was obvious, suggesting that even though the pollen viability is similar, other factors may also be a contributing factor such as genetic background of pollen source, storage period and the age of pollen-producing palms. Nevertheless, the present approach effectively improves breeding efficiency by reducing the number of individual crosses required, making it cost-effective and importantly minimises the impact of depending on the flower cycle to make the necessary crosses in a breeding programme.

Keywords: breeding, crossing, fingerprinting, mixed pollination.

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INTRODUCTION

The oil palm is a monoecious species as it bears both male and female inflorescences on the same palm in an alternating cycle. The reported period of each floral cycle is around four to six months, largely influenced by genetic and environmental factors (Purseglove, 1972). Male and female inflorescences can have more than 100 spikelets

each with the female inflorescence consisting of about 30 flowers per spikelet compared to the male inflorescence which can carry up to 1,200 flowers per spikelet (Rajanaidu et al., 2000). The male inflorescence produces up to 40 g of pollen which remains viable for at least six days. The pollen can be stored for up to one year at -5°C for subsequent use in controlled pollination (Hardon & Davies, 1969). The female flowers are usually receptive to the pollen for about 36–48 hr after anthesis (Latiff, 2000). Oil palm open pollination is carried out by wind and weevil *Elaeidobius kamerunicus*, the main pollinating insect in Malaysia, introduced from West Africa in 1981 (Syed et al., 1982).

Oil palm yields the highest amount of oil per unit area (more than 4 t/ha) and has the largest market share among the major oil crops. Malaysia with

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5.65 million hectares of land under oil palm cultivation; produced 18.55 million tonnes of palm oil and 2.12 million tonnes of palm kernel oil, making it the second most significant producer of the commodity (Parveez et al., 2024). However, as arable land is limited, the only feasible option to improve production to meet the ever-growing demand is the development of new and improved planting materials. In line with this, oil palm breeders have long realised the need for continuous production of improved commercial *dura* × *pisifera*, D×P (*tenera*) planting materials, which in turn requires the development of elite *dura* and *pisifera* lines with good combining ability. Oil palm breeders have made considerable improvement, where average yield increments of about 1.5%/yr have been estimated (Soh et al., 2002). Oil palm genetic improvement has been realised due to carefully designed breeding and selection strategies. Generally, in oil palm breeding, pollen from a selected palm (usually *pisifera*) is crossed to a few mother palms (*duras*) using the North Carolina Model 1 breeding design (Rajanaidu et al., 2000). On average, the female flowers grow in large inflorescence and mature in 5–6 months after pollination. The combining ability of the parental palms can be evaluated by the performance of the progenies produced in the breeding scheme. A minimum of four years of yield recording at maturity (around six years after planting) is required before a decision can be made to select specific palms for seed production and/or further improvement via subsequent rounds of crossing. In general, an oil palm selection cycle in a conventional breeding programme can take at least 10 years. It is also difficult to fix desirable genes in a population, which often requires numerous breeding and selection cycles (Rajanaidu et al., 1999).

The above factors make conducting breeding trials for oil palm an extremely slow, laborious and expensive exercise. As such, to alleviate some of these constraints, this study describes a novel approach, where pollen sourced from several intended parental palms can be mixed and used for controlled pollination on a selected maternal palm. Since only a single pollen grain in the mix contributes to the fertilisation of the individual flower in the inflorescence, the resulting seeds and seedlings derived from the bunch can be differentiated based on the unique DNA profile of the paternal palms. A single nucleotide polymorphism (SNP) panel that can facilitate such an approach has been reported for oil palm (Leslie et al., 2019; Ting et al., 2023). This approach improves breeding efficiency by reducing the number of individual crosses required and minimises delays caused by waiting for the female flower cycle, if crossing is to be carried out separately for each pollen source.

MATERIALS AND METHODS

Parental Palms and Crossing Programme

In total, 12 crosses were generated to verify the mixed pollen strategy in breeding programmes, and these involved nine *dura* (maternal) and seven *pisifera* (pollen source) palms. *Dura* palms were of the Deli *dura* lineage, while the *pisifera* pollen parents were of the AVROS, Lame, Yangambi and Nigerian sources. The pollens were harvested carefully from the individual paternal palms and stored separately in sealed and labelled containers at a temperature of –20°C to maintain their viability for extended periods, ranging from months to years. The viability of the selected pollens exceeded 75% (with one exception) to avoid selection bias, which may affect the number of progenies derived from a particular pollen source (Table 1). An independent mix of two, three and four pollen sources were carried out and each source of mixed pollens was used to pollinate the designated female flower of a single *dura* palm. The amount of pollen used was 0.1 g/pollen source and mixed together before puffing on the anthesising female inflorescences in one application.

The young inflorescences were initially bagged at least a week before anthesis, and hand-pollinated at the first sign of anthesis by injecting pollen through a small hole in the bag. The resulting progenies were labelled and carefully tracked for fingerprinting analysis in the nursery. The crossing scheme is shown in Table 2.

The resulting bunches from the specific crosses were harvested five months after pollination, labelled and processed for seed production as described by Rao and Choong (2014). For germination, the seeds were kept in a hot room with a temperature of 40°C for 60 days, to allow germination. Subsequently, the germinated seeds were transferred to a small polybag in the pre-nursery, with proper tracking. After three months, leaf samples were collected from individual seedlings using the leaf sampling kit (Singh et al., 2007) and genotyped with the TRUE-TO-TYPE Version 1 – 24 SNP panel (Leslie et al., 2019) and TRUE-TO-TYPE Version 2 – 108 SNP panel (Ting et al., 2023).

Genotyping of Samples with the TRUE-TO-TYPE SNP Panel Version 1 and 2

The SNP assay for both versions of the SNP panel was carried out as described previously (Leslie et al., 2019). The parental DNA were all assayed in duplicate, to ensure consistency of the SNP calls and SNP genotype for the parental palms and each seedling assayed was recorded in an Excel Sheet. Data analysis was carried out using Cervus 3.0

TABLE 1. THE LIST OF *Pisifera* PALMS USED AS POLLEN SOURCES AND THE VIABILITY OF RESPECTIVE POLLENS

No.	Pollen	Viability (%)	Genetic background	Date collected
1	0.243/43	80.0	La Me	17.03.16
2	0.395/421	80.0	AVROS	04.12.15
3	0.395/175	77.6	Yangambi	25.10.13
4	0.395/324	60.0	Nigeria	15.04.13
5	0.174/480	82.2	AVROS	09.12.16
6	0.174/655	80.0	AVROS	11.02.18
7	0.395/182	80.0	AVROS	14.01.16

TABLE 2. LIST OF CROSSES GENERATED TO EVALUATE THE MIXED POLLEN APPROACH. THE NUMBER AND SPECIFIC *Pisifera* PALMS USED AS THE PATERNAL SOURCE IN THE POLLEN MIX ARE DESCRIBED

No.	Progeny	Female parent	Male parents		
1	PK7422	0.484/411	0.243/43	0.395/421	
2	PK7401	0.484/997	0.243/43	0.395/421	
3	PK7558	0.484/1,003	0.243/43	0.395/421	
4	PK7569	0.484/351	0.243/43	0.395/421	
5	PK7570	0.484/1,003	0.243/43	0.395/421	0.395/175
6	PK7425	0.484/875	0.243/43	0.395/421	0.395/175
7	PK7446	0.484/857	0.243/43	0.395/421	0.395/175
8	PK7453	0.484/518	0.243/43	0.395/421	0.395/175
9	PK7464	0.484/518	0.243/43	0.395/421	0.395/175
10	PK7471	0.484/993	0.174/480	0.174/655	0.395/421
11	PK7468	0.484/875	0.174/480	0.174/655	0.395/421
12	PK7476	0.484/871	0.174/480	0.174/655	0.395/421

software (Marshall et al., 1998) for the assignment of individual seedlings to their respective parental pairs in each cross, without any prior information. The analysis was then repeated by fixing the female parent for each progeny, and data was only considered acceptable if both analyses agreed. In the Cervus parent analysis, a logarithm of the odds (LOD) score exceeding the threshold established in the simulation phase was used to indicate that the candidate was the true parent. To account for genotype errors, a typing error of one among the 108 loci tested was considered acceptable, if the LOD score was positive. Further analysis to discriminate individual progenies to their related pedigrees was also carried out to confidently assign each seedling to the respective parental pair.

RESULTS AND DISCUSSION

Fruit bunches were harvested for all 12 crosses, approximately five months after pollination, and seeds from these were successfully germinated from all the experimental crosses. The germination rate exceeded 50%, thus sufficient number of samples were obtained for analysis. The two versions of the SNP panel, the first consisting of a set of 24 SNP

markers and a second version consisting of 108 SNP panels, were utilised for DNA fingerprinting in this study. The first version (TRUE-TO-TYPE SNP Panel version 1), which is the 24 SNP panel, consists of SNPs that had been selected based on physical genomic location to minimise genetic linkage and minor allele frequency and maximise genetic informativity within and between populations (Leslie et al., 2019). Version 1 SNP panel is efficient for assigning palms to known parents and weed out illegitimates. However, a larger SNP panel is required for correct parental identification from a pool of possible candidates. A 108 SNP panel (Version 2) proved optimal for high accuracy (99.4 %) parental prediction (Ting et al., 2023). These two panels were developed from a range of diverse materials consisting of germplasm and advanced breeding lines to ensure that only palms with the desired genetic lineage are planted in breeding trials and commercial plantations. The availability of these SNP panels and their amenability to high throughput processing provides the required platform to evaluate the strategy of discriminating seedlings generated from mixed pollen crosses to their respective paternal source and hence assign each seedling to the correct pedigree, which is essential to make informed decisions in breeding programmes.

All 1,811 seedlings (43–415 progeny/cross) generated from the 12 DxP crosses and the pool of 16 candidate parental palms (Table 3), were genotyped with both SNP panels. The result was analysed using Cervus 3.0 software (Marshall et al., 1998) for the assignment of individual palms to their respective parental pairs in each cross. The result obtained for both panels is shown in Figure 1 and it is clear that the 108 SNP panels (Version 2) contained the optimal number of SNPs to correctly assign seedlings to their respective parental pairs. Data obtained from the 108 SNP panel assigned 99.0% of the progeny with high confidence to a paternal palm in the “pollen- mix”, compared to 88.0% of the progeny palms obtained using the 24 SNP panel. The analysis further revealed that only a small number of samples could not be assigned to any parental palms in both 108 and 24 SNP panels, which is about 0.4% and 0.5%, respectively. A limitation of using a low number of SNP markers was also evident where more than 11.0% of the progenies were assigned to incorrect males (paternal palms that were not represented in the pollen mix) when using the 24 SNP panel. This was reduced to only about 0.7% with the 108 SNP panel. The major limitation of the 24 SNP panel is that the number of SNPs employed, which are biallelic, is not sufficient to discriminate parental palms used in the crossing scheme based on the genotype profiles. The 108 SNP panel can discriminate more effectively closely related paternal palms, albeit with some mismatch. To achieve higher discriminative power a panel consisting of a larger number of SNP

markers is needed (Ting et al., 2023). However, the slight gain achieved needs to be balanced with the increasing cost of genotyping with additional SNP markers. In a breeding programme, the seedlings produced are often in excess of the numbers required in a breeding plot for a particular cross. As such, discarding the less than 1.0% of samples that could not be assigned with confidence may be more cost-effective than achieving near 100.0% accuracy using the mixed pollen strategy with DNA profiling to track and assign genetic lineage.

TABLE 3. NUMBER OF SAMPLES ANALYSED FROM 12 PROGENIES DEVELOPED USING THE POLLEN MIX APPROACH

No.	Progeny	No. of samples for SNP analysis
1	PK7422	43
2	PK7401	88
3	PK7558	141
4	PK7569	195
5	PK7425	88
6	PK7570	200
7	PK7446	89
8	PK7453	124
9	PK7464	122
10	PK7471	415
11	PK7468	101
12	PK7476	175
Total		1,811

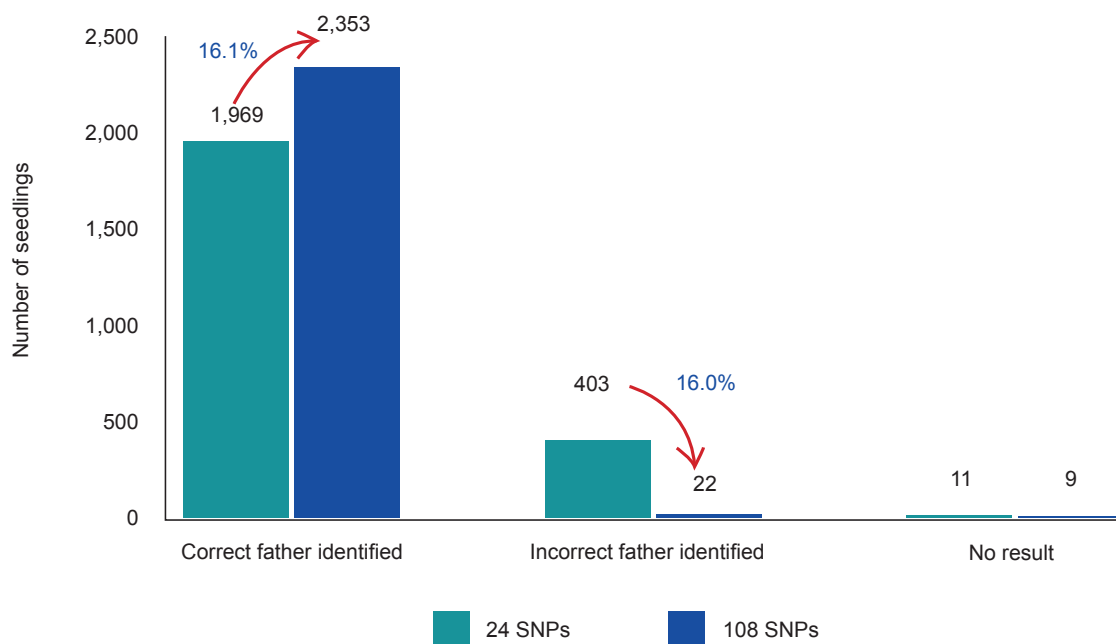


Figure 1. The comparison of fingerprinting output between 24 and 108 SNP panels.

Subsequent analysis was carried out to evaluate the efficiency of mixing two, three and four pollen sources to generate the respective controlled crosses. Only results obtained from the 108 SNP panel are discussed in detail in this study. In the “two pollen mix”, four independent crosses were created using different maternal palms (Table 1). The two paternal palms were 0.243/43 and 0.395/421 of La Me and AVROS genetic backgrounds, respectively. The viability of pollens from both sources was about 80% and the “two pollen mix” was used to generate progenies PK7422, PK7401, PK7558 and PK7569. The number of seedlings used for DNA fingerprinting with the two SNP panels ranged from 43–194/progeny. The uneven number of seedlings genotyped from each progeny was a factor in the germination rate observed for each cross and damage by rodents to some seedlings in the nursery before sampling. The assignment of individual seedlings to their respective parent is summarised in Figure 2. The percentage of seedlings assigned to paternal palms in each cross ranged between 17%–39% for 0.243/43 and 61%–83% for 0.395/421. Seedlings were successfully discriminated and assigned with high confidence to the respective pollen source, although a larger number of seedlings appear to be contributed by pollen from 0.395/421. This suggests that even though the pollen viability and storage period for both sources are similar, other factors such as genetic background and the age of pollen-producing palm could have contributed to the results observed. The results further suggest that in a “two pollen mix”

system, genotyping 200 seedlings should provide sufficient palms for each pedigree to plant in a breeding plot, where the minimum requirement is generally about 32 palms/progeny (planted in two replicates).

A similar independent exercise was also carried out for the “three pollen mix”, where four progenies namely PK7570, PK7425, PK7446, and PK7453 were generated. The three pollen sources included the two described above and with the addition of pollen from the Yangambi *pisifera* palm 0.395/175. As described in Table 1, the viability of 0.395/175 pollen at 77.6% was slightly lower compared to the other two pollen sources in the mix. The distribution of individual seedlings to their respective parent is shown in Figure 3. As expected, the assignment of seedlings to 0.395/175 (range from 5%–8%) is lower compared to the other two pollen sources, likely caused by lower pollen viability and longer storage period (there was a difference of two years in the storage facility). Similar to results in the two-pollen mix, a higher number of seedlings were assigned to pollen 0.395/421 at a range of 63%–80%, while the percentage of seedlings assigned to pollen 0.243/43 is within the range of 12%–30%. The results suggest that even if three different sources of pollen are mixed, seedlings can still be assigned to all three paternal sources, albeit at different frequencies. Based on the results above if three pollen mixes were utilised, about 400 seedlings need to be tested to get sufficient numbers for each pedigree to meet the minimum requirement of 32 palms for the standard breeding programme.

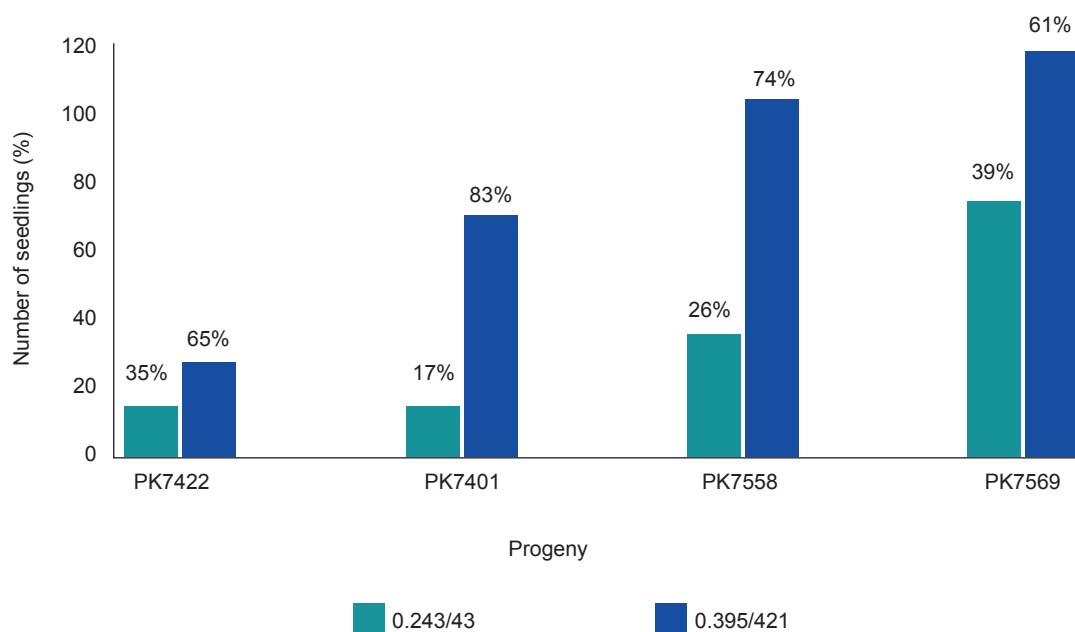


Figure 2. The assignment of individual seedlings to their respective parent for a mix of two pollen crosses using 108 SNPs.

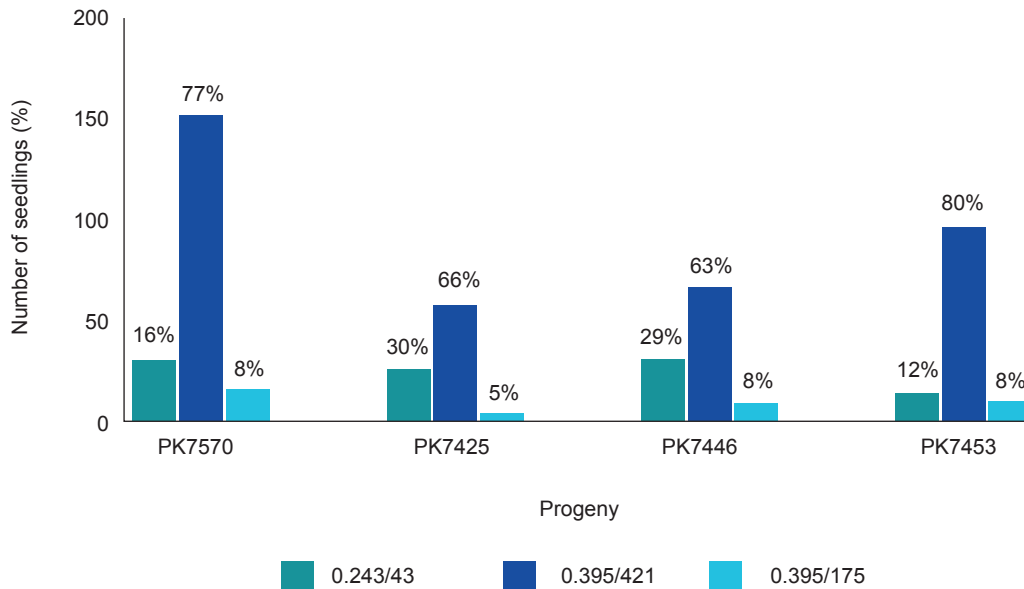


Figure 3. The assignment of individual seedlings to their respective parent for a mix of three pollen crosses using 108 SNPs.

The analysis was further extended to the “four pollen mix”, where an additional four independent crosses were generated. Progeny PK7464 was obtained using the pollen sourced from the three palms described above with an additional pollen coming from palm 0.395/324 (Table 1). The pollen sourced from 0.395/324 had a lower viability (60%), and was included to obtain a general idea of the effect of low viability pollen in the study. As expected, of the 112 seedlings genotyped, only one seedling (1%) was assigned to this specific paternal palm. The assignment of seedlings to the other three pollen sources, namely 0.243/43, 0.395/421 and 0.395/175 pollens, was 19%, 74% and 7%, respectively (Figure 4). Interestingly, the percentage of seedlings assigned to the three-pollen source was similar to that obtained in the “three pollen mix” study above. The data obtained here and from the “three pollen mix” study clearly suggests that pollen with lower viability is unable to compete for pollination and produces less offspring in the mixed pollen study.

The other three crosses generated in the “four pollen mix” study involved a different set of paternal palms namely 0.174/480, 0.174/655, 0.395/421 and 0.395/182. The viability of all selected pollen exceeded 80%, with the difference being that pollen 0.174/655 was a more recent collection compared to other pollen which was harvested and stored 15 months earlier. The DNA fingerprinting result revealed that the majority of the seedlings were assigned to the paternal palm 0.174/655 in the range of 53%–66% (Figure 4). The percentage of seedlings attributed to the other pollen source was in the range

of 2%–27%, with the largest number of seedlings obtained contributed by 0.395/182 followed by 0.395/421 and 0.174/480. However, the association between the length of pollen storage and their ability to compete to produce offspring in mixed pollen study needs further verification as other factors (genetic background and storage conditions) also need to be factored in to make a definitive conclusion. The fact that in a mixed pollination experiment, one donor is favoured for pollination over the other has been documented, likely due to pre-zygotic selection (Björkman et al., 1995). The analysis further revealed that a maximum mix of four pollen different pollen sources may be the limit to get offspring for each pedigree if implemented in a breeding programme. The number of seedlings that need to be generated and genotyped to assign to each of the four pedigrees also has to be relatively high (>1,600) if sufficient numbers are to be obtained for a breeding trial.

It is clear that the 108 SNP panel described by Ting et al. (2023) has sufficient discriminative power as a DNA fingerprinting tool to assign samples in a mixed pollen study to their respective lineage with high confidence. The fact that seedlings of different crosses were easily distinguished, suggests that the SNP panel is also an excellent tool to facilitate identification and subsequent removal of undesired seedlings in breeding and commercial nurseries. More importantly, the strategy of using mixed pollen with DNA profiling to segregate seedlings to their pedigree, not only saves cost and time in conventional breeding but also ensures the fidelity of the controlled crosses, which is

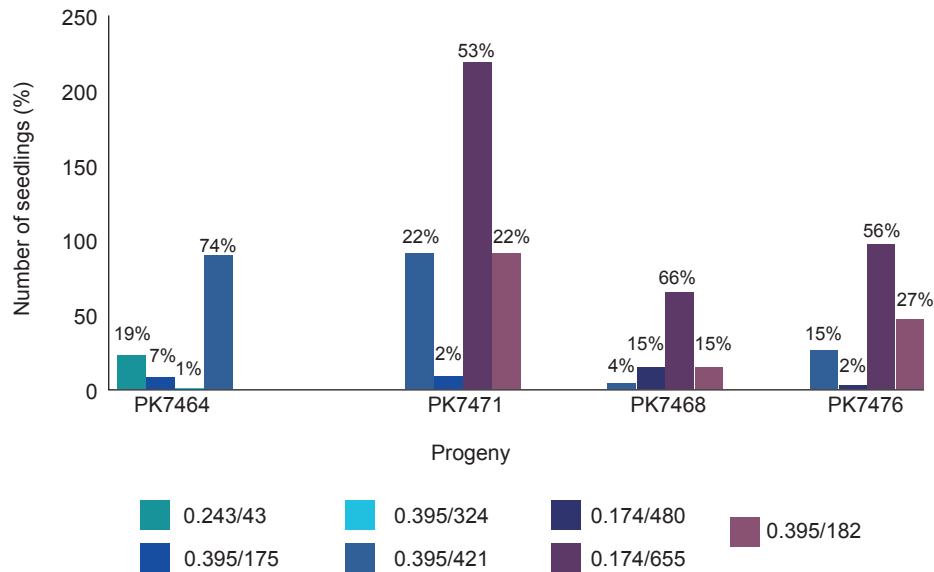


Figure 4. The assignment of individual seedlings to their respective parent for a mix of four pollen crosses using 108 SNPs.

important to make sure the correct conclusions and selections are made in breeding programmes (Corley, 2005). Interestingly, the platform also provides an interesting opportunity to establish a DNA database of all breeding materials, which collectively can improve the management and quality control of propagated materials in breeding and commercial nurseries as well as in tissue culture laboratories.

The strategy of mixing pollen from different paternal sources for making a controlled cross and subsequently assigning seedlings to the paternal source is described here for the first time in oil palm which is a perennial crop with a very laborious and costly breeding programme (Zolkafli et al., 2021). In other crops, the use of mixed pollen strategy has been mostly used to decipher the evolution of flowers and plant mating (Pannell & Labouche, 2013). It has also been described to investigate the effects of self-pollen contamination on fruit set (Matsumoto et al., 2022), evaluating the effects of pollen load composition and deposition pattern on pollen performance (Németh & Smith-Huerta, 2002), and studies focussed on fertilisation study of incongruous pollen for interspecific crosses of lilies (Prosevičius et al., 2012). The current approach described here further complements existing studies that have described an optimum set of SSR markers required for the general fingerprinting of oil palm (Sarimana et al., 2021; Singh et al., 2007; Zolkafli et al., 2021). The strategy described here can be potentially expanded to other cross-pollinating crops such as pine (Elliott et al., 2005), olive (Díaz et al., 2007), coconut (Azevedo et al., 2018) and maize (Xu et al., 2017), where an optimal set of SSR

and SNP have already been developed for routine DNA fingerprinting.

Undeniably the study has shown that the mixed pollen strategy with DNA profiling is an effective approach and can expedite the breeding programme by reducing the number of crosses required and minimising the time required to wait for the female flower cycle. However, to ensure success and to obtain a sufficient number of seedlings attributed to a particular pedigree, issues such as low germination rate and destruction of seedlings in nurseries by rodents or other factors have to be minimised. Limited sample size could lead to a downward bias of the mixed pollen approach and limit the potential of this approach to improve the efficiency of the breeding exercise.

CONCLUSION

The prospect of implementing a mixed pollen approach is very promising and it offers a promising new strategy to implement in oil palm breeding programmes. The vast majority (>99%) of the progenies in all mixed pollen crosses were successfully discriminated to their related pedigrees using a panel of 108 SNP markers in the fingerprinting analysis. The number of seedlings contributed by a particular pollen source in the mix appears to be influenced by pollen viability and to some extent length of pollen storage as well as the genetic background, and possibly the age of pollen-producing palms. All these factors may need to be considered in implementing the pollen mix approach. There is no denying that

the adoption of molecular marker technology is a viable option in the breeding programmes of a perennial crop like oil palm. In line with this, the present study provides a novel strategy for using genomics tools to help develop new and improved oil palm varieties cost-effectively and efficiently.

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