

# SureSawit™ TRUE-TO-TYPE - A HIGH THROUGHPUT UNIVERSAL SINGLE NUCLEOTIDE POLYMORPHISM PANEL FOR DNA FINGERPRINTING, PURITY TESTING AND ORIGIN VERIFICATION IN OIL PALM

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## ABSTRACT

*A high performing True-to-Type single nucleotide polymorphism assay, consisting of a minimal set of single nucleotide polymorphism markers was developed for oil palm. The single nucleotide polymorphism panel was developed from a range of diverse materials consisting of germplasm and advanced breeding lines. Analyses demonstrated that the single nucleotide polymorphism panel is effective at uniquely fingerprinting and discriminating individual palms from unrelated pedigrees. In addition, the single nucleotide polymorphism panel is a very useful tool in breeding programmes, as it can effectively discriminate between individual progeny in half-sibling, full sibling and selfed crosses. Furthermore, illegitimate palms present in half-sibling, full sibling and selfed crosses are easily identified. The single nucleotide polymorphism panel is an excellent quality control tool for use in tissue culture, as clonal materials could be differentiated based on their lineage. The fact that ramets of different clonal lines were easily distinguished, indicates that it will also be an excellent tool to identify potential culture mix-ups that occur either in the laboratory, nursery or during field planting.*

**Keywords:** SNP markers, DNA fingerprinting, SureSawit™ True-to-Type, purity testing.

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## INTRODUCTION

Palm oil supply in the worldwide market exceeds 60 million tonnes (Oil World, 2018; Kushairi *et*

*al.*, 2018), reflecting the economic value of this commodity crop. In South-east Asia (especially Malaysia and Indonesia), oil palm as a commodity crop fosters economic development and is a source of employment and livelihood for large sectors of the rural community. The progress achieved in South-east Asia in cultivating oil palm over the last 100 years (Soh *et al.*, 2017) has been impressive, considering the narrow genetic base of the planting materials (Hartley, 1988). Currently, efforts are ongoing to widen the gene pool by incorporating

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germplasm prospected from its centre of origin in Africa into breeding programmes (Rajanaidu *et al.*, 1988; 2017; Kushairi *et al.*, 2011). However, genetic improvement of oil palm is going to be a slow and labourous process as the long lived outcrossing monoecious palm has a breeding cycle of 10-12 years (Mayes, 1977) and requires large tracts of land for field planting. Nevertheless, important recent breakthroughs, such as sequencing of the whole genome (Singh *et al.*, 2013a) and analyses of the hypomethylated oil palm gene space (Low *et al.*, 2014) have made available a wide repertoire of molecular markers that can facilitate genetic studies in oil palm.

The wide scale use of DNA marker technologies such as marker assisted selection (MAS), is now seen as a viable option in oil palm breeding programmes (Singh *et al.*, 2017). This is due to an increase in the number of molecular biologists, the availability of genomics tools, and the establishment of appropriate laboratory infrastructure in many commercial plantation companies. One of the most basic applications of DNA markers in oil palm breeding is the testing for legitimacy within progeny of controlled crosses. The ability to assure 100% legitimacy in families is critical for breeding programmes, as in some instances, parental palms are selected based on the performance of their offspring in progeny tests. Should illegitimate palms be present, incorrect conclusions regarding the desirability of the parents can be drawn (Corley, 2005). Illegitimacy in oil palm breeding has been frequently highlighted and is inherent to the breeding process (Corley, 2005; Kwan *et al.*, 2017).

Complicating this is the fact that illegitimacy rates can vary greatly between backgrounds and crosses within a given background. The presence of an illegitimate palm in a breeding trial will have an adverse effect on the efforts to precisely determine the parental palms that have the best combining ability. Apart from determining legitimacy, DNA markers can also facilitate classification of specific breeding lines apart from being useful to authenticate certain accessions.

Historically, simple sequence repeat (SSR) markers were the most popular choice for determining legitimacy of oil palm crosses (Thongthawee *et al.*, 2010; Hama-Ali *et al.*, 2015). Undeniably, SSR markers were popular due to their high discriminatory power and ease of use, where the assay can be performed without the use of high-end expensive equipment. However, almost all publications on SSR define a unique set of markers (panel) for DNA fingerprinting that is appropriate within the constraints of the genetic background being tested. Furthermore, SSR markers have limitations in terms of the number of samples that can be analysed at any one time due to the high cost and labour burden of SSR assays, and the difficulty

of error free data capture and analyses. Hence, there is a necessity for a high throughput, cost-effective DNA marker panel with higher resolution than the existing marker systems. The superior DNA marker panel that is also amenable to automation and with higher accuracy, will likely be adopted more readily by the oil palm community to improve throughput and overcome labour and cost limitations.

DNA fingerprinting technology based on single nucleotide polymorphisms (SNP) promises to achieve these aims. SNP fingerprinting technology is universally being adopted in forensic science for its superior genetic resolution over older DNA marker technologies, and its amenability to high throughput processing (Sobrinho *et al.*, 2005). Furthermore, the bi-allelic nature of SNP results in less ambiguity in correctly calling the genotypes, compared to the multi-allelic SSR markers. In agriculture, SNP are now routinely used for DNA fingerprinting of maize (Tian *et al.*, 2015; Xu *et al.*, 2017), soya (Song *et al.*, 2015; Patil *et al.*, 2017), rice (Singh *et al.*, 2013b; Thomson *et al.*, 2017), rapeseed (Slankster *et al.*, 2012) and alfalfa (Annicchiarico *et al.*, 2016). Here we report the development of a high performing True-to-Type SNP panel which is effective in discriminating individual palms from unrelated pedigrees from a wide range of genetic backgrounds of *Elaeis guineensis*. The SNP panel will be particularly useful in breeding, tissue culture and supply chain quality control applications.

## MATERIALS AND METHODS

### Plant Materials and DNA Extraction

Palms from 10 unrelated pedigrees (n = 1547) were selected for inclusion in the study (Table 1). DNA was prepared from leaf samples using the cetyl trimethylammonium bromide (CTAB) method with minor modifications (Doyle and Doyle, 1990).

TABLE 1. PALMS USED FOR DEVELOPING THE TRUE-TO-TYPE SIMPLE NUCLEOTIDE POLYMORPHISM ARRAY

| Category                 | Palms |
|--------------------------|-------|
| Linked half-sibs         | 817   |
| Germplasm                | 192   |
| Bi-parental Family 1 & 2 | 104   |
| DxP <sub>1</sub>         | 125   |
| T128                     | 89    |
| DxP <sub>2</sub>         | 78    |
| DxP <sub>3</sub>         | 99    |
| TxP                      | 22    |
| Ortet & ramets           | 21    |
| Total                    | 1 547 |

## Selection of SNP Loci

Initial discovery of SNP was based on analyses of 16X coverage Illumina next-generation sequencing data (150 bp paired end reads) generated from over 300 *E. guineensis* palms representing advanced breeding lines and MPOB's diverse germplasm collection, including palms derived from Angola, Cameroon, Gambia, Ghana, Guinea, Madagascar, Nigeria, Senegal, Sierra Leone, Suriname, Taisha, Tanzania and Democratic Republic of Congo (formerly known as Zaire). High density SNP genotyping microarrays were designed to genotype approximately 50 000 SNP in a first phase and approximately 100 000 SNP in a second phase (Illumina Infinium iSelect platform). In the first and second phases, 1152 and 528 palms were genotyped respectively. Using these genotyping data, a panel of 768 SNP was selected based on physical genomic location to minimise genetic linkage and minor allele frequency to maximise genetics informativity within and between populations.

The 768 single nucleotide polymorphism panel was used to genotype 380 palms from various pedigrees by fluorescent competitive allele specific polymerase chain reaction (PCR) assays (LGC KASP Genotyping Services). A subset of 135 high performing assays was then used to genotype

a panel of 1547 palms (LGC KASP Genotyping Services).

## The True-to-Type Single Nucleotide Polymorphism Panel

SNP were identified based on optimal genetic resolution across the diverse germplasm sample cohort. SNP were then further selected based on physical genomic location to minimise linkage and to include SNP from distant locations on each of the 16 *E. guineensis* chromosomes. This then resulted in the True-to-Type minimal based on single nucleotide polymorphism panel.

## RESULTS AND DISCUSSION

### Selection of 768 SNP for DNA Fingerprinting

An initial panel of 768 SNP was identified based on whole genome sequencing of over 300 palms representing the MPOB *E. guineensis* germplasm collection, followed by two phases of genotyping using custom Illumina iSelect genotyping arrays as described above. A fluorescent competitive allele specific PCR assay was developed for each SNP, and validation of technical performance of the assays

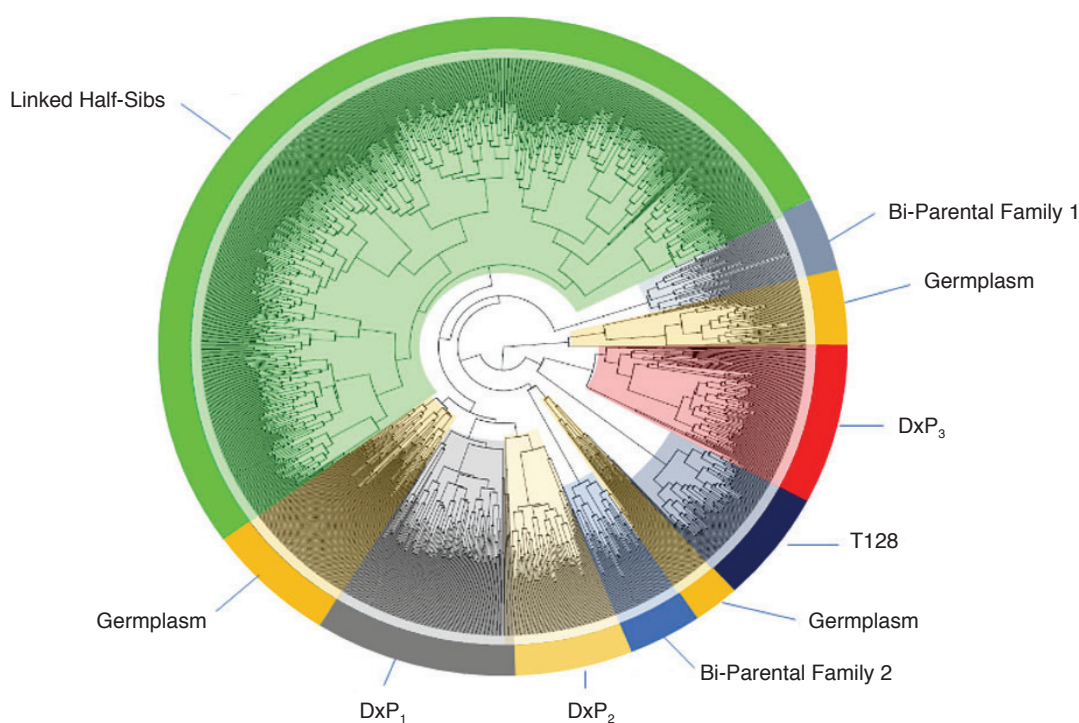


Figure 1. Dendrogram analysis using genotypes of a 135 single nucleotide polymorphism panel to discriminate 1526 palms from various backgrounds. Each individual palm is resolved into a unique genotype. While each palm is unique, the 135 single nucleotide polymorphism panel is able to cluster palms according to their background category (Table 1). Ortet and ramet samples were excluded for this analysis. TxP samples were distributed across the clusters as one of the parental palms was a germplasm sample.

was performed by comparison to next-generation sequencing-based genotypes of seven palms from different germplasm populations.

**Verification of the True-to-Type Single Nucleotide Polymorphism Panel**

The single nucleotide polymorphism panel was first evaluated for its ability to uniquely fingerprint and discriminate individual palms from unrelated oil palm pedigrees. Initially, 380 palms were genotyped for the 768 based on SNP that were discovered in MPOB’s germplasm sequencing project, followed by genotyping of 1547 palms for a reduced panel of 135 highly performing and discriminating based on SNP. A minimal single nucleotide polymorphism panel representing the final True-to-Type single nucleotide polymorphism panel was selected from the 135 SNP set.

Analysis of genotype data from the 135 single nucleotide polymorphism panel resulted in unique fingerprints from each of the 1526 palms (excluding the 21 ortet and ramet samples), giving 100% fingerprint resolution (Figure 1). Analysis of genotype data from the True-to-Type single nucleotide polymorphism panel revealed unique fingerprints from 1525 of the 1526 palms, resulting in a 99.93% fingerprint resolution (Figure 2).

Interestingly in Hop (*Humulus lupulus*), a minimum number of SNP markers (less than 10) was also as robust as a larger panel of 1006 SNP markers to effectively differentiate 116 varieties (Henning *et al.*, 2015). Similarly, a minimum set of SNP markers was found suitable for cultivar identification and for discriminating a collection of common and durum wheat (Mangini *et al.*, 2018). The identification and verification of a core set of SNP markers for routine fingerprinting and origin verification of a complex genome like oil palm will be very useful in breeding and cloning efforts towards developing new and improved varieties.

**Application of the True-to-Type Single Nucleotide Polymorphism Panel**

*Discriminate between individual progeny in full sibling families involving two parents.* In order to determine the discriminatory power of the single nucleotide polymorphism panel, a bi-parental *dura* x *pisifera* family (DxP<sub>1</sub>) including the two parental palms and 123 F<sub>1</sub> progeny was genotyped with the 135 single nucleotide polymorphism panel, as well as the minimal True-to-Type panel. Dendrogram analyses of the fingerprints generated by genotyping the 135 single nucleotide polymorphism panel (Figure 3) and the True-to-Type single nucleotide

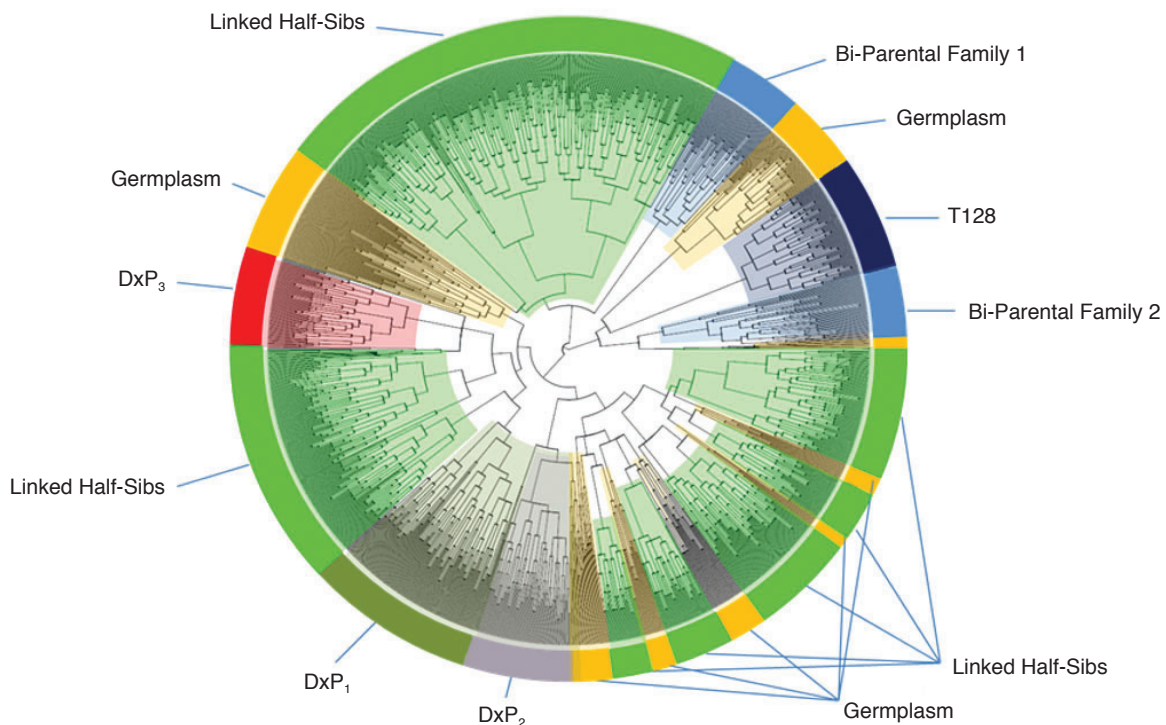


Figure 2. Dendrogram analysis using genotypes of the minimal True-to-Type single nucleotide polymorphism panel to discriminate the same 1526 palms shown in Figure 1. The minimal True-to-Type panel provided unique genotype signatures for 1525 of the 1526 palms (99.93% resolution), and also clustered palms according to their background category (Table 1). Ortet and ramet samples were excluded for this analysis. TxP samples were distributed across the clusters due to one of the parental palms being a germplasm material.

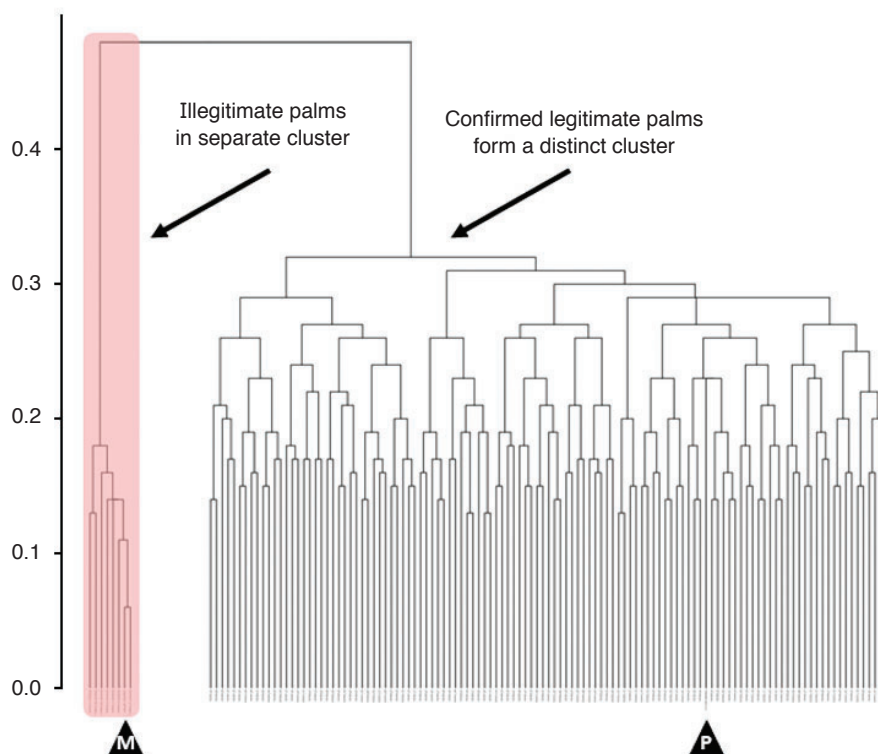


Figure 3. Dendrogram analysis using genotypes of a 135 single nucleotide polymorphism panel in 123 progeny palm from bi-parental cross  $DxP_1$  and the two parental palms (125 palms total). The single nucleotide polymorphism panel resolves each palm into a unique node. Furthermore, seven palms are divided into a distinct cluster indicating that they are not derived from the intended bi-parental cross, but instead are likely to be the result of unintended selfing of the maternal palm. Arrow heads labelled 'P' and 'M' indicate the position of the paternal and maternal palms, respectively.

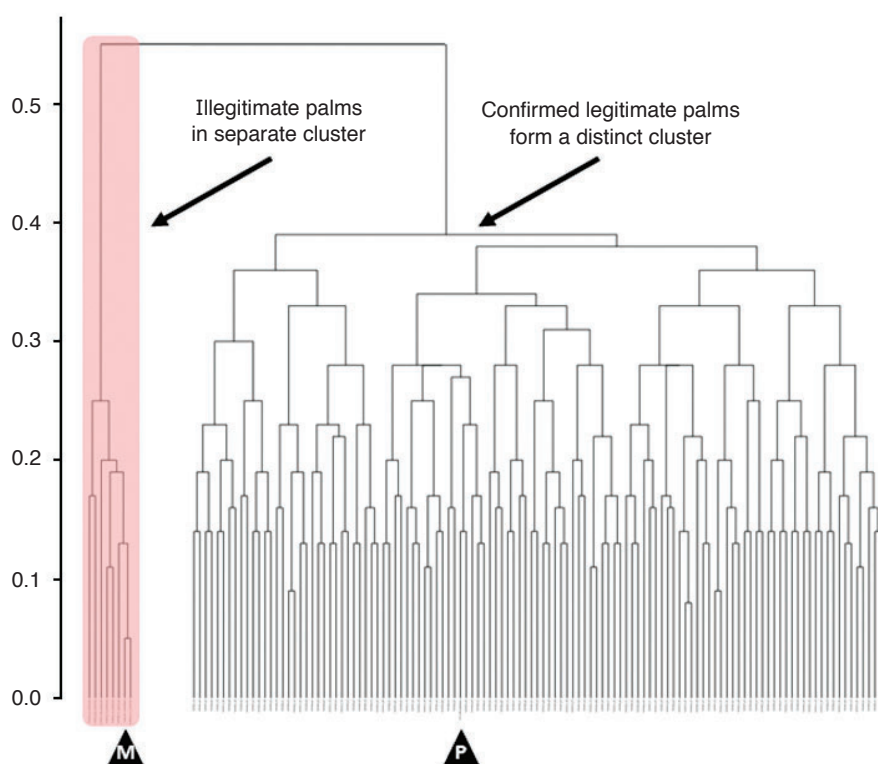


Figure 4. Dendrogram analysis using genotypes of the minimal True-to-Type single nucleotide polymorphism panel in 123 progeny palms from bi-parental cross  $DxP_1$  and the two parental palms (125 palms total). The single nucleotide polymorphism panel resolves each palm into a unique node. The same seven illegitimate palms identified by the 135 single nucleotide polymorphism panel (Figure 3) are also separated into a distinct cluster by the True-to-Type single nucleotide polymorphism panel. Arrow heads labelled 'P' and 'M' indicate the position of the paternal and maternal palms, respectively.

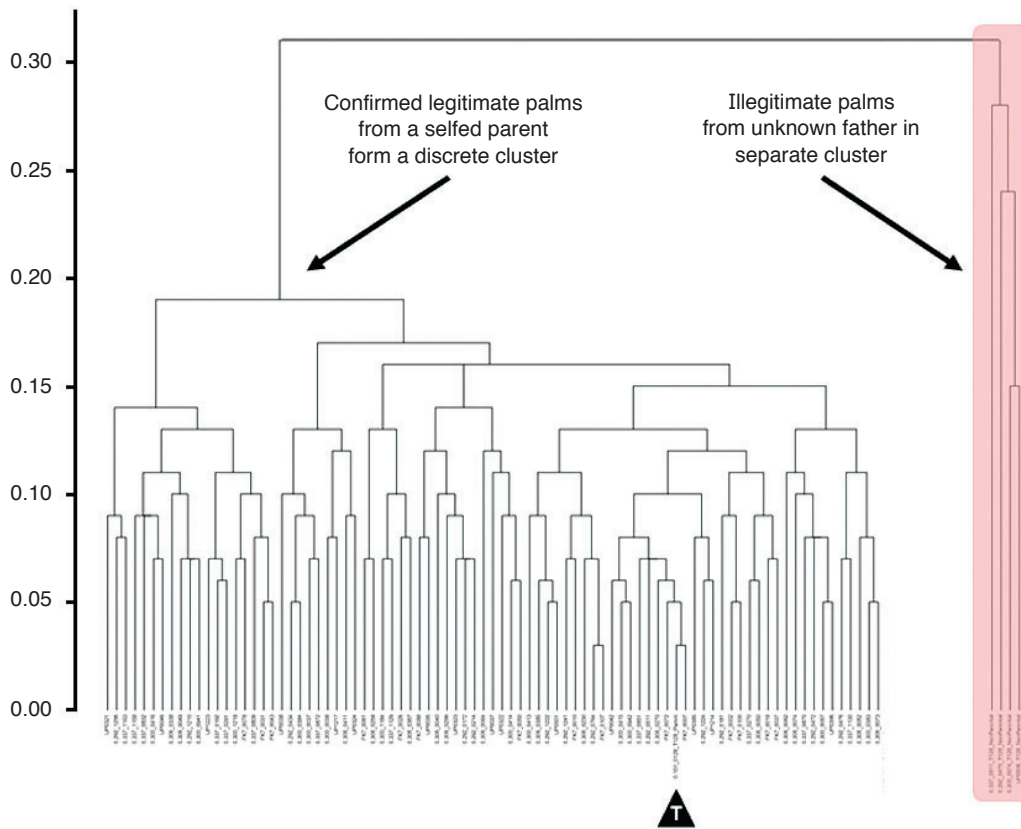


Figure 5. Dendrogram analysis using genotypes of a 135 single nucleotide polymorphism panel to discriminate 88  $F_1$  palms from an intended T128 selfed mapping cross and the selfed parent. The single nucleotide polymorphism panel resolves each palm into a unique node and identifies four illegitimate palms that are not derived from the intended selfed cross. The arrow head labelled 'T' indicates the position of the selfed parent.

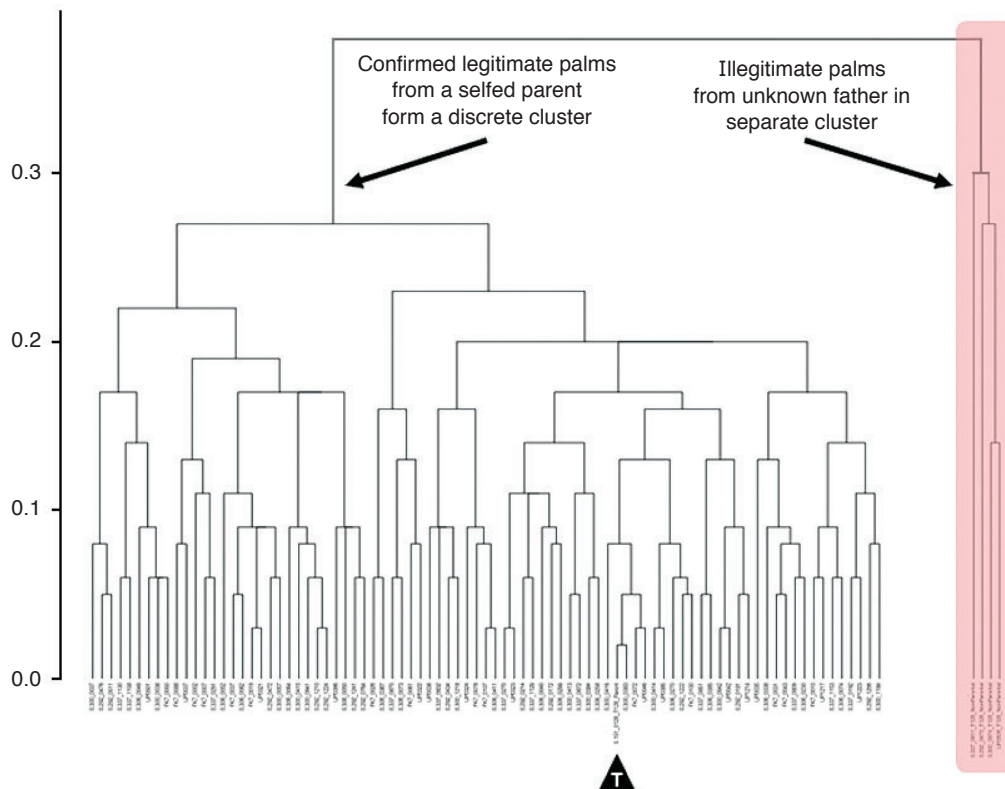


Figure 6. Dendrogram analysis using genotypes of the minimal True-to-Type single nucleotide polymorphism panel to discriminate 88  $F_1$  palms from an intended T128 selfed mapping cross and the selfed parent. The single nucleotide polymorphism panel resolves each palm into a unique node and identifies the same four illegitimate palms identified by the 135 single nucleotide polymorphism panel. The arrow head labelled 'T' indicates the position of the selfed parent.

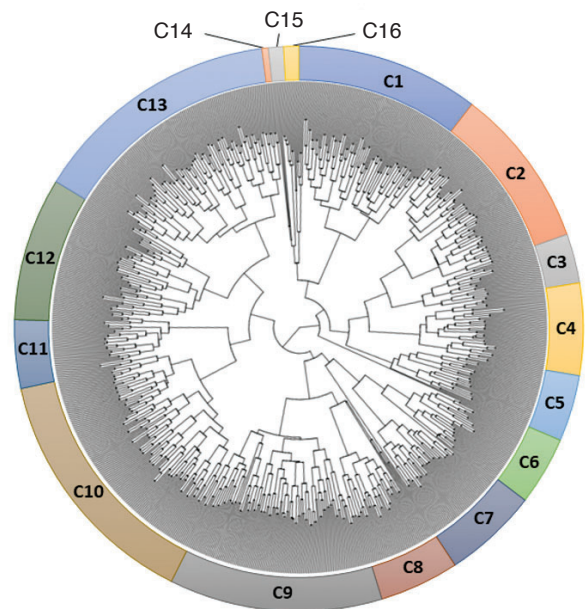


Figure 7. Dendrogram analysis using genotypes of a 135 single nucleotide polymorphism panel to discriminate 817 palms from 22 linked half-sibling families. The single nucleotide polymorphism panel resolves each palm into a unique node, even among highly related linked half-sibling populations. C1-C16 indicates the clustering of unique nodes into higher order related groups.

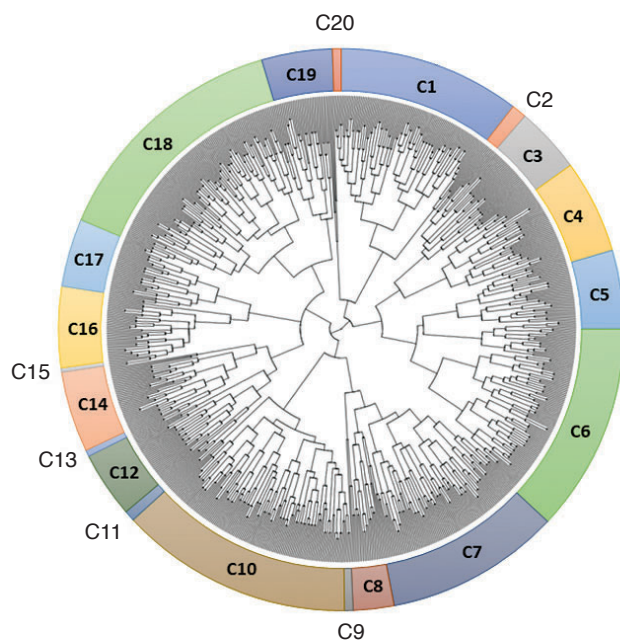


Figure 8. Dendrogram analysis using genotypes of the minimal True-to-Type single nucleotide polymorphism panel to discriminate 817 palms from 22 linked half-sibling families. The single nucleotide polymorphism panel resolves each palm into a unique node, even among highly related linked half-sibling populations. C1-C20 indicates the clustering of unique nodes into higher order related groups. The distribution of progeny from each cross into these clusters is summarised in Table 2.

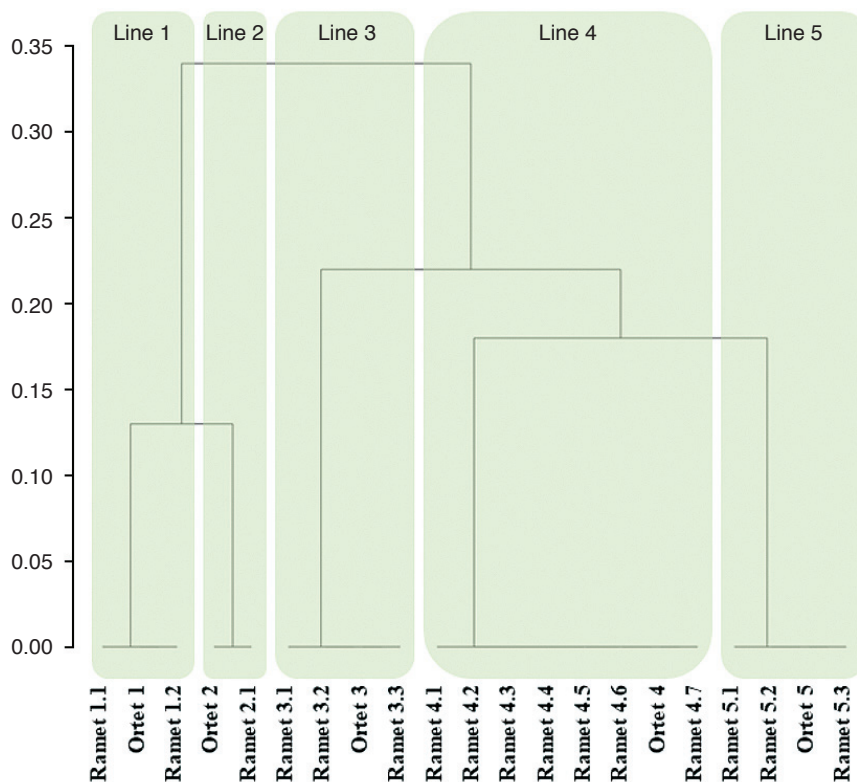


Figure 9. Dendrogram analysis using genotypes of the minimal True-to-Type single nucleotide polymorphism panel to discriminate ortets and ramets from five clonal lines. Ramets derived from each ortet are clustered into a unique, clonal line-specific cluster, together with the ortet from which they were derived. Members of each cluster are genetically identical at each single nucleotide polymorphisms (SNP).

polymorphism panel (Figure 4) each resulted in 125 unique nodes. This demonstrated that the True-to-Type assay could discriminate between individual palms in this bi-parental DxP<sub>1</sub> family with 100% resolution. The 116 palms which were true descendants of the intended bi-parental *dura* and *pisifera* parents, and seven off-type palms that grouped into an independent cluster including the maternal parental palm were also identified by cluster analysis. Therefore, these illegitimate palms are likely the result of unintended selfing of the mother palm.

**Discriminate between individual progeny in full sibling families involving a single selfed parent.** Eighty-eight F<sub>1</sub> palms from the selfing of a Nigerian T128 *tenera* palm and the selfed parent were genotyped with both the 135 single nucleotide polymorphism panel (Figure 5) and the True-to-Type single nucleotide polymorphism panel (Figure 6). This resulted in 89 unique fingerprints for each panel, indicating that the True-to-Type panel is able to differentiate the individual palms in a selfed family with 100% resolution. In each case, cluster analysis could clearly differentiate the 84 F<sub>1</sub> offsprings of the selfed *tenera* palm from the four palms of unknown paternal source (Figures 5 and 6).

**Discriminate between individual progeny in half-sibling families involving more than two parents.** MPOB breeders had created 22 half-sibling families which were generated from the crossing of five related *pisifera* palms and 22 closely related Deli *Dura* palms. The 135 single nucleotide polymorphism panel (Figure 7) and the True-to-Type single nucleotide polymorphism

panel (Figure 8) were used to genotype 817 palms from these 22 families. Both panels generated 820 unique fingerprints, demonstrating that the True-to-Type single nucleotide polymorphism panel is able to discriminate between individual palms in highly related linked half-sibling populations with 100% resolution. The clustering of palms by the True-to-Type single nucleotide polymorphism panel is summarised in Table 2. The minimal single nucleotide polymorphism panel also appears to generally cluster palms according to parentage. For example, Cluster 1 (C1) includes all 41 palms of the cross of maternal palm 1 and paternal palm 20, and also includes 44 of 45 half-sibling progenies of the cross of maternal palm 2 (full sib of maternal palm 1) and the same paternal palm 20 (Figure 8 and Table 2). One offspring from the palm 2 x palm 20 progeny is clustered in the small Cluster 20 (C20) that includes one palm from each of four crosses. These may represent illegitimate palms that were not derived from the intended cross. Half-siblings from other crosses to paternal palm 20 are mostly grouped into independent clusters, including 100% of the palm 16 x palm 20 progeny in Cluster 16 (C16), 94% of 15 x 20 in Cluster 17 (C17) and 97% of the 19 x 20 progeny in Cluster 19 (C19). Similarly, Cluster 10 (C10) and 18 (C18) include maternal palms which are full sibs. However, the exception is Cluster 6 (C6) (all three palms are not full sibs) and 7 (C7) (where only two of three palms are full sibs). Interestingly, the True-to-Type single nucleotide polymorphism panel appears to provide extremely high genetic resolution across a broad range of genetic backgrounds and has the ability to identify illegitimate palms among progeny of various controlled crosses. Increasing the number of SNP included in the panel can likely

TABLE 2. CLUSTERING OF FAMILIES BY TRUE-TO-TYPE ASSAY

| M  | P  | C1   | C2  | C3  | C4  | C5  | C6  | C7  | C8  | C9 | C10 | C11 | C12 | C13 | C14 | C15 | C16  | C17 | C18  | C19 | C20 | Total |
|----|----|------|-----|-----|-----|-----|-----|-----|-----|----|-----|-----|-----|-----|-----|-----|------|-----|------|-----|-----|-------|
| 1  | 20 | 100% |     |     |     |     |     |     |     |    |     |     |     |     |     |     |      |     |      |     |     | 41    |
| 2  | 20 | 98%  |     |     |     |     |     |     |     |    |     |     |     |     |     |     |      |     |      |     | 2%  | 45    |
| 3  | 21 |      |     | 94% |     |     |     |     |     |    |     |     |     | 6%  |     |     |      |     |      |     |     | 33    |
| 4  | 21 |      |     |     | 98% |     |     |     | 2%  |    |     |     |     |     |     |     |      |     |      |     |     | 44    |
| 5  | 21 |      |     |     |     | 95% |     |     |     |    |     |     | 3%  |     |     |     |      |     |      |     | 3%  | 39    |
| 6  | 21 |      |     |     |     |     | 97% |     |     |    |     |     |     |     |     |     |      |     |      |     | 3%  | 39    |
| 7  | 22 |      | 4%  |     |     |     | 96% |     |     |    |     |     |     |     |     |     |      |     |      |     |     | 26    |
| 8  | 21 |      | 14% |     |     |     | 81% |     |     |    |     |     |     |     |     |     |      |     |      |     | 2%  | 43    |
| 9  | 23 |      |     |     |     |     |     | 93% | 7%  |    |     |     |     |     |     |     |      |     |      |     | 2%  | 41    |
| 10 | 23 |      |     |     |     |     |     | 71% | 24% | 3% | 3%  |     |     |     |     |     |      |     |      |     |     | 34    |
| 11 | 23 |      |     |     |     |     |     | 71% | 25% |    |     |     |     |     |     |     |      |     |      |     |     | 28    |
| 12 | 24 |      |     |     |     |     |     |     |     | 2% | 98% |     |     |     |     |     |      |     |      |     | 4%  | 41    |
| 8  | 24 |      |     |     |     |     |     |     |     | 5% | 92% |     |     |     |     |     | 3%   |     |      |     |     | 37    |
| 13 | 24 |      |     |     |     |     |     |     |     |    | 87% | 13% |     |     |     |     |      |     |      |     |     | 39    |
| 14 | 24 |      |     |     |     |     |     |     |     |    |     | 84% |     |     | 16% |     |      |     |      |     |     | 31    |
| 15 | 24 |      |     |     |     |     |     |     |     |    |     | 8%  |     |     | 92% |     |      |     |      |     |     | 36    |
| 16 | 20 |      |     |     |     |     |     |     |     |    |     |     |     |     |     |     | 100% |     |      |     |     | 38    |
| 15 | 20 |      |     |     |     |     |     |     |     |    |     | 3%  |     |     |     |     |      | 94% | 3%   |     |     | 34    |
| 9  | 24 |      |     |     |     |     |     |     |     |    |     |     |     |     |     |     |      |     | 100% |     |     | 35    |
| 17 | 24 |      |     |     |     |     |     |     |     |    |     |     |     |     |     |     |      |     | 100% |     |     | 38    |
| 18 | 24 |      |     |     | 2%  |     |     |     |     |    |     |     |     |     |     |     |      |     | 98%  |     |     | 42    |
| 19 | 20 |      |     |     |     |     |     |     |     |    |     |     |     | 3%  |     |     |      |     |      |     | 97% | 33    |

Note: M - maternal palm ID; P - paternal palm ID; C1-C20 - dendrogram cluster numbered as shown in Figure 8; Total - number of palm analysed per indicated cross. Percentages were calculated as the number of palm from a given cross present in each cluster divided by the total number of palms associated with that cross. Shading indicates lowest (yellow) to highest (green) percentage of palms per cluster.

assist in resolving the exact genetic background of any given palm. Such resolution would require panels comprising much higher numbers of SNP at a substantially higher testing cost. Near perfect discrimination of large number of full sib families and tracking illegitimate progeny with a minimal single nucleotide polymorphism panel that is affordable to end-users (breeders and tissue culturists) has also been previously demonstrated in forest species such as white spruce (Godbout *et al.*, 2017).

***Discriminate between a set of ramets having a first clonal lineage, from a second set of ramets having a second clonal lineage.*** The single nucleotide polymorphism panel was also tested on tissue culture clones. Genotyping 16 ramets from five clonal lines plus five originating ortets with the True-to-Type assay revealed five unique fingerprints. Dendrogram analysis of these five fingerprints showed five unique nodes (Figure 9). Although each line was genetically distinguishable, the ortet and derived ramets in each line clustered together with identical True-to-Type single nucleotide polymorphism fingerprints. This indicates that the assay can easily differentiate ramets derived from the different clonal lineages. Similarly, True-to-Type assay can also precisely assign an unknown ramet to its clonal lineage. The True-to-Type assay reported here appears to be more effective than the SSR markers described previously for fingerprinting of oil palm clones (Singh *et al.*, 2007) as the genotype calls are easier to call and assign than the multiallelic SSR markers.

## CONCLUSION

The availability of a SureSawit™ True-to-Type test, developed based on the results from this study, will be a useful tool to differentiate both cultivated and wild oil palm genotypes. The single nucleotide polymorphism panel could also clearly differentiate individuals in both selfed and intercrossed populations, where portioning of the genetic variability differs. In a very practical application, it will enable breeders to identify and exclude illegitimate palms from a wide range of breeding populations. The array will also allow the industry to more accurately rank the desirability of parental pairs of palms, which will accelerate the development of new oil palm varieties. Undeniably, the management of breeding, seed production, and nursery practices will be significantly improved with the availability of this single nucleotide polymorphism panel, which can also be used as a tool by enforcement authorities to track lineage in cases of dispute. SureSawit™ True-to-Type testing will be offered as a service to the oil palm industry.

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