Microsatellites for parentage analysis in an oil palm breeding population

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ABSTRACT

Oil palm is an important oil crop in tropical regions. Breeding for improved varieties yielding more oil per area planted is a slow and difficult process. Establishing control-pollinated oil palm progenies for breeding purposes is not without problems as it takes a long time and many steps from pollination, seed collection, germination, to field planting. At each of these steps errors could arise. A breeding programme requires 100% certainty of correct parentage in the progeny trials. The allele frequencies at eight oil palm microsatellite loci were determined among the parents and progenies in an oil palm breeding programme. Paternity and/or parentage likelihoods were calculated for the offspring. Even though the oil palm breeding populations in Southeast Asia are assumed to have low genetic diversity because of their recent introduction from just a few founder trees, the eight loci combined had sufficient power to detect errors with great confidence. Three clear planting errors and one presumed pollination error were detected among 245 individuals in 6 full sib families. The overall genetic structure of the oil palm breeding population was evaluated using the same loci. A combination of four loci was already sufficient to reach a non-exclusion level below 1% for the detection of planting errors. To detect pollination errors confidently, 7 or 8 loci would be necessary.

Key words: microsatellites, oil palm, DNA fingerprinting, paternity, parentage.

INTRODUCTION

The African oil palm (*Elaeis guineensis* Jacq.) is an important oil producing crop in tropical regions. The history of oil palm breeding points to a rather narrow genetic base in the cultivated varieties. Presently, oil palm breeders are selecting for a number of agronomic traits, such as yield, disease resistance, oil quality characteristics, slow height growth rate and broad ecological adaptations, for which restricted genetic variability has been found in *E. guineensis* germplasm (Simmonds, 1993).

Breeding for improved oil palm varieties can be done but is not without difficulties because of the large size of the plants and consequently large plantation area needed and the small number of progenies that can be planted, the long generation time, and the difficult access to the flowers. In plant breeding programmes, it is important to know the parentage of families and individuals for a variety of reasons. Oil palm breeders make extensive use of

family performance, usually selecting the best individual plants within the best families. Such selection can only be effective if families are correctly identified, and are truly families; that is, all individuals have the same parentage. Progeny testing, in which individual parent palms are evaluated on the basis of the performance of their offspring, will not be valid if some of the offspring are illegitimate or the result of selfings (Luyindula *et al.*, 2005).

To ensure that the parents used for commercial hybrid seed production are not related, oil palm breeders rely on pedigrees (Rosenquist, 1986). For a pedigree to be useful, it is essential that the crosses were made correctly. However, controlled pollination of oil palms is difficult and prone to various sources of error. Detailed descriptions of methods and the necessary checks and quality control procedures have been published (Donough *et al.*, 1993; Chin, 1999; Rao and Kushairi, 1999). Possible problems with controlled pollination include damage to bags by rats, squirrels, oil palm spines or simply from repeated use. Pollen-bearing weevils will enter damaged bags, causing contamination by illegitimate pollen. In some female inflorescences, the accompanying male flowers may produce viable pollen, so that some self-pollination could occur. Because of the obvious risk of errors, pollination is usually subject to careful supervision. However errors may also occur during pollen collection or storage, in labeling bunches on the palm, in the seed store or during the germination stage and in the tree nursery and ultimately field planting of the progeny trials (Corley, 2005).

Molecular markers have been used to trace errors in progenies established for plant breeding (e. g. Riaz *et al.*, 2007). DNA based markers are free of environmental influence and can be readily detected at any stage and in any part of the plant through direct genome analysis. Therefore, DNA based markers provide an efficient method for genetic resources characterization, through which genetic diversity and organization at different levels

can be assessed (Karp and Edwards, 1997; Lanaud and Lebot, 1997).

Microsatellites have been widely used for DNA fingerprinting of plants because they are co-dominant markers, are ubiquitously distributed in the genome and are highly polymorphic (Fujimori *et al.*, 2003). Sixty-four microsatellite loci covering all 16 chromosomes were selected among the microsatellites mapped on the published genomic linkage map (Billote *et al.*, 2005) and were screened for their potential use. The allele frequencies at eight loci were subsequently determined among a subset of the parents in a breeding programme and paternity and/ or parentage likelihoods were calculated for some presumed offspring.

MATERIALS AND METHODS

Plant materials

A total of 245 progeny trees from six families each consisting of 40 to 45 plants of oil palm (*E. guineensis*) were sampled from the breeding plantation of Univanich Palm Oil Public Company Ltd., Krabi, Thailand. Mature leaves were collected and total genomic DNA was extracted from them using the Genomic DNA Mini Kit (Geneaid, Taiwan). The DNA quantity and quality was checked on a 1.0% agarose gel. Genomic DNA was stored at -20 °C until required.

Aside from the progeny families, 132 parent trees used in the active breeding programme were sampled as well, including the presumed maternal and paternal parents of the progenies. These trees were classified as "Yangambi", "Ekona", "AVROS", "TOPI" and "Combined" origins.

Microsatellite primers design

Primers for microsatellite markers had previously been developed for the construction of an oil palm genetic linkage map (Billote *et al.*, 2005). Sixty-four of the microsatellite loci covering the sixteen chromosomes of oil palm were selected and oligonucleotides were synthesized (Ocimum Biosciences, India). A panel of 48 parental trees was

screened for the number of alleles that could be detected at each of the 64 loci. Eight loci that showed a high level of polymorphism were selected for the parentage analysis study. Details of the eight primer pairs are presented in Table 1. Two of the loci that mapped to the same linkage group were separated by 64 cM and were considered essentially unlinked.

PCR-amplification

DNA fragments were amplified by polymerase chain reaction (PCR) using the microsatellite specific primers. PCR-reaction mixtures contained 25 ng of plant genomic DNA, 1.7 mM of Mg^{2+}, 10 pmoles of each primer, 200 µM of dNTPs, 1× *Taq* Buffer with $(NH_4)_2SO_4$, 0.035 units of *Taq* DNA polymerase (Fermentas® UAB, Lithuania), in a total volume of 10 µl. The PCR was done on a thermocycler (Biometra® T1 Thermocycler) using the following cycling parameters: 94 °C for 3 min., followed by 40 cycles at 94 °C for 30 sec., 55-60 °C for 30 sec., 72 °C for 30 sec. and a final incubation at 72 °C for 5 min. The reaction products were checked by agarose gel electrophoresis.

The different alleles were identified by separating the PCR fragments on denaturing polyacrylamide gels (4.5% AccuGel[TM] 19:1, National Diagnostics, U.S.A., containing 8 M urea and 1× TBE), at 75 W constant power for one and a half to two and a half hours. The polyacrylamide gels were stained with silver nitrate (Bassam *et al.*, 1991). The alleles were scored as co-dominant multi-allelic loci by comparison to a set of reference alleles identified during the study.

Population genetic analysis of the candidate parent palms

The number of alleles, polymorphism information content, heterozygosity and deviations from Hardy-Weinberg equilibrium (HWE) were estimated using the programme CERVUS (Marshall *et al.*, 1998). The Bonferroni correction was applied when testing the significance of HWE deviations. Departure from expected segregation ratios were

tested using the χ^2 test. For the identified subpopulations, deviation from HWE was tested using the exact test as implemented in GENEPOP version 4.0.10 (Rousset, 2008) using 1000 dememorization steps, 100 batches and 1000 iterations per batch for the Markov chain parameters.

Individual parental trees were assigned to ideal populations based on their genetic fingerprints, assuming HWE within populations and differentiation between populations. The programme STRUCTURE 2.3.3 (Falush *et al.*, 2007) tests different numbers of populations (K) to find the highest likelihood. The programme was run ten times for each number of subpopulation (K) value, ranging from 1 to 10, using the admixture and correlated allele frequency model with 50,000 replicates for length burn-in and 500,000 replicates for number of MCMC after burn-in during analysis. The final population subgroups were determined based on Ln P(D), Var[Ln P(D)] and K (Evanno *et al.*, 2005) for the model choice criterion to refer as true number of populations (K). The number of populations considered by STRUCTURE is not necessarily the same as the number of populations sampled.

The programme DARwin5 (Perrier *et al.*, 2003) was used to visualize phylogenetic relationships among the parental trees. DARwin5 calculates a similarity matrix based on the number of shared alleles detected at each microsatellite locus and calculates a genetic distance based on the number of shared alleles among diploid organisms. A Weighted Neighbour Joining tree was constructed based on simple matching of alleles shared at each locus in a diploid organism. Bootstrap values were calculated based on 200 resampling of the dataset.

Parentage testing

CERVUS (Marshall *et al.*, 1998) analyses data from co-dominant genetic markers such as microsatellites (STRs), SNPs and allozymes. It assumes that markers are autosomal and that the species is diploid. It also assumes that markers are inherited independently of each other, in other words

Table 1   Information of the microsatellites primers used for six families of 245 oil palm population. Linkage group assignment according to Billotte *et al.*, 2005

Primer	LG	Position	Repeat motif	GENBANK Accession No.	PCR Product size	Sequence 5' – 3'
MEGCIR3428	1	65	(GA)15	AJ578667	177	F-CATTTTGACAGCTCGTGATG R-TTGGCCGCTATATCTCCAAC
MEGCIR3301	3	46	(GA)18	AJ578641	185	F-GTTAGCTGGCCGGTCTGTAG R-AACATTAGTCCCAGAAGGTTGG
MEGCIR0894	7	13	(GA)18	AJ578562	153	F-TGCTTCTTGTCCTTGATACATAGA R-TCTCCACATGAACTCCATCG
MEGCIR3111	8	104	(GA)16	AJ578623	214	F-TTTTCGCAAGAGATCCTGACA R-CAGATGGCCGCTGAATTTAC
MEGCIR3825	12	92	(GA)21	AJ578736	211	F-CACATTGGAGAGCACTTGGA R-TCTCTCTGTGGTTTTAATGGAAGA
MEGCIR3546	14	27	(GA)15	AJ578680	198	F-CGTCGACCTCTTCATACATTAAAC R-GCATTGCACATACCAGCAAC
MEGCIR3633	14	91	(GA)23	AJ578693	165	F-GCATTTCAACATCTCCCAAATC R-AGCAGTATATGATAATCTGCAAGGC
MEGCIR0782	16	57	(GA)20	AJ578538	227	F-CTTTCAATGCCCTAGCGTTC R-GTCGAAAGAAGCGAGATGCT

that they are unlinked. The programme compares parent-offspring genetic data and calculates a likelihood ratio of the hypothesized relationship (parent-offspring) to the null hypothesis (unrelated) for each candidate parent-offspring set. The programme performs a simulation to determine a critical value for the likelihood ratio that produces a desired level of confidence in the results.

RESULTS

Of the 64 primer pairs initially tested, 54 successfully amplified a clear single fragment in a panel of 48 parental trees. The number of alleles identified in this test panel of 48 individuals ranged from 1 to 8. Among the primer pairs that amplified a large number of alleles, those with more or less equal allele frequency distribution were selected. Eight loci were then used for further analysis of all sampled parents and the progeny palms.

The number of alleles among all parental trees at the 8 selected microsatellite loci ranged from 5 to 10. The observed heterozygosity (H_{obs}), expected heterozygosity (H_{exp}) and polymorphism information content (PIC) ranged between 0.439-0.689, 0.629-0.843 and 0.580-0.821 respectively.

The average non-exclusion probability for one candidate parent (NE-1P) ranged from 0.485 to 0.782, average non-exclusion probability for one candidate parent given the genotype of a known parent of the opposite sex (NE-2P) ranged from 0.317 to 0.613, and the average non-exclusion probability for a candidate parent pair (NE-PP) ranged from 0.143 to 0.431 (Table 2). The combined non-exclusion probability for all eight loci was 0.01194705 and 0.00062865 for first parent and second parent respectively.

The programme CERVUS found significant deviations from HWE for 7 loci and estimated a frequency of null alleles ranging from 4.6 to 28.3 % for the individual loci. Conformity to expected segregation ratios were tested and for 7 out of 48 family x locus combinations the χ^2 test indicated a significant deviation. To investigate the presence of population structure, the programme STRUCTURE was used. Population structure analysis indicated that the 132 parental palm trees could be grouped in 5 different groups more or less according to pedigree and origin information: "Yangambi-I", "Yangambi-II", "Ekona", "AVROS" and "TOPI" (Figure 1) with Ln P(D) = -2622.7, Var[Ln P(D)] = 184.8. The

Table 2   Allele frequency analysis results for the 132 parent trees.

Locus	k	N	Hobs	Hexp	PIC	NE-1P	NE-2P	NE-PP	NE-I	NE-SI	HW	F(Null)
MEGCIR3428	5	132	0.439	0.629	0.580	0.782	0.613	0.431	0.186	0.483	***	+0.1849
MEGCIR3301	7	132	0.545	0.784	0.750	0.600	0.420	0.234	0.079	0.379	***	+0.1780
MEGCIR0894	9	132	0.477	0.843	0.821	0.485	0.317	0.143	0.044	0.341	***	+0.2832
MEGCIR3111	10	132	0.659	0.836	0.814	0.494	0.324	0.147	0.047	0.345	*	+0.1160
MEGCIR3825	10	132	0.583	0.802	0.782	0.541	0.362	0.168	0.058	0.365	***	+0.1738
MEGCIR3546	8	131	0.595	0.818	0.792	0.534	0.359	0.178	0.057	0.357	***	+0.1556
MEGCIR3633	9	132	0.576	0.792	0.759	0.584	0.406	0.221	0.074	0.374	***	+0.1559
MEGCIR0782	6	132	0.689	0.766	0.728	0.630	0.450	0.264	0.091	0.391	NS	+0.0464

k: number of alleles at the locus, N: number of individuals typed at the locus, Hobs: the observed heterozygosity, Hexp: expected heterozygosity, PIC: polymorphic information content (Botstein *et al.*,1980), NE-1P: average non-exclusion probability for one candidate parent, NE-2P: average non-exclusion probability for one candidate parent given the genotype of a known parent of the opposite sex, NE-PP: average non-exclusion probability for a candidate parent pair, NE-I: average non-exclusion probability for identity of two unrelated individuals, NE-SI: average non-exclusion probability for identity of two siblings, HW: significance of deviation from Hardy-Weinberg equilibrium. Key: NS = not significant, * = significant at the 5% level, ** = significant at the 1% level, *** = significant at the 0.1% level. These significance levels include a Bonferroni correction, F(Null): estimated null allele frequency.

presence of 5 subpopulations also happens to be suggested by the ΔK calculated according to Evanno *et al.* (2005).

For four of the identified subpopulations we estimated population genetic parameters separately. There were too few trees grouped in the "AVROS" subpopulation to perform a meaningful test. Because there were only 18-44 individuals in each of these four subpopulations, HWE estimates were tested using the exact test implemented in GENEPOP 4 (Rousset, 2008), which calculates exact P-Values by the Markov chain method (Table 3). While for the whole population 6 loci showed significant deviations from HWE at 0.1% level, each subpopulation now

has deviations for one or two loci at the 1% level and one or two additional at 5%.

For first parent pair loci 4 individuals did not match, for the pair of progeny and second parent 5 individuals had a mismatch. For the complete trios (two parents and offspring) parent pair loci had individuals 8 mismatching. Except for the four odd individuals, CERVUS calculated 95% confidence for all trios.

Paternity analysis of 245 full sib progenies from 6 families with 8 SSR markers detected 3 individuals in one single progeny set that clearly did not belong to the family they were assigned to (parentage testing) because they had two apparent
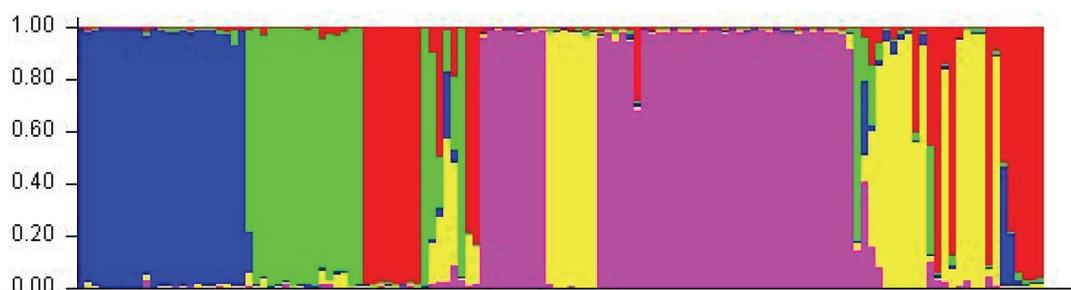

Figure 1 Genetic diversity of oil palm population (*E. guineensis*). Population structure analysis summary plot of estimates of K=5, clustering of 132 parent trees. "Yangambi I" in blue, "Yangambi II" in green, "Ekona" in red, "AVROS" in yellow and "TOPI" in pink (see colour figure on the journal website).

Table 3 Hardy-Weinberg test on 4 parent subpopulation groups.

Locus	Yangambi-I (N = 23)	Yangambi-II (N = 18)	Topi (N = 44)	Ekona (N = 18)
MEGCIR3428	0.1897	0.1816	0.2274	0.6352
MEGCIR3301	0.4943	0.0029**	0.0000***	0.3955
MEGCIR0894	1.0000	0.2436	0.0480*	0.2142
MEGCIR3111	0.0103*	0.1520	0.4015	0.0009***
MEGCIR3825	0.9388	0.0000***	1.0000	0.6954
MEGCIR3546	0.0014**	0.3208	0.5180	0.0499*
MEGCIR3633	0.0352*	0.5649	0.0009***	0.0581
MEGCIR3633	0.0001***	0.0228*	0.4324	0.5013

* = significant at the 5% level, ** = significant at the 1% level, *** = significant at the 0.1% level. Hardy Weinberg: Probability test by using program GENEPOP 4. Estimation of exact P-Values by the Markov chain method. Markov chain parameters for all tests, Dememorization: 1000, Batches: 100, Iterations per batch: 1000 (Rousset, 2008).

mismatching alleles at 1 or 2 loci in addition to single allele mismatches at other loci. One individual had a single allele mismatch at three loci, with all matching alleles derived from the same parent. No errors were detected in any of the 5 other progeny families. Two more individuals had one apparent mismatching genotype at a single locus, which could be due to mutations.

DISCUSSION

The programme CERVUS found significant deviations from Hardy Weinberg for 7 loci and estimated sometimes high frequencies of null alleles. Null alleles can be confirmed through departure from Mendelian segregation ratios in progeny sets. Detection from expected segregation ratios were tested for each locus in each progeny family. For 7 out of 48 family x locus combinations the χ^2 test indicated a significant deviation from the expected ratios. However, in all but one of these cases both parents were heterozygous and thus null alleles are not the cause of the observed segregation distortion. Another possible explanation for the observed deviations from HWE in the parental population would be the existence of population structure, the so called Wahlund effect. Population structure analysis indicated that the 132 parental palm trees could be grouped in 5 different groups more or less according to pedigree and origin information: "Yangambi I", "Yangambi II", "Ekona", "AVROS" and "TOPI" (Figure 1). The different groups were also revealed by the DARwin5 phylogenetic tree. Even though the bootstrap values for the unrooted tree were almost all below 70%, three out of the 5 subgroups formed monophyletic clusters (Figure 2). A few trees that were of assumed mixed origins in the STRUCTURE analysis formed very small distinct clusters in the phylogenetic analysis.

For four of the identified subpopulations we estimated population genetic parameters separately. Because these subpopulations contained only a small number of individuals, HWE estimates were tested using exact test in GENEPOP 4 (Rousset, 2008).

Compared to the HWE test over all 132 parental palm trees, fewer significant deviations from HWE could be detected within each of the subpopulation (Table 3) suggesting that population structure and the Wahlund effect can account for a part of the deviations from HWE. Because only limited deviations from expected segregation ratios could be found, and because a clear population structure was indicated, we concluded that the high frequency of null alleles estimated by CERVUS was not to be concerned about.

The non-exclusion probability for 8 loci combined was 0.01194705 for first parent (NE-1P). This implies that there is still a 1% chance that the 8 loci combined will not exclude an unrelated candidate parent from parentage of an arbitrary offspring when none of the parents are known. The second parent non-exclusion probability (NE-2P) for the 8 loci combined was 0.00062865 implying that when the genotype of one parent was assumed to be known, as would be common for seed harvested from a known female parent tree, more than 99.9% of the candidate parents could be excluded from paternity.

Four individual progenies were clearly identified that did not belong to the same family as the rest. Even with only 4 loci, these illegitimate offspring could already be detected. Three of these individuals were most likely the result of an error during the seed and seedling handling stages, because for some loci neither of the two alleles observed matched to any of the two assumed parents. The fourth individual showed single allele genotype mismatches at three loci, with all matching alleles derived from the same, presumably female, parent. This result could indicate either a pollination error or a planting error. An additional two individuals indicated an unexpected allele at a single locus and were probably the result of a pollination error where pollen from a closely related parent was mixed up. Alternatively, the single mismatch observed in those plants could be due to a mutation at the microsatellite locus. A total of 245 × 2 meioses were studied at 8 loci. The occurrence of two mutations would

indicate a mutation rate of 5.12×10^{-4}, which is relatively high, but would still be within the range of mutation events observed for dinucleotides repeat SSRs in maize. The mutation rate estimated at dinucleotide repeats microsatellite loci in maize was from 3.1×10^{-5} to 7.2×10^{-4} while an upper limit of 6.7×10^{-5} was estimated for other SSR repeat types (Vigouroux *et al.*, 2002). The estimated average mutation rate was 10^{-2} to 3.9×10^{-3} averaged over 15 loci for trinucleotides (TAA)n in chickpea (Udupa and Baum, 2001) and 2.4×10^{-4} per allele per generation averaged over 10 dinucleotide SSR loci in durum wheat (Thuillet *et al.*, 2002). Mutation rates at microsatellite loci per allele and per generation have been estimated to range from 6.3×10^{-6} in Drosophila (Schug *et al.*, 1997) to 10^{-2} in the human genome (Weber and Wong, 1993).

CONCLUSION

Even though the genetic diversity in commercial oil palm breeding stock is assumed to be low, microsatellite analysis can be used for correct
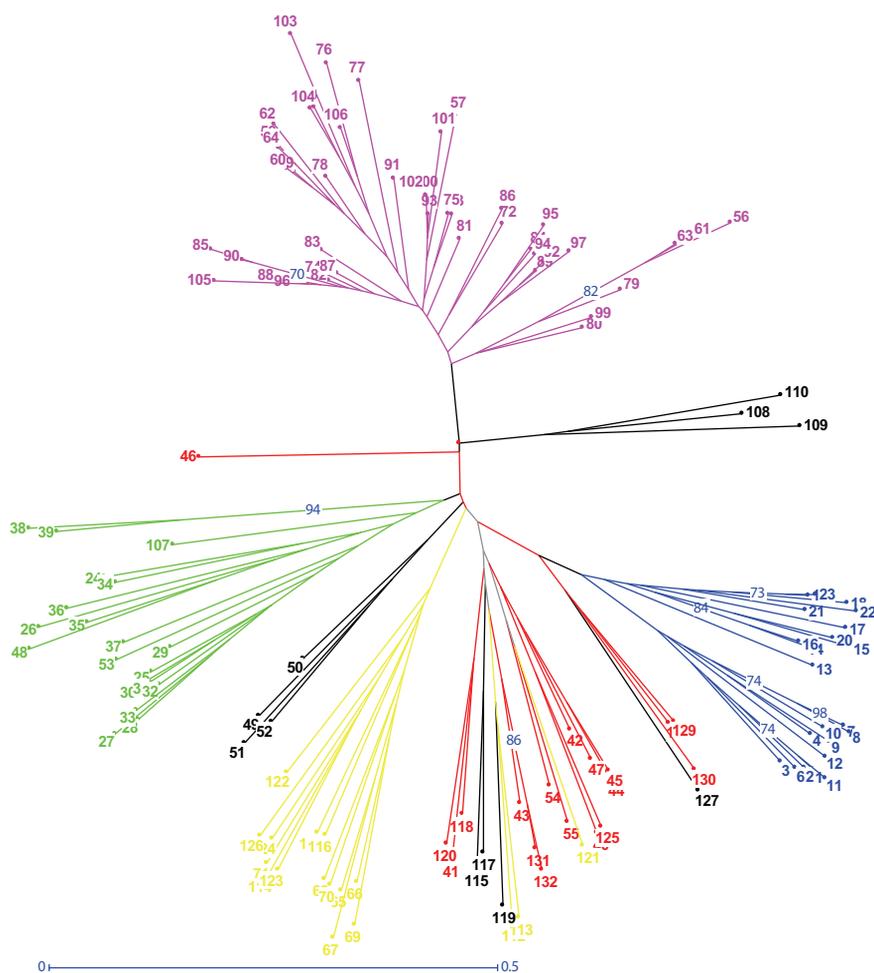

Figure 2 DARwin5 unrooted tree analysis. The colors of the tree branches indicate the five groups as identified through the STRUCTURE clustering of 132 parent trees. "Yangambi I" in blue, "Yangambi II" in green, "Ekona" in red, "AVROS" in yellow, "TOPI" in pink and unassigned trees in black (see colour figure on the journal website). Bootstrap values larger than 70% are indicated.

parentage assignment in progeny trials of oil palm. The allele frequencies at eight loci were determined among the parents and progenies in an oil palm breeding programme. Paternity and/or parentage likelihoods were calculated for the offspring. A combination of four loci was already sufficient to reach a non-exclusion level below 1% for the detection of planting errors. To detect pollination errors confidently, 7 or 8 loci were necessary.

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