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(19) **United States**(12) **Patent Application Publication**
Nelson et al.(10) **Pub. No.: US 2010/0138951 A1**(43) **Pub. Date: Jun. 3, 2010**(54) **METHODS OF PRODUCING HAPLOID AND DOUBLED HAPLOID OIL PALMS**(75) Inventors: **Stephen Peter Connor Nelson**,
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C40B 30/04 (2006.01)(52) **U.S. Cl. 800/268; 800/298; 800/278; 800/260;**
435/6; 506/9(57) **ABSTRACT**

The present invention relates to haploid oil palm plants and homozygous doubled haploid oil palm plants. The invention also relates to methods for producing and selecting haploid and doubled haploid plants. More particularly, but not exclusively, the method may be used for selecting haploid and doubled haploid oil palm plants. Haploid and doubled haploid plants are selected by a large-scale screening based on a combination of the phenotype with the use of molecular methods combined with flow cytometry techniques to identify haploid and doubled haploid plants. More particularly, a method for selecting haploid and doubled haploid plants is described comprising: (a) germinating seeds; (b) selecting seedlings with atypical phenotype; (c) assessing heterozygosity using markers; (d) isolating cells from the seedlings and determining the DNA content of the cells; and (e) isolating and purifying the DNA and using defined molecular markers to characterise the genotype of the plant. The haploid oil palm plants may be used for producing homozygous doubled haploid oil palms: doubled haploids may be intercrossed to produce uniform F₁ hybrids of superior properties.

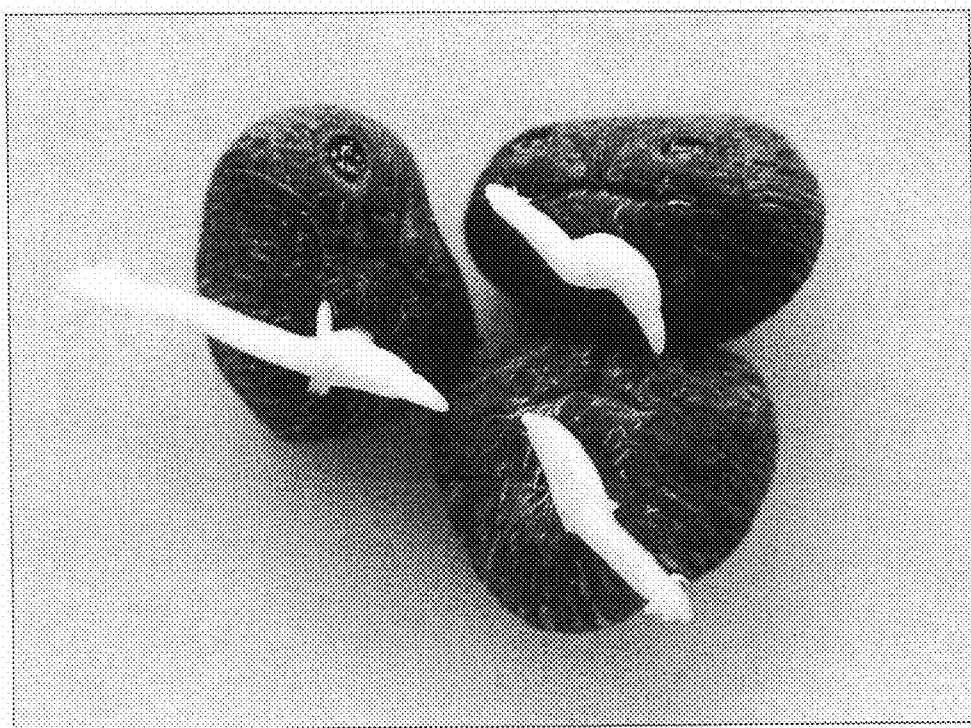


FIG. 1

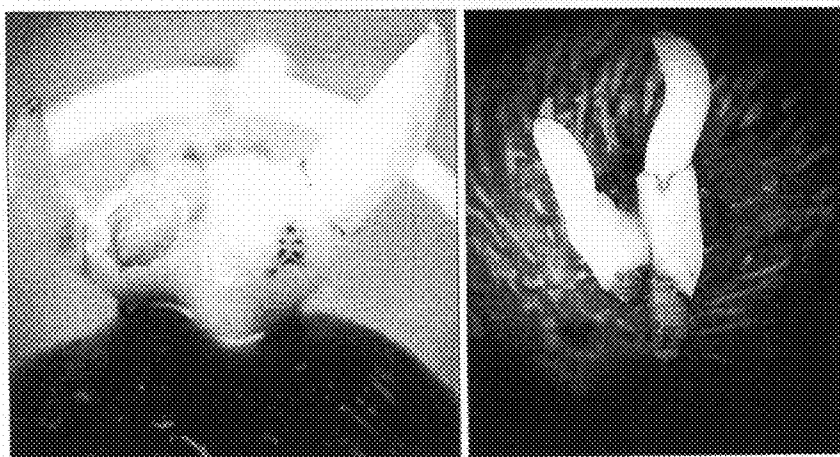
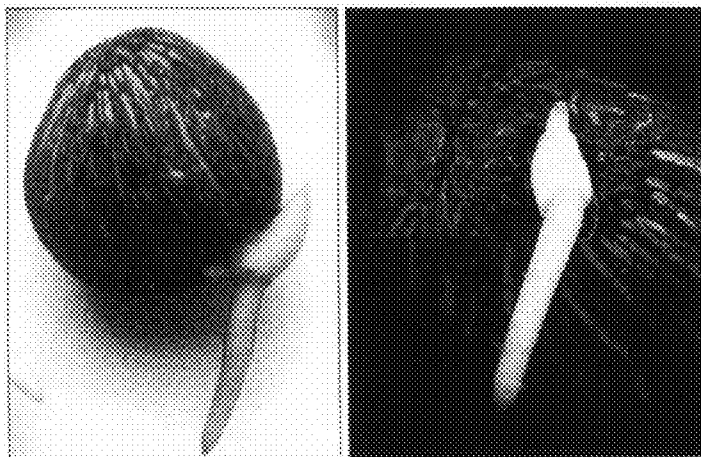


FIG. 2

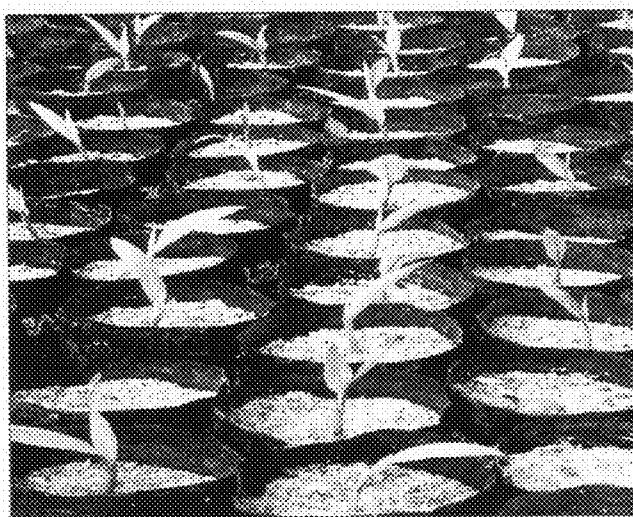


FIG. 3

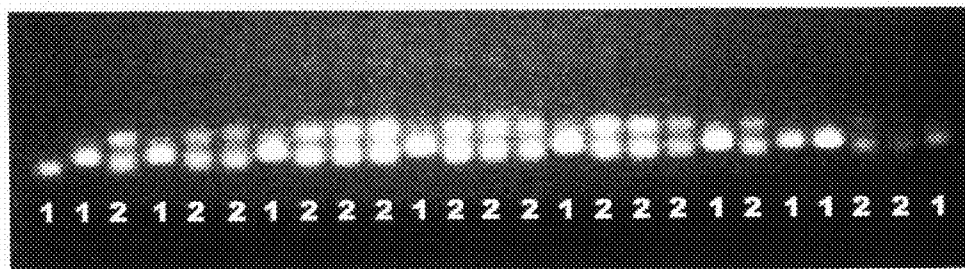


FIG. 4A

First Seedling Screen (2004-2005)

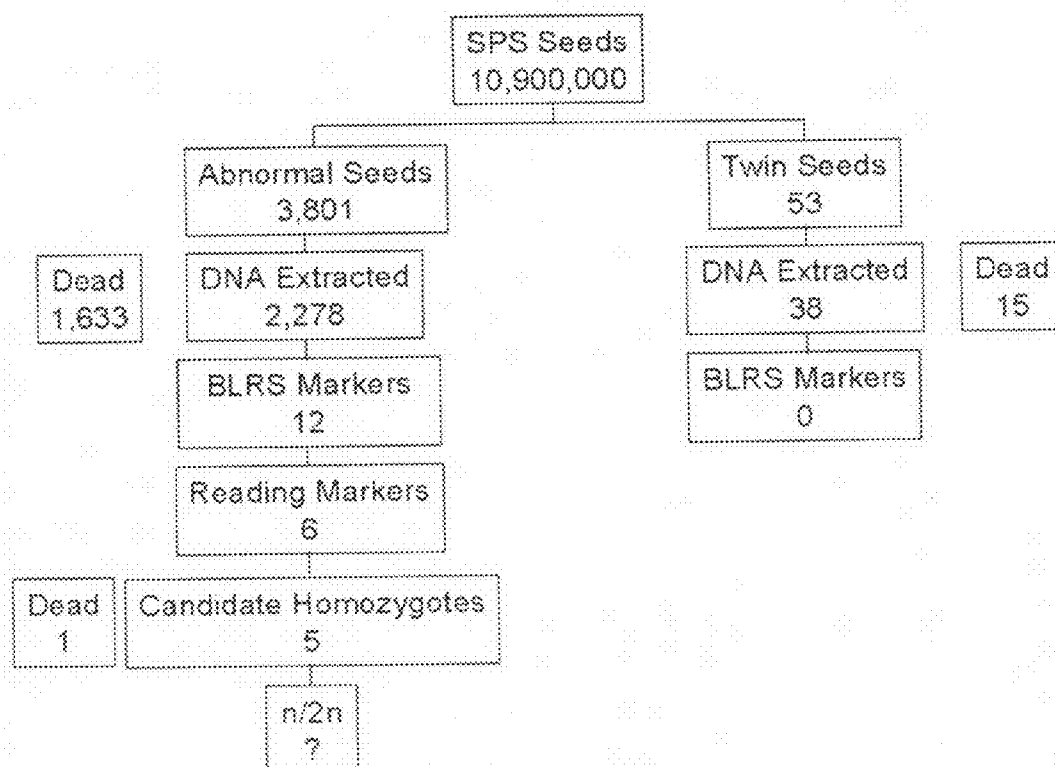


FIG. 4B

FIG. 5A

File: Sample_H042.fcs Date: 29-09-2006 Time: 12:24:22 Particles: 2813 Acq.-Time: 69 s

partec PAS

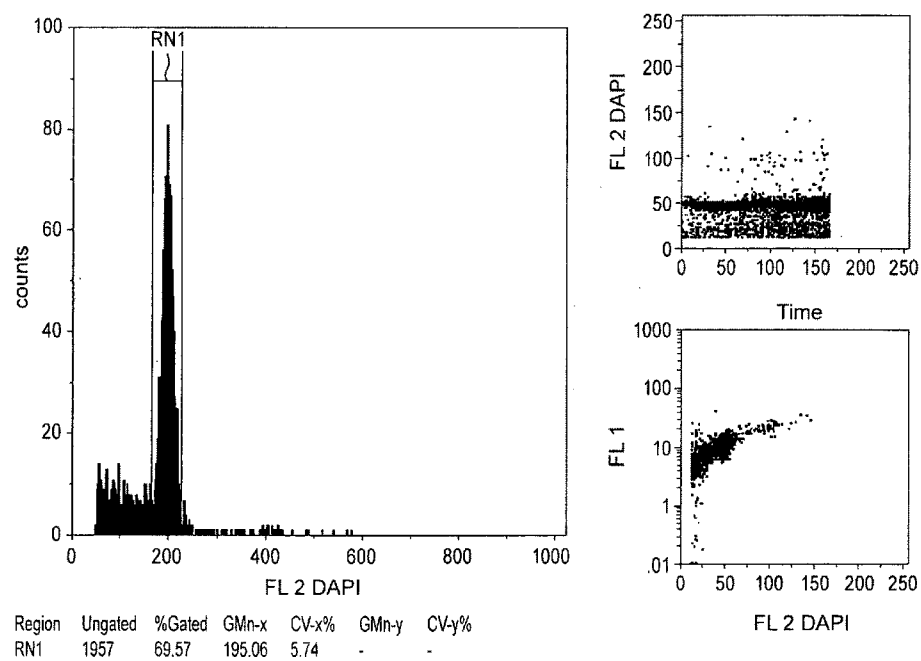
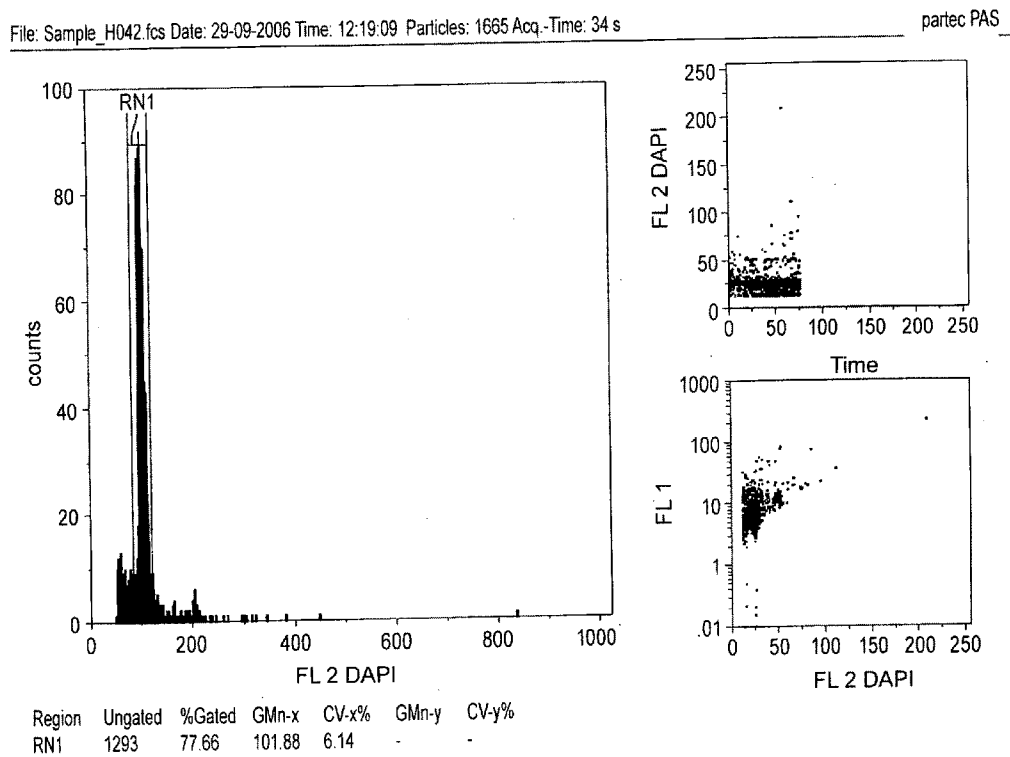


FIG. 5B

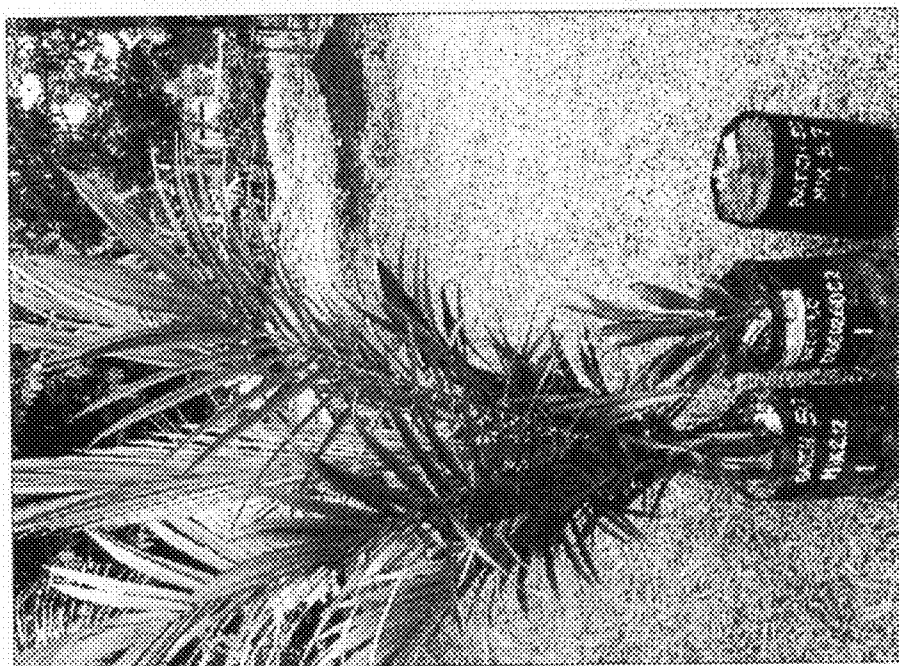


Parents of Homozygotes

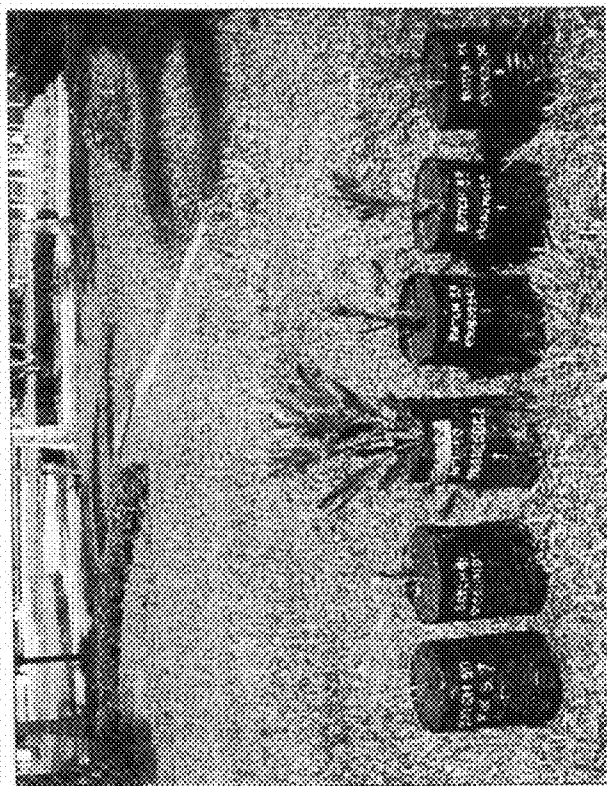
Candidates	Confirmation	Female	Male	Fruit type
50-mix5-07	homozygous	??	??	Dura
50- 03060367C	homozygous	BL1237/38-05	BL148/05-08	Pisifera
50-03060260C-2	homozygous	BL1231/05-05	BL143/04-06	Dura
53-03090761C-5	homozygous	BL1233/22-11	BL148/19-03	Dura
53-03080954C-2	homozygous	BL1222/35-11	BL148/19-03	Dura
51-03060318C	??	BL1233/22-02	BL143/04-06	??

FIG. 6

Homozygotes & Haploids



Hetero Homo



Confirmed Haploids

FIG. 7A

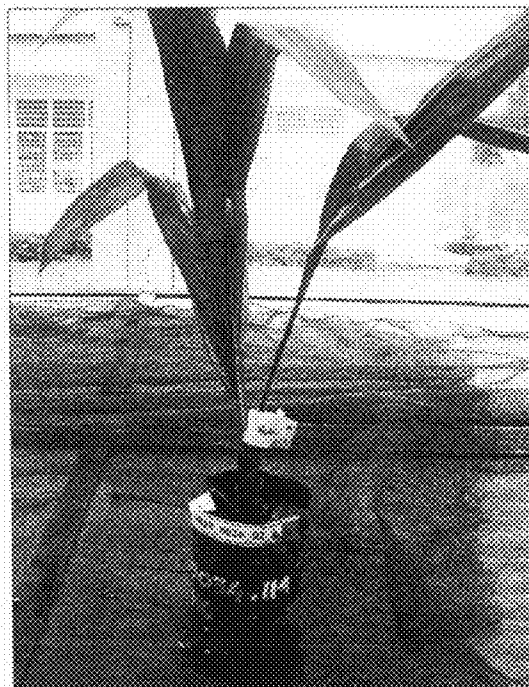
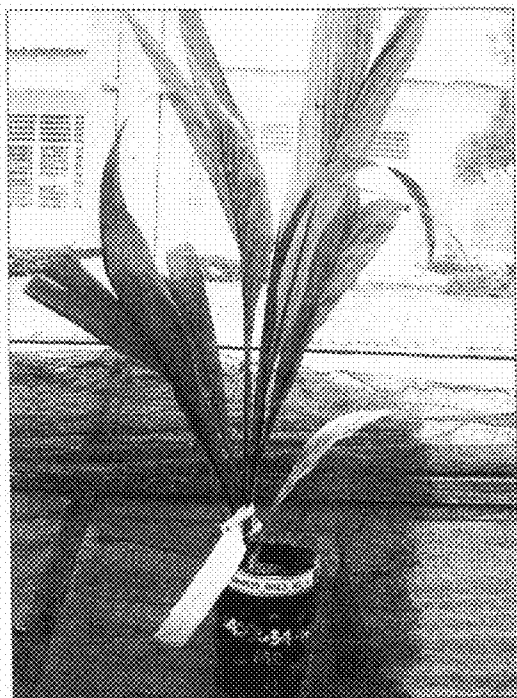


FIG. 7B

Measurement of Ploidy

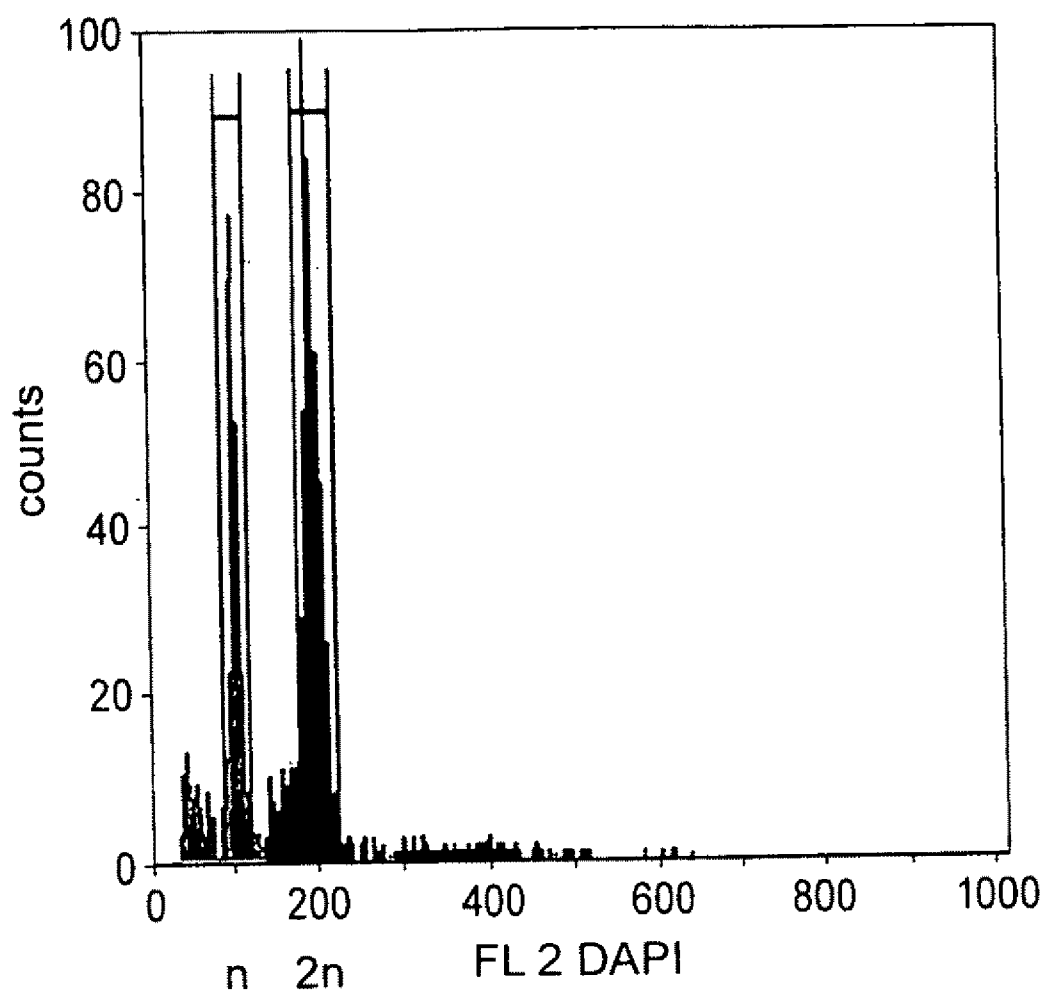


FIG. 8

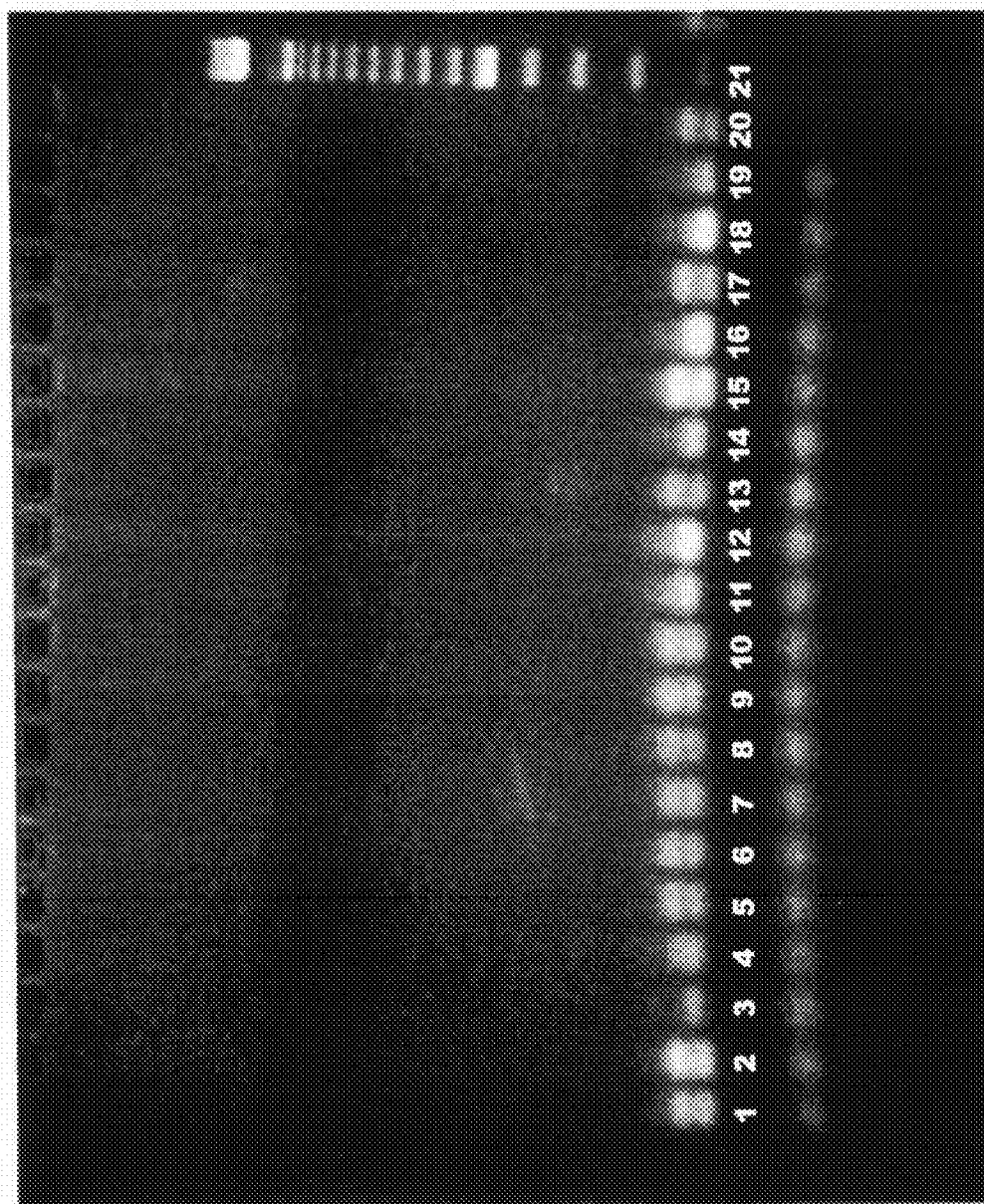


FIG. 9

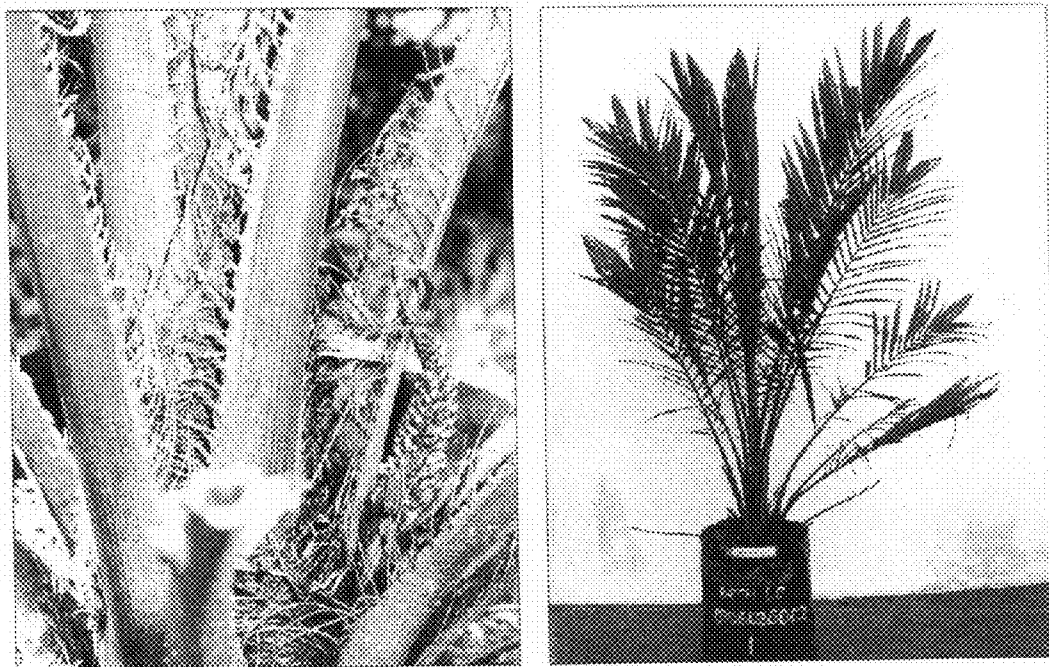


FIG. 10

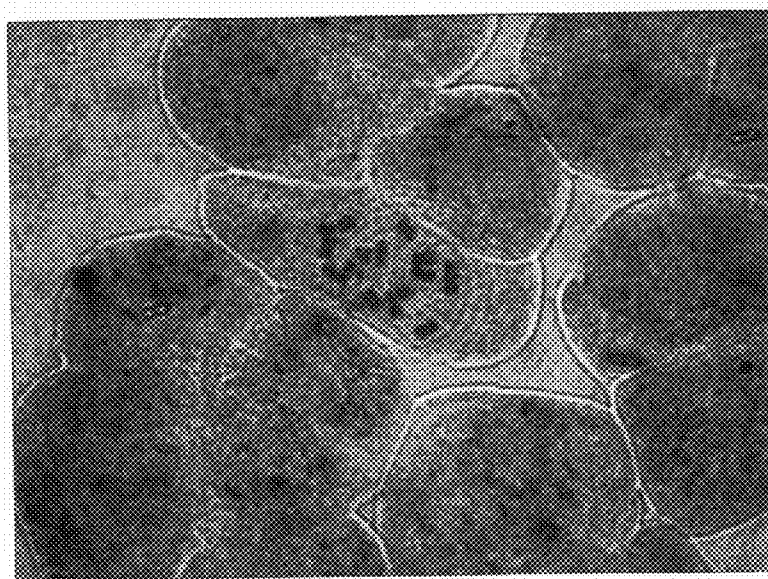


FIG. 11

METHODS OF PRODUCING HAPLOID AND DOUBLED HAPLOID OIL PALMS

FIELD OF THE INVENTION

[0001] The present invention relates to haploid palm plants and homozygous doubled haploid palm plants. The invention also relates to methods for producing and selecting haploid and doubled haploid plants. More particularly, but not exclusively, the method may be used for selecting haploid and homozygous doubled haploid oil palm and date palm plants.

BACKGROUND OF THE INVENTION

[0002] Although plant breeding programs worldwide have made considerable progress developing new cultivars with improved yield, pest and disease resistance, and other useful traits, breeding as a whole relies on screening numerous plants to identify novel, desirable characteristics. Often, very large numbers of progeny from crosses must be grown and evaluated over several years in order to select one or a few plants with a desired combination of traits.

[0003] In a typical plant breeding programme, two parent plants are crossed and the resulting progeny are screened and one or more plants that possess a desirable combination of phenotypic traits are identified and selected. The plant with desired traits may then self-fertilise or be crossed to yield a population of progeny plants that must be individually analysed to determine which plants possess the desired combination of phenotypic traits originally introduced in the first generation. If, as is often the case, the desired phenotypic traits are derived from the combined effect of several genes, then the number of progeny plants that must be screened depends on the number of genetic differences between the parent plants. Thus, the greater the number of genetically-controlled differences between parents, the larger the number of progeny that must be grown and evaluated, and the lower the probability of obtaining progeny with all the desired traits. The problem is exacerbated when some of these traits (such as yield) require the plants to reach maturity before they can be evaluated.

[0004] One possible solution to the problem of screening large numbers of progeny that segregate for the desired traits depends on the ability to produce or identify haploid plants derived from the gametic cells of parental individuals. The chromosome complements of these haploids sometimes spontaneously double to produce diploid plants or else can be doubled artificially using colchicine or by other means. In particular, though not exclusively, doubled haploids can be produced by the in vitro culture of microspores that normally give rise to pollen grains. The resultant doubled haploid plants, however they are derived, are instantly and completely homozygous. This means that the seed offspring generated from the selfing of such plants are genetically identical to the parental clone and so can be multiplied rapidly by seed. Furthermore, when two such doubled haploids are crossed sexually, the resultant seed offspring are genetically invariant and heterozygous for all loci that differ between the two parents (i.e. they are genetically uniform F_1 offspring).

[0005] The ploidy level of a somatic cell is defined as the number of genome sets of chromosomes that it contains. A genome set of chromosomes (also known as the base number, x) is most simply described as the number of heterologous chromosomes present in the nuclear genome and equals that present in the gametophytes of a diploid organism. For

example, humans are diploid organisms, having $2n=2x=46$ chromosomes in their somatic cells and $n=x=23$ in their gametes (eggs and sperm). When the ploidy level is greater than one, genetic analysis is made more difficult by the effects of dominance; when more than one copy of a gene is present only one copy, the dominant one, may influence phenotype or else both copies contribute to the expressed phenotype (partial or no dominance). With dominance, the other copy of the gene, the recessive allele, is apparently 'masked' because its presence is not apparent at the phenotypic level. Haploid organisms contain the same number of chromosomes (n) in their somatic cells as do the normal gametes of the species. The term haploid sporophyte is generally used to designate sporophytes having the gametic chromosome number (Palmer and Keller, 2005a) and in a diploid organism this complement is the same as the base number (x).

[0006] Haploids of higher plants can be distinguished from their diploid equivalent in many ways. Most obviously from the perspective of phenotype, they are usually smaller in appearance, partly because of their smaller cell size; in general terms, cell volume in plants is positively correlated to ploidy level. Several methods for the provisional assignment of the haploid status of a plant exploit this relationship. The most widely used of these phenotypic methods is the measurement of stomatal guard cell length and chloroplast content in these cells (e.g. Sari et al., 1999; Stanys et al., 2006), although none of the phenotypic predictors of haploidy is absolutely reliable. Methods providing direct measurements of genome size provide a far more reliable diagnosis of haploid status. These include direct measurement of the chromosome number using conventional chromosome counting techniques, and measurement of the DNA content using microdensitometry (e.g. Zhang et al., 1999) or more especially, flow cytometry (Coba de la Pena and Brown, 2001; Bohanec, 2003; Eeckhaut et al., 2005). The latter technique has also been applied to characterise the cell cycle stages in various tissues of oil palm material, although not for the detection of haploid plants or tissues (Srisawat and Kanchanapoom 2005; Srisawat et al., 2005). It is also possible to exploit the absolute absence of heterozygosity in haploids and doubled haploids to detect such plants using various co-dominantly inherited molecular marker methods (e.g. Chani et al., 2000; Tang et al., 2006).

[0007] Haploids may have intrinsic value because of their overall reduction in size compared with diploids. Haploids also have value in allowing the isolation of mutants, which may be masked in a diploid, particularly where the mutant allele is non-functional. Haploids also have value in transformation programmes. If haploids are transformed directly, then true breeding diploid transgenic plants can be produced in one step following doubling of chromosomes. It should be noted that a wide range of techniques for chromosome doubling are known (Kasha, 2005 and references incorporated therein) and these techniques, or modifications of them, are applicable and relevant in the context of this invention. Some studies on the development of chromosome doubling techniques in oil palm have already been reported and whilst these data relate to the doubling of diploid material (to give polyploids), the protocol described will also have utility for haploid doubling (Madon et al., 2005a).

[0008] An important use of haploids is based on the fact that marked improvements in the economics of plant breeding can be achieved via doubled haploid production, since selection and other procedural efficiencies can be markedly improved

through the provision of elite true-breeding (homozygous) progenies (Nei, 1963; Choo, 1981; Melchers, 1972; Hermesen and Ramanna, 1981; Snape, 1984). With doubled haploid production systems, homozygosity is achieved in one generation. Thus, the breeder can eliminate the numerous cycles of inbreeding that is usually necessary to achieve practical levels of homozygosity by conventional methods. Indeed, absolute homozygosity for all traits is not achievable by conventional breeding methods. Consequently, an efficient doubled haploid technology would enable breeders to reduce the time and the cost of cultivar development relative to conventional breeding practices.

[0009] Spontaneous haploids may occur in many species of plants, albeit at low frequencies. For tropical perennial crop species of commercial importance the following summary is relevant:—oil palm (none reported from any source), rubber (no spontaneous haploids reported, though two reports from anther and ovary culture quoted in Table 3-1 from Maluszynski et al., 2003b, are Chen et al., 1988, Jayasree et al., 1999), sugar cane (no spontaneous haploids, though again two reports quoted in Maluszynski et al., 2003b, are Liu et al., 1980, and Fitch and Moore 1996), coffee (reports of spontaneous haploids, eg Lashermes et al., 1994) cotton (many examples of spontaneous haploids), cacao (spontaneous haploids reported, Dublin 1972). It is important to note in the context of the current invention that in none of the cases where spontaneous haploids have been described has it been subsequently possible to accumulate significant numbers of haploids or doubled haploids to have utility for crop improvement, in other words they are rare in occurrence. For this reason, emphasis has turned to alternative means of generating haploids and doubled haploids. Haploid plants of several other species have also been created following various laboratory manipulations, including parthenogenesis, androgenesis, chromosome elimination, and tissue culture-based methods, although progress has been poor for perennial crops, particularly tropical species that habitually outcross.

[0010] The general lack of progress towards haploid and doubled haploid production in woody species (Stettler and Howe, 1966) is due mainly to the present emphasis on production methods involving an in vitro phase; there are numerous problems associated with the general intransigence of woody species to growth under such conditions

[0011] As well as having value in their own right as potential new varieties, homozygous plants also have utility for the generation of F_1 hybrid plants, where crosses are made between selected homozygous males and females. These F_1 plants often exhibit so-called hybrid vigour (heterosis), a characteristic often associated with dramatic increases in yield compared with either parent, and first described by Shull (1908). Furthermore, the production of F_1 hybrids allows the breeder to produce large quantities of seed comprising of a single genotype from homozygous parental lines. This property will have many advantages over a genetically heterogeneous mix of genotypes because of the potential to select single elite genotypes that produce high yields and/or possess other desirable characteristics. There is also potential to achieve higher yields by selecting genotypes for adaptation to specific environments and to optimise agronomic and management practices. In many crops, the only realistic alternative to producing a single genotype in commercial quantities is by asexual cloning. There are well-developed methods of vegetative propagation, using suckers, cuttings or grafts to

produce clones for some crops (for example, rubber, cocoa and coffee) but not all crops (for example, oil palm and coconuts).

[0012] According to Hermesen and Ramanna (1981):—“Just as in self-pollinators, the application of haploidy in cross-pollinated diploid crops is based on the use of DH-lines (Doubled Haploid lines). However, owing to inbreeding depression (note: homozygous individuals of a normally outcrossing species typically exhibit reduced vigour and this is known as inbreeding depression), these lines cannot be used directly but only as parental inbred lines for the production of hybrid varieties. When inbred lines are being developed via haploids, all barriers to repeated selfing, which are characteristics of natural cross-pollinators are bypassed, e.g. dioecy, self incompatibility and long juvenile periods. The time saving is particularly apparent in biennial crops and in crops with a long juvenile period. Only via haploidy can inbred lines be developed in these crops.”

[0013] As such, haploid plants (and doubled haploid plants) reveal all their genetic information or, in other words, their genotype is completely displayed by their phenotype. Resistance to pest and diseases or unfavourable external factors (drought, salinity, heavy metal toxicity etc) can thus be directly recognized and selected. Haploid plants allow the detection of mutants that are unable to pass through the embryonic phases. For similar reasons haploid plant tissue make ideal vehicles for genetic transformation, by whatever gene manipulation techniques are relevant, to give genetically modified material that on doubling give homozygous versions of the introduced gene or genes.

[0014] The agricultural applications for haploids centres on their capacity for the rapid generation of homozygous genotypes after chromosome doubling:

[0015] Reduce time for variety development, e.g. from 10 to 6 years or less;

[0016] Homozygous recombinant lines can be developed in one generation instead of after numerous back-cross generations; and

[0017] Selection for recessive traits in recombinant lines is more efficient because recessive alleles are not ‘masked’ by the effects of dominant alleles.

[0018] Introduction of “alien” genes speeded by allowing homozygotes to be developed readily.

[0019] The crossing of two homozygous elite lines (such as can be produced by doubling haploids) can generate genetically uniform, highly heterozygous ‘hybrid’ varieties, as is exemplified by the highly successful hybrid maize varieties first produced in the USA during the 1930s. It is not surprising that there have been many efforts to reproduce the yield increase gained in hybrid maize varieties in other crops, through the development of ‘hybrid lines’. For example, F_1 hybrid varieties of sunflower and sugar beet are now widely grown on a commercial basis, and hybrid lines of oilseed rape (canola) and rice are becoming increasingly available; more than half the rice grown in China is ‘hybrid’, with yields at least 20% higher than the non-hybrid equivalent. To date, there has been no corresponding progress with the highest yielding of all oilseed crops, oil palm; although oil yields of 4.8-7 t/ha are 3-8 times greater than other oil seed crops (Wahid et al., 2004). Overall, oil palm is the world’s leading source of vegetable oils and fats, on a par with soybean (Abdullah, 2005) but has nevertheless yet to benefit from the release of hybrid varieties.

[0020] The lack of progress towards the generation of hybrid varieties for oil palm is principally because the breeding system of the crop precludes the simple production of inbred lines. Oil palm is essentially an outbreeding species, but unlike corn in which a male and a female flower are produced on the same plant at the same time, each oil palm plant produces either male or female flowers at any one time, and therefore a palm can only readily be self-pollinated by methods of controlled pollination using stored pollen. Progress in converting oil palm into a hybrid crop, and thereby exploiting the potential hybrid vigour, depends upon the development of a process for the reliable production of homozygous plants. To date, however, there is no published example of any haploid, or homozygous diploid oil palm plant. There has nevertheless been extensive breeding (Wahid et al., 2004), cell culture (Abdullah 2005; Abdullah et al., 2005; Rival and Parveez, 2005; Te-chato et al., 2005), and transformation studies (US Application 20030159175) geared towards the genetic improvement of the oil palm crop.

[0021] Two main species of oil palm plant are grown commercially: *Elaeis oleifera* Kunth and *Elaeis guineensis* Jacq. The latter has three sub-types: *dura*, *tenera*, and *pisifera*. Most cultivars or planted stands are *tenera*, which produces fruit with higher oil content. Date palms are a family of species comprising *Phoenix dactylifera* and other *Phoenix* species that are interfertile within *dactylifera*.

[0022] All oil palm seeds currently used for commercial plantings are produced from parents selected from genetically heterogeneous populations of non-homozygous palms. Variation in the level of parental heterozygosity and in the genetic divergence between parental lines means that there is extensive genetic segregation amongst the resulting seed offspring. Thus, the seeds produced from palm crosses are therefore not genetically uniform. This genetic variation impedes the oil palm industry from selecting specific genotypes for high yield or other desirable traits.

[0023] The oil palm only has a single growing point, and unlike some other palm species, including date palm, does not produce suckers. For these reasons, clones cannot be produced by the standard methods of vegetative propagation. However, it is possible to produce somatic clones by tissue culture, in which small pieces of tissue (explants) from leaves, inflorescences or roots are first cultured on special nutrient media. The growing tissue may then form callus (a mass of cells without differentiation), and this may be treated to produce embryoids tissue, which themselves slowly develop into plant shoots. However, such tissue culture techniques are generally difficult and laborious to perform, and the underlying biology is poorly understood. Moreover, there is also a risk of somaclonal variation, induced by the tissue culture process itself and which has led to phenotypic abnormalities (Corley et al., 1986) that may result in complete loss of yield.

[0024] Mention should be made of the apparent claim by Maluszynski et al. (2003b, see Table 3-1) that Teixeira et al. (1994) have already published a protocol for doubled haploid production in oil palm. This claim is erroneous. In fact, the latter publication describes somatic embryogenesis from diploid floral tissue, and does not describe the culture of anthers or other reproductive tissue to produce haploid embryos and plants.

[0025] The only related work on other palm species include failed attempts to produce haploids of coconut (*Cocos nucifera*) via anther culture (Thanh-Tuyen and De Guzman,

1983a,b; Monfort, 1984, 1985; Thanh-Tuyen, 1985, 1990; Pannetier and Buffard-Morel, 1986; Griffis and Litz, 1997; Perera, 2002a,b, 2003, Perera et al. 2006), and date palm (*Phoenix dactylifera*) (Brochard, 1981; Bouguedoura, 1991; Chaibi et al., 2002). There is one report of attempts at ovule culture in coconut (Coconut Research Board, 2002). However no haploid plants were produced from any of these in vitro studies. However, there is a single example of a haploid coconut plantlet isolated from a twin seedling (Whitehead and Chapman, 1962), and cytological evidence of a haploid chromosome number ($n=16$) observed from a single embryo from the same species. There also a claim that treatment of unpollinated female date palm inflorescences with gibberellic acid induced doubled haploid "apomictic" progeny (Ben Abdallah et al., 2001). However, none of these publications describe an effective method to produce and select spontaneous haploids or doubled haploids or provide teaching relevant to the production of haploid or homozygous material of oil palm.

[0026] Oil palm is a perennial monocotyledon, with a long generation period such that breeding of the crop is a very slow process; generally taking approximately 20 years to develop and progeny test a new generation of palms for commercial seed production. There are no reports of breeders producing inbred lines by inbreeding (eight generations of selfing) because this would take a biological minimum of 40 years to achieve because of the time required to make crosses (6 months), process seed (3 months), grow seedlings in nursery (12 months), field plant seedlings—male and female inflorescences will develop after 18-24 months, collect pollen & self pollinate palm and harvest bunch (24-30 months). Currently, genetic improvement of oil palm is mainly performed by conventional means. Compared to other oil producing crops, which are predominantly annuals, the introduction of novel traits into oil palm is an extremely protracted process; it may require between 12 to 14 years to improve or to introduce a trait into oil palm. In addition to the long generation period, breeding of perennials such as oil palm require large areas for breeding trials and an extensive series of time-consuming backcrosses.

[0027] Thus, the lack of progress with oil palm is principally because the breeding system of the crop precludes the simple production of inbred lines. Oil palm is essentially an outbreeding species, but unlike maize in which a male and a female flower are produced on the same plant at the same time, each oil palm plant produces either male or female flowers at any one time, and therefore a tree cannot be easily self-pollinated. Progress in converting oil palm to a hybrid crop, and thereby exploiting the potential hybrid vigour, depends upon the development of a process for the reliable production of homozygous plants.

SUMMARY OF INVENTION

[0028] In accordance with a first aspect of the invention, there is provided a method for selecting haploid or doubled haploid oil palm or date palm plants useful for seed production, multiplication and crop improvement, the method comprising

[0029] (a) providing a population of palmplants;

[0030] (b) choosing from the population a subset of individual plants of atypical phenotype;

[0031] (c) assessing the heterozygosity of plants in the subset in a prescreen;

[0032] (d) assessing the DNA content of plants in the subset;

[0033] (e) discarding from the subset plants found to be heterozygous;

[0034] (f) classifying remaining plants in the subset as haploid or diploid according to the results of step (e).

[0035] Steps (c) and (d) may be done in either order. Similarly, step (e) may precede or follow step (d), though it cannot precede step (c) because it is dependent on the results obtained in that step.

[0036] Preferably, plants classified in step (f) as diploid are further assessed for heterozygosity using multiple molecular markers, those found to be heterozygous being discarded and the remainder classified as doubled haploids.

[0037] Preferably, step (c) to assess the heterozygosity of the chosen subset uses molecular or biochemical markers, in particular between 2 and 40, for example between 10 to 20 microsatellite markers, although similar numbers of markers using one of the many marker systems based on Single Nucleotide Polymorphisms (SNPs) could also be applied (e.g. High Resolution Melt analysis or pyrosequencing).

[0038] Preferably, the atypical phenotype is atypical growth morphology or growth pattern which may appear during the germinated seed or seedling stages, or later. More preferably, the atypical growth morphology is one or more of reduced radicle growth, altered radicle:plumule length ratio, changed radicle:plumule angle, altered colour of radicle or plumule, altered seed shape or size during germination; and altered radicle width:length ratio. The atypical phenotype of a germinated seed may also be the germination of two embryos from a single seed. Selection may also be carried out in a population of palms comprising nursery or field planted palms, when the atypical growth morphology or growth pattern may for example be one or more of slower vegetative growth, reduced ratio of leaflet width to length, reduced frond internode distance, angle of frond to plant axis, leaf colour, and precocious flowering.

[0039] By “atypical phenotype” is meant any aberrant phenotype exhibited by the haploids and doubled haploids that falls outside the normal phenotypic range expected for non-haploid material (i.e. usually diploid but polyploid for some crops). The confidence limits constituting ‘the normal range’ may change from species to species but atypical individuals might ordinarily represent less than 1% of the population screened. For example, in oil palm, candidate haploid seedlings may be selected from germinated seed just after the plumule and radicle have developed. Germination will commence after about 10 days incubation in the germination room. Cohorts of germinating oil palm seedlings typically exhibit a fairly synchronous developmental pathway and a reasonably homogenous phenotype (see FIG. 1). Abnormal germinated seed may deviate from the characteristic phenotype in one of many ways (see FIG. 2) and may include features including those as diverse as reduced radicle growth, altered radicle:plumule length ratio, changed radicle:plumule angle (typically around 180° in normal types), altered colour of radicle or plumule, changes of seed shape or size, changed radicle width:length ratio and germination of two embryos from a single seed (twin seedlings). A key element of novelty here lays in the logical reiterative nature of the selection of features that are used in the definition of atypical. Moreover, as the number of identified haploids increases, ordination approaches are used to identify those traits that are most important in discriminating the atypical set and these are used

to redefine the search criteria. This process will progressively improve accuracy of the phenotypic screen as the increasing numbers of haploids enhances statistical power and as uninformative traits are discarded from consideration, and will continue until there are no further increases in haploid frequency. Accordingly, it is a feature of the invention to use as the atypical phenotype by which plants are selected one or more atypical phenotypes shown from previous tests to correlate with haploid or dihaploid character.

[0040] Preferably, the step of further assessing the homozygosity of a chosen plant, e.g., a germinating seedling, using multiple molecular markers comprises using between 50 and 200, for example between 70 and 120, microsatellite markers. More preferably, this step is performed with a pooled sample of markers. A chosen plant is identified as a doubled haploid if it is homozygous for all molecular markers used.

[0041] Preferably, the population of plants comprises at least 1,000,000 individuals. More preferably, the population of plants comprises between 5,000,000 and 20,000,000 individuals. Still more preferably, the population of plants germinated individuals is a population of germinated seeds or seedlings.

[0042] By “providing germinated seeds or seedlings” is meant any process whereby seeds sprout and seedlings begin to grow. In the case of oil palm, it includes both the germination techniques commonly used by commercial and plant breeding seed production units: the wet heat method and the dry heat method. The former method is now less used: the whole process may be shorter (95 days against 120 days for dry heat), but some germination will take place during the heating period and so a less uniform set of seedlings will be produced. Oil palm seed is dormant when it is harvested, and under natural conditions germinates sporadically over several years. The critical requirement to break dormancy is to maintain the seed at a raised temperature of 39-40° C. for up to 80 days.

[0043] The nature of the marker system used or the order in which elements or activities are applied in the above can be adjusted according to circumstances.

[0044] The use of markers, preferably co-dominant molecular markers, allows the identification of hemizygous haploids and homozygous diploid individuals. Haploid and doubled haploids have only one allele for all loci within their nuclear genomes. Therefore, any individual exhibiting two alleles for any locus can be discarded as a potential haploid or doubled haploid plant. It is preferred according to our invention to provide a low-cost pre-screen to discard large numbers of false candidates and a high-resolution genome characterisation (see below) to confirm haploid or doubled haploid status following DNA content assessment (e.g. by flow cytometry, see below).

[0045] Flow cytometry is used for assessing the genome content of plant or animal cells, and can be used to distinguish between diploid and haploid material. By “flow cytometry” is meant any method for counting, examining and sorting analyte suspended in a stream of fluid. It allows for simultaneous multiparametric analysis of the desired characteristics of single cells flowing through an optical or electronic detection apparatus. In this step, therefore, flow cytometry is applied to the individuals exhibiting an abnormal phenotype and also high levels of homozygosity (identified in steps b and c) to distinguish between the haploids and diploids. Thus, haploid plants are first identified at the completion of this step.

[0046] A more comprehensive molecular assessment of genomic heterozygosity is used to provide genetic confirmation of the identity of haploid plants and also identifies individuals that are diploid and are derived from the chromosome doubling of haploid individuals (so-called doubled haploid plants). In both cases, the assessment is based on the fact that haploids and doubled haploids will be completely hemizygous and homozygous respectively. Preferably, microsatellite markers are used for this purpose, although many other marker systems could equally be used. By this means the status of haploids is confirmed and doubled haploid plants identified.

[0047] Novelty in this method resides partly in the reiterative nature of the phenotypic screen and the use of the ligation-cloning method to generate large numbers of markers to confirm haploid status but also in the combination of steps to create a method that systematically identifies rare haploids from amongst a large population of seed that are the product of a sexual cross, which has previously been considered impractical.

[0048] In accordance with a second aspect of the invention, there is provided a plant selected by the method according to the first aspect of the invention.

[0049] In accordance with a third aspect of the invention, there is provided a method for producing a homozygous doubled haploid oil palm plant, the method comprising:

[0050] (a) selecting a haploid oil palm plant using a method according to the first aspect of the invention;

[0051] (b) obtaining a doubled haploid oil palm plant through spontaneous chromosome doubling; or by doubling the chromosome number by application of an external stimulus to the haploid plant; or by application of an external stimulus to a cell or cells isolated from the haploid plant, followed by regeneration of a plant using tissue culture; or by pollinating or cloning the haploid plant, or by selfing the haploid plant by exploiting the occasional spontaneously doubled chromosome number in male and female reproductive cells.

[0052] In accordance with a fourth aspect of the invention, there is provided a method for producing a diploid F_1 hybrid of oil palm, the method comprising:

[0053] (a) selecting at least two homozygous doubled haploid oil palm plants using a method according to the first aspect of the invention; or obtaining at least two homozygous doubled haploid oil palm plants using a method according to the third or eighth aspect of the invention;

[0054] (b) Using two genetically different homozygous doubled haploid oil palm plants identified above and sexually crossing these to produce genetically uniform F_1 hybrid offspring.

[0055] In accordance with a fifth aspect of the invention, there is provided an oil palm plant produced by the method according to the third and fourth aspects of the invention.

[0056] In accordance with a sixth aspect of the invention, there is provided a haploid oil palm plant.

[0057] In accordance with a seventh aspect of the invention, there is provided a homozygous doubled haploid oil palm plant.

[0058] The incorporation of atypical phenotype in the method described above favours the selection of haploid plants over doubled haploid plants since elite examples of the latter may actually exhibit a normal phenotype. For this reason, we also provide a second method that preferentially

selects for doubled haploid offspring from the same starting material. This is a general method that has application both to oil and date palm and to other crops.

[0059] Thus, in accordance a eighth aspect of the invention, there is also provided a method for identifying doubled haploid plants among progeny of a single maternal parent, the method comprising:

[0060] (a) identifying at least 20 heterozygous unlinked loci in the maternal parent, preferably using co-dominant molecular markers such as microsatellites or SNP-based markers

[0061] (b) performing a preliminary screen using 1-5 of the selected markers; discarding heterozygotes; retaining the remainder as candidate doubled haploids

[0062] (c) applying flow cytometry to the retained candidates; discarding haploids; retaining diploids as potential doubled haploids

[0063] (d) applying at least a further 15 of the remaining markers to the retained candidates, and classifying individuals that are diploid and homozygous for all applied markers as doubled haploids (the probability of this occurring by independent assortment is $2^{20} = 1/1,048,576$; lower, if more than 20 markers are used)

[0064] We are unaware of any study aiming to select doubled haploid, absolutely homozygous plants amongst sexual offspring that incorporates prior characterisation of the maternal parent and selection of a fixed number of unlinked heterozygous loci as a basis for subsequent screening. This step allows for standardisation between experiments, genotypes and species since although the markers used may be different, the power of analysis remains the same (in this case a $1/1,048,576$ of finding a single false positive homozygous diploid rendered such by independent assortment). This step of the second method is therefore novel, as is the assembly of steps to create the method.

[0065] Commercial and government oil palm breeding programmes have not invested in research programmes to identify haploid genotypes despite the inherent value of haploids (or directly doubled haploids) to produce parental true breeding lines and thus F_1 hybrids. The discovery of a process to obtain haploids in any crop has the potential to transform the rate of breeding progress because well advanced and proven breeding strategies can be adopted from the cereal world crops e.g. rice, wheat, maize etc. Furthermore genetically homogeneous commercial planting material can be produced which further increases value by selecting specific F_1 hybrid crosses for particular locations, management practices, or which have certain selected traits.

[0066] Instead the oil palm industry for the last thirty years has invested millions of US dollars in the production of oil palm clones by somatic embryogenesis. This is despite the major problems with flowering abnormality which may result in zero bunch yield and the failure of researchers to fully understanding the underlying biological mechanisms which cause flowering abnormality (it is widely assumed to be an 'epigenetic' phenomenon: a modification of gene expression, passing from one cell generation to the next). Despite these difficulties the oil palm industry has remained committed to new investment in oil palm cloning techniques to produce superior genetically uniform plants: It is therefore surprising that until now a process has not been developed to screen for spontaneous haploids and double haploids which would avoid these problems. We are aware of previous unpublished

attempts by Malaysian oil palm research stations to screen seedlings in the nursery for haploids but with no apparent success.

BRIEF DESCRIPTION OF FIGURES

[0067] In order that the present invention may be fully understood and readily put into practical effect, there will now be described by way of non-limitative examples only preferred embodiments of the present invention, the description being with reference to the accompanying illustrative figures.

[0068] In the figures:

[0069] FIG. 1 shows normal seedlings after germination;

[0070] FIG. 2 shows abnormal seedlings after germination;

[0071] FIG. 3 shows seedlings after transfer to a nursery house;

[0072] FIG. 4A shows an example gel used to identify individuals that are homozygous (one band) and heterozygous (two bands) for a selected marker;

[0073] FIG. 4B is a flow chart showing a hierarchical screen to identify homozygous plants;

[0074] FIG. 5 shows a representative flow cytometry histogram of samples from a diploid (a) and a haploid (b) genotype;

[0075] FIG. 6 is a table showing parents of confirmed haploids;

[0076] FIG. 7A shows haploids and corresponding heterozygous diploid plant;

[0077] FIG. 7B shows images of a typical diploid heterozygous oil palm (bottom) and two doubled haploids (top) sown on the same day.

[0078] FIG. 8 shows the DNA content of haploid and diploid plants as measured using flow cytometry.

[0079] FIG. 9 shows a photograph of gel showing use of molecular markers to identify haploids/homozygous diploids (one band) from heterozygous diploids (two bands).

[0080] FIG. 10 shows confirmed haploid 50-03060260-0002 with first inflorescence two years and seven months after planting (left photograph of inflorescence and right photograph of haploid seedling).

[0081] FIG. 11 is a photomicrograph of cells of a haploid oil palm according to the invention.

DEFINITIONS

[0082] Following are definitions of words used in the specification and claims:

[0083] "plant" includes whole plants at any stage of development, for example seeds, germinated seeds, seedlings, nursery and field-planted palms; and progeny of same.

[0084] "haploid" means any cell containing the gametic chromosome number, or any tissue or plant comprising such cells.

[0085] "homozygous" characterises any cell containing two or more identical sets of chromosomes, or any tissue or plant composed of such cells

[0086] "plantlet" means any small plant which is not fully grown.

[0087] Origin of Materials Used

[0088] The oil palm germplasm (*Elaeis guineensis* Jacq) used in the following experiments was obtained in Indonesia (Sumatra) where the first stage of the procedures (selection of material of atypical phenotype) was carried out. The historic origin of the oil palm (*Elaeis guineensis*) is understood to be

West Africa, where it has been cultivated for many years: the species was introduced from West Africa to the Pacific region in the first half of the last century, since when it has been widely cultivated throughout that region.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example

[0089] The following example is presented to further illustrate and explain the present invention and should not be taken as limiting in any regard. It shows the application of the invention to oil palm: but is similarly applicable to date palm.

[0090] All references herein mentioned are hereby incorporated by reference.

[0091] (1a) Seed Processing

[0092] 1 The mesocarp was mechanically removed from oil palm seeds and the seeds were air dried for 24 hours at ambient temperature and then for 24 hours in an air-conditioned room at 25° C. to a seed moisture content of 15-18%. The seeds were then stored, usually for one to three months in an air-conditioned room (25° C.) in plastic bags or trays (although it is possible to store seeds for up to one year in this way).

[0093] 2 The seeds were soaked for three days to increase their moisture content to 18-20% and then heat treated in plastic bags or trays for 40 to 60 days at 38-40° C.

[0094] 3 After heating, the seeds were soaked for five days to raise their moisture content to >22% and then dried at ambient temperature for approximately four hours

[0095] 4 The seed were transferred to a germination room where under ambient temperatures germination usually starts after 7 to 10 days and continues for two to three months.

[0096] (1b) Morphological Screen

[0097] There were two large-scale morphological screens of oil palm seedlings for morphological off-types. The first consisted of 10,900,000 germinated seeds, of which 3,854 were identified as being morphologically deviant (3,801) or twin-seeded (53), with the remaining individuals all being deemed 'normal' (see FIGS. 1 and 2 for examples of both types). Thus, in this instance 99.96% of seeds evaluated were classified as exhibiting, a normal phenotype and 0.035% being aberrant. In the second screen, approximately 10,000,000 commercial seedlings were screened, together with approximately 1,000,000 seedlings taken from breeding experiments. This trial generated 5,704 morphological candidates, of which 5,601 were phenotypically abnormal and 103 were twin-seeded. In this screen, therefore, 99.95% of seedlings were classed as normal and 0.05% as aberrant prior to transfer to the nursery house (FIG. 3).

[0098] (1c) Molecular Pre-Screen

[0099] Molecular Pre-Screen to Exclude Heterozygous Individuals.

[0100] The protocol applied to perform a molecular pre-screen of seedlings showing abnormal phenotypes to discard heterozygotes comprised the following stages:

[0101] 1. DNA extraction

[0102] 2. Amplification of microsatellite markers by PCR

[0103] 3. Separation of PCR products by agarose gel electrophoresis

[0104] 4. Scoring of results to discard individuals with one or more heterozygous loci

[0105] Each stage is described below:

[0106] 1. DNA Extraction

[0107] Around 0.5 cm of the radicle (around 50 mg) was removed from the seedling and used to extract DNA using the Qiagen 96 DNeasy extraction kit according to the manufacturer's instructions as described below, although other systems for DNA extraction could also be used.

[0108] A. Preparation

[0109] 1. For new kits, add 100% ethanol to AP3/E buffer and AW buffer

[0110] 2. Set water bath to 65° C.

[0111] 3. Preheat AE and AP1 buffer to 65° C.

[0112] 4. If AP1 buffer has a cloudy appearance, heat to 65° C. and shake until the solution becomes clear

[0113] B. Protocol

[0114] 1. Add 50 mg plant material into each tube in two collection microtube racks. Retain the Clear cover.

[0115] 2. Add one tungsten carbide bead into each microtube.

[0116] 3. Prepare the lysis solution: (400 µl AP1+1 µl RNase+1 µl Reagent DX)/reaction plus 15% of each component.

[0117] 4. Disrupt the sample using MM 300, 30 Hz for 1.5 minutes.

[0118] 5. Pulse centrifuge to 3000 rpm.

[0119] 6. Remove and discard caps, add 130 µl AP2 buffer into each collection microtube.

[0120] 7. Close the microtubes with new caps. Place a clear cover (from step 1) over the 96 well plate. Shake the plate vigorously for 15 s. Pulse centrifuge to 3000 rpm.

[0121] 8. Incubate the racks for 10 min at -20° C.

[0122] 9. Remove and discard the caps. Transfer 400 µl of each supernatant to new plate of collection microtubes (provided). Do not transfer pellet and floating particles. Hold the strips and use the lowest pipette speed. Recover the tungsten beads.

[0123] 10. Add 1.5 volume (typically 600 µl) of AP3/E buffer.

[0124] 11. Close the microtubes with new caps and mix vigorously.

[0125] 12. Pulse centrifuge (3000 rpm) to collect solution).

[0126] 13. Place 96 well plates on top of S-Blocks provided.

[0127] 14. Transfer 1 ml of sample into each well of the 96 well plate.

[0128] 15. Seal with Airpore Tape sheet and centrifuge for 4 min at 6000 rpm.

[0129] 16. Add 800 µl of Buffer AW to each sample.

[0130] 17. Centrifuge for 15 min at 6000 rpm.

[0131] 18. Add 100 µl of buffer AE to each sample and seal with new AirPore sheets.

[0132] 19. Incubate for 1 min at room temperature (15-25° C.).

[0133] 20. Centrifuge for 2 min at 6000 rpm.

[0134] 2. Amplification of Microsatellite Markers by PCR

[0135] Primers

[0136] The following microsatellite markers were used:

	Forward primer	Reverse primer
Marker 1	GAGATTACAAAGTCCAAACC (SEQ ID NO: 1)	TCAAATTAAGAAAGTATGC (SEQ ID NO: 16)
Marker 2	ACGCATGCAGCTAGCTTTTC (SEQ ID NO: 2)	CGCGTGAAAGATATGAATCAAC (SEQ ID NO: 17)
Marker 3	CACGCACGCAGTTTATTCTT (SEQ ID NO: 3)	GGATGTATGCTTTACCTCCGAAT (SEQ ID NO: 18)
Marker 4	CCCCTTTTGCTTCCCTATTT (SEQ ID NO: 4)	CTCCTTTTCCCACATCACAGA (SEQ ID NO: 19)
Marker 5	GACACAAGCAAAAACAAAAGCA (SEQ ID NO: 5)	ATTCTGAAAGGAGGGGGA (SEQ ID NO: 20)
Marker 6	ATATGTGTGGGTGTGCGTGT (SEQ ID NO: 6)	TGCCTCTGGTTGTTAGTCTGG (SEQ ID NO: 21)
Marker 7	TCTCTCTCTCTCTCTATGTGGTGT (SEQ ID NO: 7)	TGGCAATCAGCACACATTCT (SEQ ID NO: 22)
Marker 8	GCAGCTCTTTCCACACCTCT (SEQ ID NO: 8)	TGTGGTCTCCTGAGGAAGATG (SEQ ID NO: 23)
Marker 9	TTTCCCCATCACAGAATTG (SEQ ID NO: 9)	CCCCTTTTGCTTCCCTATTT (SEQ ID NO: 24)
Marker 10	TAGCCGCACTCCCACGAAGC (SEQ ID NO: 10)	CCAGAATCATCAGACTCGGACAG (SEQ ID NO: 25)
Marker 11	AGCTCTCATGCAAGTAAC (SEQ ID NO: 11)	TTCAACATACCGTCTGTA (SEQ ID NO: 26)
Marker 12	CCTTCAAGCAAAGATACC (SEQ ID NO: 12)	GGCACCAACACAGTAA (SEQ ID NO: 27)

-continued

	Forward primer	Reverse primer
Marker 13	GTAGCTTGAACCTGAAA (SEQ ID NO: 13)	AGAACCACCGGAGTTAC (SEQ ID NO: 28)
Marker 14	GCTCGTTTTTTGTTTAGGTGA (SEQ ID NO: 14)	TTTCTCCATAGTCCGTTAC (SEQ ID NO: 29)
Marker 15	CCTCGGGTTATCCTTTTACC (SEQ ID NO: 15)	TGGCTGGCTTCGGTCTTAG (SEQ ID NO: 30)

Note:

Markers 10-15 were obtained from Billotte et al (2005)

[0137] Reaction Mixtures

[0138] In all cases, 10 μ l a PCR reaction mixture contained the following reagents; 1.0 μ l of 10 \times PCR buffer (Bioline), 0.3 μ l $MgCl_2$ (10 mM), 0.4 μ l dNTPs (10 mM of each), 0.2 μ l of each primer pair (10 μ M), 1-5 ng of DNA (extracted as above) and 1 U of Tag DNA polymerase (5 U μ l⁻¹ Bioline).

[0139] PCR Conditions

[0140] The following conditions were used for the Polymerase Chain Reaction for all microsatellite markers: an initial 94° C. denaturing step for 2 min followed by 35 cycles of; 94° C. for 30 sec, 52° C. for 30 sec and 72° C. for 45 sec, with a final extension step of 72° C. for 7 min.

[0141] 3. Separation of PCR Products by Agarose Gel Electrophoresis

[0142] Agarose gel electrophoresis and ethidium bromide staining were routinely used to fractionate and visualise products generated by microsatellite PCR.

[0143] (1) Reagents

TBE running buffer:	0.089 M Tris base, 0.089 M boric acid (pH 8.3) and 2 mM Na_2EDTA
Loading buffer:	0.23% (w/v) bromophenol blue 60 mM EDTA 40% (w/v) sucrose
Ethidium bromide stain:	1% (w/v) ethidium bromide
Ladder 100 bp (Gibco Life Science BRL)	

[0144] (2) Gel Preparation and Loading

[0145] 1.0-1.5% (w/v) agarose (was prepared in 1 \times TBE buffer and subjected to heating in a microwave (700 W) for 2 \times 1 min at full power to create a gel solution. The gel solution was cooled to approximately 55° C. prior to the addition of ethidium bromide (3.5 μ l per 100 ml gel). The ends of a suitable gel tray rig (midi-gel tray for 100 ml gels, maxi-gel tray for 250 ml gels) were sealed with masking tape and an appropriate number and type of combs placed in position. Combs with 16 \times 20 μ l wells were most often employed. The gel solution was carefully poured into the prepared tray and allowed to cool for at least 20 min. Combs and tape were then removed and the gel tray submerged into a tank containing 1 \times TBE buffer.

[0146] Generally, 5 μ l of sample were mixed with 2 μ l of bromophenol blue buffer prior to loading. The loading buffer serves two functions; first, it increases the specific gravity of the sample thereby preventing diffusion of DNA from the top of the well into the surrounding buffer, and second, it indicates the progress of product as they migrate through the gel

by electrophoresis (the blue dye migrates at approximately the same position as DNA fragments 200 bp in length). To estimate the size of the amplicons, 4 μ l of 100 by Gibco's ladder (Gibco Life Science BRL) were loaded together with the analysed samples.

[0147] Electrophoresis of mid-gels (100 ml) was performed at 120 Volts in 1 \times TBE buffer for approximately 1 h. Following electrophoresis, gels were removed from the rig and post-stained in 5 mg/l aqueous ethidium bromide solution for 40 min, destained in distilled water for 2 min and then viewed under Ultra Violet Illumination using a UVP Bio-Doc-system. Images of the gels were captured by the UVP Bio-Doc system as jpeg format and used for scoring.

[0148] 4. Scoring of Results to Discard Individuals with One or More Heterozygous Loci

[0149] PCR products generated by each microsatellite-genotype combination were evaluated for the presence of one or two distinct bands after fractionation by agarose gel electrophoresis (stages 1-3 above). Any genotype that yielded two products for any of the microsatellite loci was deemed to be heterozygous and so discarded as a possible candidate haploid or doubled haploid plant. The remaining individuals were sent forward to step (d) of the pipeline (flow cytometry)

[0150] Results

[0151] There were over 2000 phenotypically abnormal seedlings identified from the two morphological screens described above that were randomly selected for the molecular screen: In addition, there were a further 150 individuals with the normal phenotype. There were also 24 diploid tenera clones used as controls (all diploid heterozygotes).

[0152] When these were screened using up to 15 of microsatellite markers (1-15), 117 genotypes (see table in flow cytometry section for identification codes) exhibited a single allele for all loci (an example is shown in FIG. 4, using marker 09) and so were deemed to be highly homozygous.

[0153] Accordingly, these individuals were considered as candidate haploids/doubled haploids and progressed to step d (flow cytometry).

[0154] FIG. 4 shows Band profiles generated by marker 09 across 25 oil palm genotypes. Individuals showing two alleles (marked '2') were discarded from the screen.

[0155] (1d) Assessment of Nuclear Genome Content by Flow Cytometry**[0156] Flow Cytometry**

[0157] Individuals identified as morphologically abnormal and highly homozygous (stages b and c) were subjected to flow cytometry to establish their ploidy level using the following protocol.

[0158] Sample Preparation

[0159] The cell nuclei were isolated from fresh plant material (leaves or roots), by chopping the plant material (a few cm²/20-50 mg)) with a sharp razor blade in an ice-cold buffer, in a plastic petri dish. The DNA buffer (stored at 4° C.) is based on:

[0160] Arumuganathan, K. and Earle, E. D. Estimation of Nuclear DNA Content of Plants by Flow Cytometry. Plant Molecular Biology Reporter, Vol 9(3) 1991, Pages 229-233.

[0161] 5 mM Hepes

[0162] 10 mM Magnesium sulphate heptahydrate

[0163] 50 mM Potassium chloride

[0164] 0.2% Triton X-100

[0165] 2% DTT (Dithiothreitol)

[0166] 2 mg/litre DAPI

[0167] pH 8

[0168] DAPI, a fluorescent dye that selectively binds to form a complex with double-stranded DNA and give a product that fluoresces at 465 nm, was introduced to the solution. DAPI has specific DNA-binding properties, with preference for adenine-thymine (AD-rich sequences. After chopping, the buffer (ca. 2 ml.), containing cell constituents and large tissue remnants, is passed through a nylon filter of 40 micrometer mesh. This method will produce thousands of nuclei from a leaf piece of a few cm².

[0169] The solution containing stained nuclei was passed through the flow cytometer. Controls are required of known ploidy (DNA content) as reference—for oil palm, tissue from

diploid tenera palms were used because the shell thickness must be heterozygous and therefore the palm cannot be haploid.

[0170] The fluorescence of the stained nuclei, passing through the focus of a light beam from a high-pressure mercury lamp, was measured by a photomultiplier and converted into voltage pulses.

[0171] These voltage pulses were electronically processed to yield integral and peak signals that can be processed by a computer. When the samples are run with the appropriate filter-settings for excitation and emission, DNA histograms can be produced.

[0172] Material

[0173] Flow cytometer: CyFlow ML (Partec GmbH, Otto Hahnstrasse 32, D-4400 Munster, Germany) with a high-pressure mercury lamp, OSRAM HBO 100 long life. Objective: 40xN.A. 0.8 air (Partec)

[0174] Filter combination with DAPI:

[0175] Heat protection filter KG-1

[0176] Excitation-filters: UG-1 and BG-38.

[0177] Dichroic mirrors: TK 420 and TK 560.

[0178] Emission-filter: GG 435

[0179] Software: Flomax version 2.4 d (Partec)

[0180] Results

[0181] Of the 117 genotypes identified as highly homozygous in step c, 83 were identified as haploid by flow cytometry, with remaining 34 individuals being diploid (see Table 1 below)

TABLE 1

Ploidy level (x or 2x; haploid or diploid) of homozygous clones identified using markers 1-15			
Candidate	DNA sample code	Σ Primers Used in screening	Flow cytometry Result
50-Mix5-7	11260406301	9 Primer	x
50-03060367C	07280501801	15 Primer	x
50-03060260C-2	07280501901	15 Primer	x
53-03080954C-2	09270500101	10 Primer	x
53-03090761C-5	09280504501	10 Primer	x
BATCH 51; 03060318C; 1	060728_0010_01_a	15 Primer	x
BATCH 53; 03090761C; 5	060728_0018_01_a	15 Primer	x
0623/172; 05095508C; 1	060728_0021_01_a	15 Primer	x
BATCH 50; 03060260C; 2	060728_0027_01_a	15 Primer	x
0611/32; 05050248C; 1	060728_0032_01_a	15 Primer	x
0611/16; 05050228C; 1	060728_0034_01_a	15 Primer	x
BATCH 53; 03080954C; 2	060728_0035_01_a	15 Primer	x
06 412; 04059061B; 3	060728_0050_01_a	14 Primer	2x
0628/152; 05100720C; 1	060729_0021_01_a	15 Primer	x
0628/185; 05100351C; 1	060729_0063_01_a	15 Primer	x
BATCH 51; 03060626C; 1	060729_0127_02_a	15 Primer	x
BATCH 67; 0409034MC; 2	060729_0130_02_a	14 Primer	2x
BATCH 67; 0409034MC; 4	060729_0131_02_a	15 Primer	2x
BATCH 67; 0409034MC; 15	060729_0132_02_a	15 Primer	2x
BATCH 65; 0409034MC; 7	060729_0134_02_a	15 Primer	2x
BATCH 65; 0409034MC; 35	060729_0138_02_a	15 Primer	2x
BATCH 65; 0409034MC; 56	060729_0139_02_a	15 Primer	2x
BATCH 65; 0409034MC; 50	060729_0141_02_a	15 Primer	2x
BATCH 65; 0409034MC; 47	060729_0142_02_a	15 Primer	2x
0628/53; 05090595C; 1	060731_0043_01_a	15 Primer	x
0627/125; 05090717C; 2	060731_0065_01_a	15 Primer	x
0627/12; 05080220C; 1	060731_0080_01_a	15 Primer	x
0627/6; 05080095C; 1	060731_0086_01_a	14 Primer	x
0631/Normal; 05039033B; 31	060731_0265_01_a	14 Primer	x
64-0409021MC-34	02130604301	15 Primer	2x
64-0410040MC-1	02130604801	15 Primer	2x
51-03060626C	02130605301	15 primer	x
64-0410040MC-20	02140600401	15 primer	2x

TABLE 1-continued

Ploidy level (x or 2x; haploid or diploid) of homozygous clones identified using markers 1-15			
Candidate	DNA sample code	Σ Primers Used in screening	Flow cytometry Result
64-0410040MC-16	02140600801	15 primer	2x
65-0409021MC-2	02140601001	15 primer	2x
06 412B-04059061B-3	02170605501	15 Primer	2x
06 412B-04129091B	02170605801	15 Primer	2x
0550-15/05010827C	02200602401	15 Primer	x
0550-17/05010442C-1	02200602601	15 Primer	x
0550-23/05020059C	02200603101	15 Primer	x
0550-33/05020568C	02200603401	15 Primer	x
0550-36/05020420C-2	02200603701	15 Primer	x
0550-40/05010880C	02200607501	14 Primer	x
0551-36/05020511C	02200607601	15 Primer	x
0551-32/05020361C-1	02210600401	15 Primer	x
0552-4/05010836C-2	02210600901	15 Primer	x
0552-38/05020501C	02210603101	14 Primer	x
0552-39/05020415C	02210603201	15 Primer	x
0552-31/05020858C	02210603701	15 Primer	x
0552-91/05020375C	02210603901	15 Primer	x
0552-111/05020626C	02210607201	15 Primer	x
0552-128/05020558C-1	02210607701	15 Primer	x
0601-35/05020946C	02210608201	15 Primer	x
0601-42/05030201C-6	02210609501	15 Primer	x
0601-51/05030224C-2	02220600201	15 Primer	x
0607-21/05040317C-3	02220601801	14 Primer	x
0606-32/05040240C	02220606201	13 Primer	x
0601-77/05020961C	02230600701	15 Primer	x
0601-62/05030147C	02230601401	15 Primer	x
0601-54/05030462C	02230601901	15 Primer	x
0551-21/05020271C-1	02200605801	14 Primer	x
0601-9/05020843C-2	02230603101	15 Primer	x
0602-17/05020631C-1	02230605501	16Primer	x
0607-111/05040970C-1	03010600201	15 primer	x
0607-81/05040578C-1	03010600501	15 primer	x
0607-73/05040573C-1	03010605101	15 Primer	x
0607-89/05040748C-3	03010605501	15 primer	x
0607-102/05050016C-2	03010606601	15 primer	x
0608-15/05040519C-3	03010606901	15 primer	x
0608-45/05041003C-1	03150603401	15 Primer	x
0610-60/05041024C-2	03150604401	15 primer	x
0610-124/05055039C-1	03150604601	15 primer	x
0609-54/05050089C-2	03150604701	15 primer	x
0610-41/05050352C-1	03150606701	15 primer	x
0609-58/05050255C-1	03220600201	15 primer	x
0610-82/05050099C-2	03220601401	15 primer	x
0610-77/05050353C-1	03220602701	15 primer	x
0610-121/05055090C-1	03220603301	15 Primer	x
0610-81/05050099C-1	03220605901	15 primer	x
0609-100/05055311C-1	03290600301	15 primer	x
0610-11/05040938C-1	03290601101	15 primer	x
0610-68/05050376C-3	03290602001	15 primer	x
0610-58/05050344C-1	03290602201	15 primer	x
0610-73/05050594C-3	03290603301	15 primer	x
0611-84/05050714C-4	03290605001	15 primer	x
0611-70/05050223C-1	03290606701	15 primer	x
0611-73/05050351C-1	03290608001	15 Primer	x
0610-67/05050376C-2	04050600501	15 primer	x
0610-40/05050102C-2	04050600901	15 primer	x
0611-99/05050544C-1	04050602601	15 primer	x
0611-110/05055011C-1	04050603601	15 primer	x
0612-2/05050017C-1	04050609101	15 primer	x
0612-70/05050530C-1	04050609201	15 primer	x
0612-76/05050512C-1	04050610301	15 Primer	x
0611-109/05055144C-1	04120600101	15 Primer	x
0611-31/05050220C-1	04120600601	15 Primer	x
0611-38/05050284C-4	04120600901	15 Primer	x
0611-40/05050171C-1	04120601101	14 Primer	x
0612-80/05050713C-1	04120603101	15 Primer	x
65-0409034 MC-66	060829__0001__02__a	15 Primer	2x
65-0409034 MC-68	060829__0002__02__a	15 Primer	2x
65-0409034 MC-72	060829__0003__02__a	14 Primer	2x

TABLE 1-continued

Ploidy level (x or 2x; haploid or diploid) of homozygous clones identified using markers 1-15			
Candidate	DNA sample code	Σ Primers Used in screening	Flow cytometry Result
65-0409034 MC-111	060829_0005_02_a	15 Primer	2x
65-0409034 MC-94	060829_0011_02_a	14 Primer	2x
65-0409034 MC-120	060829_0012_02_a	15 Primer	2x
65-0409034 MC-144	060829_0013_02_a	15 Primer	2x
65-0409034 MC-133	060829_0015_02_a	15 Primer	2x
65-0409034 MC-187	060829_0020_02_a	15 Primer	2x
65-0409034 MC-193	060829_0021_02_a	14 Primer	2x
65-0409034 MC-199	060829_0023_02_a	15 Primer	2x
65-0409034 MC-135	060829_0025_02_a	15 Primer	2x
65-0409034 MC-114	060829_0026_02_a	13 Primer	2x
65-0409034 MC-147	060829_0027_02_a	15 Primer	2x
65-0409034 MC-36 B	060829_0030_02_a	15 Primer	2x
65-0409034 MC-39 A	060829_0031_02_a	15 Primer	2x
65-0409034 MC-73 A	060829_0034_02_a	15 Primer	2x
65-0409034 MC-71 A	060829_0035_02_a	14 Primer	2x

[0182] Example histograms are shown in FIG. 5.

[0183] Genome Characterization

[0184] Genome characterisation was used for two purposes. First, to confirm the lack of heterozygosity among plants identified as haploids by the morphological assessment, molecular screen and by flow cytometry. Second, to identify doubled haploids on the basis of being diploid and lacking any detectable heterozygosity. The method used to assess both sets of plants was identical and is described below.

[0185] Marker Strategy

[0186] Genome characterisation (for heterozygosity) was performed using 96 microsatellite loci. Rather than screening all primers against all candidate samples using labelled primers, a pooling strategy (as proposed by Cryer et al., 2005) was adopted that avoids the need for large numbers of expensive, labelled SSR primers. The method involves amplifying each microsatellite locus for all haploid candidates using unlabelled primers, bulking and ligating the products together into a vector, and then performing a second amplification using a fluorescently labelled vector primer to expose allelic forms. The number of alleles at each locus for all individuals could

then be assessed by fractionation on the capillary sequencer. Validity of the results obtained by this method was verified by comparing profiles generated using the pooled strategy with those obtained on 10 representative samples (diploid and haploid) using a subset of 24 labelled microsatellite markers fractionated and detected by conventional capillary electrophoresis.

[0187] Genome Characterisation Using Bulk Ligation of PCR Products

[0188] The first step in the screen involved amplifying 12 candidate samples; using 96 microsatellite markers listed in Table 2 (below). For all reactions, the 10 μ l microsatellite reaction mixture contained the following reagents; 1.0 μ l of 10 \times PCR buffer (Bioline), 0.3 μ l $MgCl_2$ (10 mM), 0.4 μ l dNTPs (10 mM of each), 2.0 μ l of each primer pair (1 μ M), 1-5 ng of DNA (extracted at BLRS) and 1 U of Taq DNA polymerase (5 U μ l Bioline). The thermal cycler was programmed with an initial 94° C. denaturing step for 2 min followed by 35 cycles of; 94° C. for 30 s, 52° C. for 30 sec and 72° C. for 45 sec, with a final extension step of 72° C. for 7 min. PCR products were assessed for size by electrophoresis through a 1% w/v agarose gel for 30 min at 120 V.

TABLE 2

Microsatellite markers used for bulk ligation screen (Billote et al. 2005).		
Marker	Forward primer	Reverse Primer
MARKER 16	GACCTTTGTCAGCATACTGGTGTG (SEQ ID NO: 31)	GCAGGCCTGAAATCCCAAT (SEQ ID NO: 127)
MARKER 17	ATGCATGTGATTTTATTAGGTGAGA (SEQ ID NO: 32)	CGACCCTCAGTCAATCAGTAAG (SEQ ID NO: 128)
MARKER 18	AAGCTAGCGACCTATGATTTTAG (SEQ ID NO: 33)	AAACAAGTAATGTGCATAACCTTTC (SEQ ID NO: 129)
MARKER 19	CCCACCACCCCTAGCTTCTC (SEQ ID NO: 34)	ACCCCGGTCCAAATAAAATC (SEQ ID NO: 130)
MARKER 20	AGAGAGAGAGAGTGCGTATG (SEQ ID NO: 35)	GTCCCTGTGGCTGCTGTTTC (SEQ ID NO: 131)

TABLE 2-continued

Microsatellite markers used for bulk ligation screen (Billote et al. 2005).		
Marker	Forward primer	Reverse Primer
MARKER 21	GGGTAGCAAACCTTGTATTA (SEQ ID NO: 36)	ACTTCCATTGTCTCATTATTCT (SEQ ID NO: 132)
MARKER 22	CGAGGCCCAAAACATTCAC (SEQ ID NO: 37)	GGTCCCGATCCCGTCTACTG (SEQ ID NO: 133)
MARKER 23	TTGCGGCCCATCGTAATC (SEQ ID NO: 38)	TCCCTGCAGTGTCCCTCTTT (SEQ ID NO: 134)
MARKER 24	AGGGAATTGGAAGAAAAGAAAG (SEQ ID NO: 39)	TCCTGAGCTGGGGTGGTC (SEQ ID NO: 135)
MARKER 25	AGCAAGAGCAAGAGCAGAACT (SEQ ID NO: 40)	CTTGGGGCTTCGCTATC (SEQ ID NO: 136)
MARKER 26	TAGCCATGCCGCCACCACTT (SEQ ID NO: 41)	CAATCCATTAGCGTGCCTTCT (SEQ ID NO: 137)
MARKER 27	CTTACCCCGCTCCTCTCCT (SEQ ID NO: 42)	CGAAATGCCCTTCTTTACACTA (SEQ ID NO: 138)
MARKER 28	CCTTATATCGCACGGTTCC (SEQ ID NO: 43)	TTCTTGGGGTCTCGCTACGG (SEQ ID NO: 139)
MARKER 29	GCAAGATGCAATGGAGTTCA (SEQ ID NO: 44)	CAAACCGCAGCAAGTCAGA (SEQ ID NO: 140)
MARKER 30	GC AAAATTCAAAGAAAACCTTA (SEQ ID NO: 45)	CTGACAGTGCAGAAAATGTTATGT (SEQ ID NO: 141)
MARKER 31	CGTTCATCCCACCACCTTTC (SEQ ID NO: 46)	GCTGCGAGGCCACTGATAC (SEQ ID NO: 142)
MARKER 32	GAATGTGGCTGTAAATGCTGAGTG (SEQ ID NO: 47)	AAGCCGCATGGACAACCTAGTAA (SEQ ID NO: 143)
MARKER 33	ACATTCCTCTATTATTCTCAC (SEQ ID NO: 48)	GTTTTGTTGGTATGCTTGT (SEQ ID NO: 144)
MARKER 34	AAGCCAACTTCACAGATATGTTGAT (SEQ ID NO: 49)	ATGAGCCTAACAAAGCACATTCTAA (SEQ ID NO: 145)
MARKER 35	AGTGAGGTATGGTTGATTAGGA (SEQ ID NO: 50)	TATTGATAGCATTTGGGATTAG (SEQ ID NO: 146)
MARKER 36	CTCCGATGGTCAAGTCAGA (SEQ ID NO: 51)	AAATGGGGAAGGCAATAGTG (SEQ ID NO: 147)
MARKER 37	GCCGTTCAAGTCAATTAGAC (SEQ ID NO: 52)	TTTGGGAGCAAGCATTATCA (SEQ ID NO: 148)
MARKER 38	TGCTTCTTGTCCTTGATACA (SEQ ID NO: 53)	CCACGTCTACGAAATGATAA (SEQ ID NO: 149)
MARKER 39	CACCACATGAAGCAAGCAGT (SEQ ID NO: 54)	CCTACCACAACCCAGTCTC (SEQ ID NO: 150)
MARKER 40	TTTTATTTTCCCTCTCTTTTGA (SEQ ID NO: 55)	ATTGCGTCTCTTTCCATTGA (SEQ ID NO: 151)
MARKER 41	CATATGGCGCACAGGCAC (SEQ ID NO: 56)	GCAATACAAGAGACCCCAAAT (SEQ ID NO: 152)
MARKER 42	AGTTGGTTTGCTGATTTG (SEQ ID NO: 57)	TGTTGCTTCTTTGATTTTC (SEQ ID NO: 153)
MARKER 43	GCTGAAGATGAAATTGATGTA (SEQ ID NO: 58)	TTCAGGTCCACTTTCATTTA (SEQ ID NO: 154)
MARKER 44	ATGACCTAAAAATAAATCTCAT (SEQ ID NO: 59)	ACAGATCATGCTTGCTCACA (SEQ ID NO: 155)

TABLE 2-continued

Microsatellite markers used for bulk ligation screen (Billote et al. 2005).	
Marker Forward primer	Reverse Primer
MARKER 45 GGTGCAAGAGAGGAGGAATG (SEQ ID NO: 60)	TTTGGTAGTCGGGCGTTTTA (SEQ ID NO: 156)
MARKER 46 GTTTGGCTTTGGACATG (SEQ ID NO: 61)	TCCATCACAGGAGGTATAG (SEQ ID NO: 157)
MARKER 47 TGTTTTGTTTCGTGCATGTG (SEQ ID NO: 62)	GGCTGACATGCAACTAAC (SEQ ID NO: 158)
MARKER 48 CGGTTTGTGCGCATCTATG (SEQ ID NO: 63)	GTCGTCAGGGAACAACAGT (SEQ ID NO: 159)
MARKER 49 CAATCATTGGCGAGAGA (SEQ ID NO: 64)	CGTCACCTTTCAGGATATG (SEQ ID NO: 160)
MARKER 50 GAGCATGACGAAACAAAGG (SEQ ID NO: 65)	GCAACATGTTTGATGCATTAATAGTC (SEQ ID NO: 161)
MARKER 51 TCCAAGTAGCAAATGATGAC (SEQ ID NO: 66)	TGCCCTGAAACCTTGA (SEQ ID NO: 162)
MARKER 52 GAAGGGGCATTGGATTT (SEQ ID NO: 67)	TACCTATTACAGCGAGAGTG (SEQ ID NO: 163)
MARKER 53 AACACTCCAGAAGCCAGGTC (SEQ ID NO: 68)	GGTTTAGGTATTGGAAGTATAGAC (SEQ ID NO: 164)
MARKER 54 GATCCCAATGGTAAAGACT (SEQ ID NO: 69)	AAGCCTCAAAGAAGACC (SEQ ID NO: 165)
MARKER 55 TGTGGTTTGAGGCATCTTCT (SEQ ID NO: 70)	GCCCACCAAAGAAGTAGT (SEQ ID NO: 166)
MARKER 56 TAGCCGCACTCCACGAAGC (SEQ ID NO: 71)	CCAGAATCATCAGACTCGGACG (SEQ ID NO: 167)
MARKER 57 TCAAAGAGCCGCACAACAAG (SEQ ID NO: 72)	ACTTTGCTGCTTGGTGACTTA (SEQ ID NO: 168)
MARKER 58 GGGGATGAGTTTGTTTGTTC (SEQ ID NO: 73)	CCTGCTTGGCGAGATGA (SEQ ID NO: 169)
MARKER 59 TCTAATGCTCCAAGGTACA (SEQ ID NO: 74)	GGCTTGGTCCACGATCTT (SEQ ID NO: 170)
MARKER 60 AGCTCTCATGCAAGTAAC (SEQ ID NO: 75)	TTCAACATACCGTCTGTA (SEQ ID NO: 171)
MARKER 61 TCCTCACTGCTCCTCTAATC (SEQ ID NO: 76)	ACTCCCTATGGACCTTAGTC (SEQ ID NO: 172)
MARKER 62 AGGAGGGCGAACGAGAAACA (SEQ ID NO: 77)	CGACTGCTGATGGGAAGAG (SEQ ID NO: 173)
MARKER 63 CTACGGACTCACACCTATAT (SEQ ID NO: 78)	ATGGTTCATCAATGAGATC (SEQ ID NO: 174)
MARKER 64 GTGAGCGATTGAGGGGTGTG (SEQ ID NO: 79)	GGGGCTTGATTGAGTATTTCCA (SEQ ID NO: 175)
MARKER 65 AGGGCAAGTCATGTTTC (SEQ ID NO: 80)	TATAAGGGCGAGGTATT (SEQ ID NO: 176)
MARKER 66 GAAGCCTGAGACCGCATAGA (SEQ ID NO: 81)	TTCGGTGATGAAGATTGAAG (SEQ ID NO: 177)
MARKER 67 TTTCTTATGGCAATCACACG (SEQ ID NO: 82)	GGAGGGCAGGAACAAAAGT (SEQ ID NO: 178)

TABLE 2-continued

Microsatellite markers used for bulk ligation screen (Billote et al. 2005).		
Marker	Forward primer	Reverse Primer
MARKER 68	GTTTATCATTTTGGGGTCAG (SEQ ID NO: 83)	CGGTGTCCCTCAGGATGTA (SEQ ID NO: 179)
MARKER 69	CATGCACGTAAAGAAAGTGT (SEQ ID NO: 84)	CCAAATGCACCCTAAGA (SEQ ID NO: 180)
MARKER 70	AATCCAAGTGGCCTACAG (SEQ ID NO: 85)	CATGGCTTTGCTCAGTCA (SEQ ID NO: 181)
MARKER 71	TGTAGGTGGTGGTTAGG (SEQ ID NO: 86)	TGTCAGACCCACCATT (SEQ ID NO: 182)
MARKER 72	AGCAAGACACCATGTAGTC (SEQ ID NO: 87)	GACACGTGGGATCTAGAC (SEQ ID NO: 183)
MARKER 73	AAAAGCCGATAGTGGGAACA (SEQ ID NO: 88)	ATGCTGAGAGGTGGAAAATAGAG (SEQ ID NO: 184)
MARKER 74	GTCCATGTGCATAAGAGAG (SEQ ID NO: 89)	CTCTTGGCATTTCAGATAC (SEQ ID NO: 185)
MARKER 75	AGCCAATGAAGGATAAAGG (SEQ ID NO: 90)	CAAGCTAAAACCCCTAATC (SEQ ID NO: 186)
MARKER 76	CAATTCCAGCGTCACTATAG (SEQ ID NO: 91)	AGTGGCAGTGGAACACAGT (SEQ ID NO: 187)
MARKER 77	GGGCTTTCATTTTCCACTAT (SEQ ID NO: 92)	GCTCAACCTCATCCACAC (SEQ ID NO: 188)
MARKER 78	GACAGCTCGTGATGTAGA (SEQ ID NO: 93)	GTTCTTGGCCGCTATAT (SEQ ID NO: 189)
MARKER 79	ACTTGTAACCCCTCTTCTCA (SEQ ID NO: 94)	GTTTCATTACTTGGCTTCTG (SEQ ID NO: 190)
MARKER 80	CCTTCAAGCAAAGATACC (SEQ ID NO: 95)	GGCACCAAACACAGTAA (SEQ ID NO: 191)
MARKER 81	CCACTGCTTCAAATTTACTAG (SEQ ID NO: 96)	GCGTCCAAAACATAAATCAC (SEQ ID NO: 192)
MARKER 82	GGGAGAGGAAAAAATAGAG (SEQ ID NO: 97)	CCTCCCTGAGACTGAGAAG (SEQ ID NO: 193)
MARKER 83	AGCAGGGCAAGGCAATACT (SEQ ID NO: 98)	TTCAGCAGCAGGAAACATC (SEQ ID NO: 194)
MARKER 84	GCCTATCCCTGAACTATCT (SEQ ID NO: 99)	TGCACATACCAGCAACAGAG (SEQ ID NO: 195)
MARKER 85	CATCAGAGCCTTCAAACCTAC (SEQ ID NO: 100)	AGCCTGAATTGCCTCTC (SEQ ID NO: 196)
MARKER 86	ATTCATTGCCATTCCCTTCA (SEQ ID NO: 101)	TTGTCCCTCTGTTCCTCA (SEQ ID NO: 197)
MARKER 87	ATTGCAGAGATGATGAGAAG (SEQ ID NO: 102)	GAGATGCTGACAATGGTAGA (SEQ ID NO: 198)
MARKER 88	TCTCCAAATCACTAGAC (SEQ ID NO: 103)	ATCTGCAAGGCATATTC (SEQ ID NO: 199)
MARKER 89	ACGTTTGGCAACTCTC (SEQ ID NO: 104)	ACTCCCTCTTTGACAT (SEQ ID NO: 200)
MARKER 90	TCCACTCTGGCAACTCC (SEQ ID NO: 105)	AAGGATGGGCTTTGTAGT (SEQ ID NO: 201)
MARKER 91	TTTAGAGGACAAGGAGATAAG (SEQ ID NO: 106)	CGACCGTGTCAGAGTG (SEQ ID NO: 202)

TABLE 2-continued

Microsatellite markers used for bulk ligation screen (Billote et al. 2005).		
Marker	Forward primer	Reverse Primer
MARKER 92	AGCAAAATGGCAAAGGAGAG (SEQ ID NO: 107)	GGTGTGTGCTATGGAAGATCATGT (SEQ ID NO: 203)
MARKER 93	GTAGCTTGAACCTGAAA (SEQ ID NO: 108)	AGAACCACCGGAGTTAC (SEQ ID NO: 204)
MARKER 94	AAGCCACCAGGATCATC (SEQ ID NO: 109)	GTCATTGCCACCTCTAACT (SEQ ID NO: 205)
MARKER 95	TTACTTGCTAAGCTCTCTAGC (SEQ ID NO: 110)	TGGCTGTTTAATCTGTCTG (SEQ ID NO: 206)
MARKER 96	CTATATTTGGTTGGCTTGA (SEQ ID NO: 111)	ACTCATTTCAATCTCAGTGTC (SEQ ID NO: 207)
MARKER 97	TGCTACGTGCTGAAATA (SEQ ID NO: 112)	TATTTACAGGTCGCTTCA (SEQ ID NO: 208)
MARKER 98	CCTCCACTTCTCTTCATCTT (SEQ ID NO: 113)	CTTCCTCAAGCTCAACAAT (SEQ ID NO: 209)
MARKER 99	GATGTTGCCGCTGTTTG (SEQ ID NO: 114)	CATCCCATTTCCCTCTT (SEQ ID NO: 210)
MARKER 100	ATGCTCCACCAAGTTTA (SEQ ID NO: 115)	CACATCCTAGCATCATTG (SEQ ID NO: 211)
MARKER 101	AAGCAATATAGGTTCAAGTTC (SEQ ID NO: 116)	TCATTTTCTAATCCAAACAAG (SEQ ID NO: 212)
MARKER 102	GCTCGTTTTTGTGTTAGGTGA (SEQ ID NO: 117)	TTTTCTCCATAGTCCGTTAC (SEQ ID NO: 213)
MARKER 103	CAGCACACAAATGACAT (SEQ ID NO: 118)	CACCTTTCCTTTTGTGTC (SEQ ID NO: 214)
MARKER 104	CCTATTCCTTACCTTTCTGT (SEQ ID NO: 119)	GACTTACTATCTTGGCTCAC (SEQ ID NO: 215)
MARKER 105	CCTTGCAATCCACTATT (SEQ ID NO: 120)	AGTTCTCAAGCCTCACA (SEQ ID NO: 216)
MARKER 106	CCTCCTTTGGAATTATG (SEQ ID NO: 121)	GTGTTTGATGGGACATACA (SEQ ID NO: 217)
MARKER 107	ATTGGAGAGCACTTGATAG (SEQ ID NO: 122)	TTCTCTTCCTTCTCACTTGT (SEQ ID NO: 218)
MARKER 108	AGCCAGATGGAATACAC (SEQ ID NO: 123)	GTGCGATAAGAGGAGAGT (SEQ ID NO: 219)
MARKER 109	TAGTTTTCCCATCACAGAGT (SEQ ID NO: 124)	ACAATATTAGACCTTCCATGAG (SEQ ID NO: 220)
MARKER 110	GTGCAGATGCAGATTATATG (SEQ ID NO: 125)	CCTTTAGAATTGCCGTATC (SEQ ID NO: 221)
MARKER 111	ACAATAACCTGAGACAACAAGAAC (SEQ ID NO: 126)	ATACATCCCTCCCTCTCT (SEQ ID NO: 222)

[0189] Two bulks were constructed for each of the 12 individuals with each bulk containing 48 markers. The bulked PCR products were then purified using QIAquick PCR Purification columns (QIAGEN) as per manufacturer's instructions. The purified products were then ligated into a pDrive cloning vector (QIAGEN) to allow a universal binding site for the second round PCR. The pDrive vector was selected

because of its high efficiency for ligation and due to the fact that it contains the M13 forward and M13 reverse primer-binding sites. The linear vector is designed to exploit the behaviour of Taq polymerase, which produces a single adenosine nucleotide overhang on resulting PCR fragments, by containing a complementary base (U-base) at the points of insertion. With a simple ligation reaction the adenosine base

from the PCR product and the U-base from the vector ligate together resulting in the recircularisation of the plasmid. This was achieved by adding 5 μ l of 2 \times Ligation Master Mix, 4 μ l of PCR product and 1 μ l of the pDrive vector (50 ng μ L⁻¹) into a 0.2 ml eppendorf tube. Reagents were collected by pulse centrifugation and the ligation reaction was performed at 4° C. for approximately 15 h. The ligation product was diluted 1:10 with nanopure water and this formed the template for the second PCR involving a single microsatellite locus specific primer in combination with a fluorescently labelled universal primer M13 (either forward or reverse). The forward M13 (-40) was labelled with the fluorescent dye (FAM) and the reverse with HEX (both supplied by SIGMA ALDRICH). The PCR conditions were the same as in the initial amplification step this time using the diluted ligation product as the DNA template. Products were diluted 1 in 5 and arranged in bulks based on the expected size of fragment and the fluorescent dye used, which allowed numerous samples to be assessed in a single run of the capillary sequencer. These products were separated by capillary electrophoresis on an ABI Prism 3100 sequencer. The sequencer uses a linear flowing media, namely POP-6TM polymer (Applied Biosystems), to separate fragments in the capillaries and the fluorescence emitted from the incorporated labelled primer is recorded by the software program Genescan Version 3.1TM (Applied Biosystems). The output file allows comparisons of the genetic profiles of individuals by portraying peaks that represent the AFLP-DNA fragments. This fragment analysis was performed using ABI PRISM Genotyper® 3.6NT software (Applied Biosystems), which allows analysis of the size of the fragments (in base pairs) and can also assess the strength of the amplified product. Allele sizes were assessed using ABI PRISM Genotyper® 3.6NT software (Applied Biosystems). Any individual that generated two allelic peaks for any microsatellite marker was deemed partly heterozygous and so discarded as not being a possible haploid or doubled haploid (depending of flow cytometry results).

[0190] Results

[0191] Genome Characterisation

[0192] A subset of 8 of the 24 candidates identified after the molecular screen including both diploid and haploid individuals (listed in the table below) was subjected to more

extensive molecular characterization with 80 additional microsatellite markers using the bulked ligation technique described above (e).

TABLE 3

	Genotype reference	Sample code	Ploidy level
2	BATCH 53; 03090761C; 5	060728_0018_01_a	Haploid
4	BATCH 50; 03060260C; 2	060728_0027_01_a	Haploid
7	BATCH 53; 03080954C; 2	060728_0035_01_a	Haploid
13	BATCH 67; 0409034MC; 4	060729_0131_02_a	Haploid
16	BATCH 65; 0409034MC; 35	060729_0138_02_a	Diploid
18	BATCH 65; 0409034MC; 50	060729_0141_02_a	Diploid
22	0627/125; 05090717C; 2	060731_0065_01_a	Haploid
24	0629/97; 05100048C; 3	060731_0105_01_a	Haploid

[0193] As expected, all individuals identified as haploids by flow cytometry contained only a single allele across all 80 loci surveyed. Thus, these individuals are hemizygous for 95 loci in total (including the 15 markers used in the screen) and this was deemed to confirm their haploid status. In contrast, the two diploids were heterozygous for many of the loci screened.

[0194] Verification of the Bulk Ligation Technique

[0195] When profiles of ten individuals were subjected to microsatellite analysis by conventional capillary electrophoresis and using labeled microsatellite primers, the profiles obtained indicated identical scores for allelic status across 24 markers.

[0196] Identification of Doubled Haploids

[0197] Here, we screened all 34 diploid candidates that were homozygous for all 15 markers as described above and applied a further 32 fluorescent labeled microsatellite markers (listed below) using conventional capillary electrophoresis through a ABI Prism 3100 DNA sequencer. There were two genotypes (65-0409034 MC-144 and 65-0409034 MC-114) that were homozygous for all markers. Thus, these plants were homozygous for a total of 47 microsatellite markers and so were deemed to be doubled haploid oil palm plants.

[0198] The Microsatellites Used for This Characterization Step Were as Follows:

Microsatellite Marker	Forward Primer Sequence	Reverse Primer Sequence
MARKER 16	GACCTTTGTCAGCATACTTGGTGTG (SEQ ID NO: 31)	GCAGGCCTGAAATCCCAAAT (SEQ ID NO: 127)
MARKER 21	GGGTAGCAAACCTTGTATTA (SEQ ID NO: 36)	ACTTCATTGTCTCATTATTCT (SEQ ID NO: 132)
MARKER 23	TTGCGGCCCATCGTAATC (SEQ ID NO: 38)	TCCCTGCAGTGTCCTCTTT (SEQ ID NO: 134)
MARKER 29	GCAAGATGCAATGGAGTTCA (SEQ ID NO: 44)	CAAACGCAGCAAGTCAGA (SEQ ID NO: 140)
MARKER 36	CTCCGATGGTCAAGTCAGA (SEQ ID NO: 51)	AAATGGGGAAGGCAATAGTG (SEQ ID NO: 147)
MARKER 44	ATGACCTAAAAATAAAATCTCA (SEQ ID NO: 59)	ACAGATCATGCTTGCTCACA (SEQ ID NO: 155)

- continued

Microsatellite Marker	Forward Primer Sequence	Reverse Primer Sequence
MARKER 48	CGGTTTGTGCGCATCTATG (SEQ ID NO: 63)	GTCGTCAGGGAACAACAGT (SEQ ID NO: 159)
MARKER 51	TCCAAGTAGCAAATGATGAC (SEQ ID NO: 66)	TGCCCTGAAACCCCTTGA (SEQ ID NO: 162)
MARKER 54	GATCCCAATGGTAAGACT (SEQ ID NO: 69)	AAGCCTCAAAAGAAGACC (SEQ ID NO: 165)
MARKER 55	TGTGGTTTGAGGCATCTTCT (SEQ ID NO: 70)	GCCCCACAAAAGAAAGTAGT (SEQ ID NO: 166)
MARKER 62	AGGGAGGCGAACGAGAAACA (SEQ ID NO: 77)	CGACTGCTGATGGGAAGAG (SEQ ID NO: 173)
MARKER 67	TTTCTTATGGCAATCACACG (SEQ ID NO: 82)	GGAGGCGAGGAACAAAAGT (SEQ ID NO: 178)
MARKER 68	GTTTATCATTTTGGGGTCAG (SEQ ID NO: 83)	CGGTGTCCCTCAGGATGTA (SEQ ID NO: 179)
MARKER 69	CATGCACGTAAAGAAAGTGT (SEQ ID NO: 84)	CCAAATGCACCCTAAGA (SEQ ID NO: 180)
MARKER 71	TGTAGGTGGTGGTTAGG (SEQ ID NO: 86)	TGTCAGACCCACCATTA (SEQ ID NO: 182)
MARKER 72	AGCAAGACACCATGTAGTC (SEQ ID NO: 87)	GACACGTGGGATCTAGAC (SEQ ID NO: 183)
MARKER 74	GTCCATGTGCATAAGAGAG (SEQ ID NO: 89)	CTCTTGGCATTTCAGATAC (SEQ ID NO: 185)
MARKER 77	GGGCTTTCATTITCCACTAT (SEQ ID NO: 92)	GCTCAACCTCATCCACAC (SEQ ID NO: 188)
MARKER 78	GACAGCTCGTGATGTAGA (SEQ ID NO: 93)	GTTCTTGGCCGCTATAT (SEQ ID NO: 189)
MARKER 79	ACTTGTAACCCCTCTTCTCA (SEQ ID NO: 94)	GTTTCATTACTTGGCTTCTG (SEQ ID NO: 190)
MARKER 81	CCACTGCTTCAAATTTACTAG (SEQ ID NO: 96)	GCGTCCAAAACATAAATCAC (SEQ ID NO: 192)
MARKER 84	GCCTATCCCTGAACTATCT (SEQ ID NO: 99)	TGCACATACCAGCAACAGAG (SEQ ID NO: 195)
MARKER 88	TCTCCAAATCACTAGAC (SEQ ID NO: 103)	ATCTGCAAGGCATATTC (SEQ ID NO: 199)
MARKER 89	ACGTTTGGCAACTCTC (SEQ ID NO: 104)	ACTCCCTCTTTGACAT (SEQ ID NO: 200)
MARKER 92	AGCAAAATGGCAAAGGAGAG (SEQ ID NO: 107)	GGTGTGTGCTATGGAAGATCATAGT (SEQ ID NO: 203)
MARKER 96	TCTATATTGGTTGGCTTGA (SEQ ID NO: 111)	ACTCATTTCAATCTCAGTGTC (SEQ ID NO: 207)
MARKER 98	CCTCCACTTCTTTCATCTT (SEQ ID NO: 113)	CTTCCTCAAGCTCAAACAAT (SEQ ID NO: 209)
MARKER 104	CCTATTCCTTACCTTTCTGT (SEQ ID NO: 119)	GACTTACTATCTTVGGCTCAC (SEQ ID NO: 215)
MARKER 105	CCTTGCATTCCACTATT (SEQ ID NO: 120)	AGTTCTCAAGCCTCACA (SEQ ID NO: 215)
MARKER 110	GTGCAGATGCAGATTATATG (SEQ ID NO: 125)	CCTTTAGAATTGCCGTATC (SEQ ID NO: 216)

-continued

Microsatellite Marker	Forward Primer Sequence	Reverse Primer Sequence
MARKER 111	ACAATAACCTGAGACAACAAGAAAC (SEQ ID NO: 126)	ATACATCCCCCTCCCCTCTCT (SEQ ID NO: 222)
MARKER 112	GAACCTGGCGTGTAAC (SEQ ID NO: 223)	TGGTAGGTCTATTGAGAGT (SEQ ID NO: 224)

[0199] FIG. 7B shows images of a typical diploid heterozygous oil palm (bottom) and two doubled haploids (top) sown on the same day.

[0200] Generating Doubled Haploids from Haploids

[0201] Haploid cells will sometimes undergo “spontaneous doubling” whereby failure of complete mitosis gives a doubling of the chromosomes. If this occurs early in development, the seed, plantlet and plant derived is a doubled haploid. If no such doubling occurs then a haploid is obtained and in most circumstances, such haploid plants are intrinsically infertile, in that the process of meiosis is unable to generate gametes capable of fertilisation. In order to produce a fertile plant from which sexual progeny can be produced it is necessary either to double the chromosome number of a haploid by application of an external stimulus, or to rely on the rare process by which a haploid cell can spontaneously double. The former method is the most usually adopted, and usually involves the application of a chemical agent capable of inhibiting mitosis and thereby inducing the formation of a diploid cell. There are several chemicals known to induce such a chromosome doubling process and of these colchicine is the best known, and most commonly utilised. Other similar agents include microtubule inhibitors such as the herbicides trifluralin, and oryzalin. Such chemicals can either be applied to a whole plant and fertile seeds may be produced on that plant, or they can be applied in vitro to isolated cells from which an intact plant can be regenerated using conventional tissue culture techniques. For a full description of available chemical and other methods and their means of application see Kasha (2005) and references therein.

[0202] An alternative to the external application of stimuli is the exploitation of spontaneous doubling. For example, in a haploid, the nucleus of an individual cell may occasionally fail to divide normally at mitosis and thus form a diploid cell that ultimately gives rise either to a diploid sector(s) that may encompass most or all of the main shoot axis or (if it occurs in the first embryonic division) a doubled haploid plant. In either case, the selfed seed secured from such individuals will be completely homozygous and genetically identical to the parent. This process can occur during the formation of reproductive cells and in this case it is possible that fertile gametes (pollen or egg cells) may be produced. If both male and female gametes form on the same plant then successful fusion of gametes can take place and an embryo will develop. Such an embryo will be a homozygous diploid, and will breed true in all future selfed generations; all its selfed progeny will be genetically identical. In oil palm, the inflorescences are usually either male or female (though hermaphrodite inflorescences are known to occur occasionally) and therefore selfing of a particular haploid plant may require the storage of pollen from a male inflorescence until a suitable female inflores-

cence is available for pollination. Such procedures are commonly used in oil palm breeding.

[0203] In the present example, we have found that haploid oil palm plants obtained by the process of the invention produce their first inflorescences after approximately two years of vegetative growth, and it is likely that such plants will produce a low, but usable frequency of fertile gametes, from which homozygous progeny can be isolated. One haploid plant has now started to flower

[0204] FIG. 10 shows confirmed haploid 50-03060260_0002 with first inflorescence two years and seven months after planting (left photograph of inflorescence and right photograph of haploid seedling).

[0205] Homozygosity Screen of Haploid Candidates

[0206] Six oil palms identified as haploid from flow-cytometry were screened to confirm homozygous status. This was achieved by identifying heterozygous markers from each candidate's maternal parent and recording the number of these markers that were also homozygous in the candidate. For a true haploid, the expectation is that all individual loci should contain one allele (hemizygous). A total of 96 markers (listed in Table 5) were screened on each of the mother palms and those that were shown to be heterozygous were then assessed on the progeny candidate palms. The markers used consisted of a universal anchor sequence that was used to incorporate a fluorescent dye into the PCR products, allowing the allele sizes to be assessed by fractionation through capillary electrophoresis. All six candidate palms were shown to be 100% homozygous over all the heterozygous loci identified in the parent (Table 3). The palms were therefore deemed to be completely homozygous, as expected for haploid plants.

TABLE 4

List of the haploid candidate oil palms used in the homozygosity confirmation screening and their respective female parent palms.

Haploid Candidate	Female Parent
0710/20; 06041160; 0002	BL1222/33-14
0702/122; 06030674; 0002	BL1233/21-06
0710/N; 06010987; 0028	BL10868/31-07
0650/80; 06030324; 0003	BL1230/43-22
0701/154; 06030903; 0001	BL10883/35-12
0704/251; 06031385; 0001	BL10868/13-28

TABLE 5

Microsatellite markers used for homozygosity screenings for haploid and double haploid candidates.

1	m0163
2	m0192

TABLE 5-continued

Microsatellite markers used for homozygosity screenings for haploid and double haploid candidates.	
3	m0195
4	m0353
5	m0399
6	m0409
7	m0425
8	m0433
9	m0445
10	m0773
11	m0782
12	m0783
13	m0788
14	m0803
15	m0825
16	m0844
17	m0894
18	m0905
19	m0906
20	m0910
21	m1492
22	m1977
23	m2029
24	m2110
25	m0772
26	m0779
27	m0790
28	m0878
29	m3298
30	m3346
31	m3739
32	m3750
33	m3232
34	m3260
35	m3311
36	m3387
37	m3399
38	m3400
39	m3526
40	m3544
41	m3555
42	m3557
43	m3574
44	m3607
45	m3691
46	m3716
47	m3718
48	m3737
49	m0793
50	m0795
51	m0800
52	m0801
53	m0882
54	m2347
55	m2492
56	m2577
57	m2628
58	m2813
59	m3160
60	m3194
61	m3293
62	m3296
63	m3305
64	m3363
65	m3376
66	m3427
67	m3543
68	m3567
69	m3590
70	m3869
71	m0230
72	m0328
73	m0772
74	m0779
75	m0790

TABLE 5-continued

Microsatellite markers used for homozygosity screenings for haploid and double haploid candidates.	
76	m0827
77	m0878
78	m3298
79	m3346
80	m3739
81	mEgUWA07
82	mEgUWA44
83	mEgUWA50
84	m3535
85	m3310
86	m1716
87	m3705
88	m3826
89	m0146
90	m0369
91	m0408
92	m0832
93	m0588
94	m1773
95	m0059
96	m2860

[0207] Confirmation of Haploid Status by Chromosome Squashes

[0208] Roots of one haploid confirmed above were pre-treated in iced water for 24 h and fixed in 3:1 alcohol:glacial acetic acid. Roots were then stored at 4° C. for 24 h or until required. Roots were then washed in water, softened in 1N HCl for 20 min at room temperature, washed in water (2 min) and stained in saturated aceto-orcin for 1 minute. The root tip was then isolated, squashed and mounted onto a glass slide. Mounted root squash preparations were then examined on a compound microscope. Several unbroken cells were identified that contained the expected 16 chromosomes (see photomicrograph, FIG. 11)

[0209] Identification of Doubled Haploid Oil Palm

[0210] Twenty-six palms previously scored homozygous for 15 markers at BLRS (Bah Lias Research Station, Indonesia) and identified as diploid from flow-cytometry were screened to confirm that they are homozygous and thus doubled haploids. This was achieved by identifying at least 20 unlinked heterozygous markers from each candidate's maternal parent and assessing the level of homozygosity of these markers in the candidate. A total of 111 markers were screened on each of the mother palms (96 markers were screened in one laboratory and 15 markers in another) and those that were shown to be heterozygous were then assessed on the progeny candidate palms. From this, one palm (candidate 5; ID-0644/219;05049082;0003) proved to be entirely homozygous over all 35 markers identified heterozygous in the parent (palm number BL013/12-06). Taking linkage into account, the probability of this event occurring naturally is less than 1 in 2 million and could be less than 1 in 16 million (considering the unmapped markers).

TABLE 6

Microsatellite markers identified as heterozygous in the maternal parent and homozygous in the double haploid candidate.					
0710/20; 06041160; 0002	0702/122; 06030674; 0002	0710/N; 06010987; 00028	0650/80; 06030324; 0003	0701/154; 06030903; 0001	0704/251; 06031385; 0001
1 m0195	m0425	m0195	m0195	m0425	m0788
2 m0788	m0825	m0905	m0425	m2628	m0894
3 m0825	m2628	m3360	m2628	m0588	m3160
4 m0801	m3543	m3543	m1773	m3311	m3543
5 m3360	m0146	m1773	m3739	m3544	m1773
6 m3543	m3311	m3557	mEgUWA50	m3557	m0779
7 m1773	m0779	m0779	m2518	m3737	m0878
8 m3737	m0878	m0878	m3535	m0779	m3739
9 m0779	m3739	m3739	m3310	m0878	mEgUWA44
10 mEgUWA50	m2518	mEgUWA4	m3693	m3739	mEgUWA07
11 mEgUWA07	m0059	mEgUWA7	m3705	mEgUWA44	m2518
12 m2518	m0445	m3826	m0059	mEgUWA07	OPSSR30
13 m3826	m0773	m3310	OPSSR30	m2518	m0445
14 m3693	m0782	m3705	m0353	m3826	m0773
15 m3705	m3194	m0353	m0399	m3535	m0782
16 m3298	m3293	m0399	m0409	m3693	m3194
17 m3346	m3296	m0409	m3387	m0059	m3293
18 m3739	m3387	m0906	m3399	OPSSR30	m3296
19 m0445	m3399	m3574	m3526	OPSSR32	m0163
20 m0773	m3526	m3691	m3574	m3298	m0192
21 m0782	m0163	m3718	m3691	m3346	m3607
22 m0353	m0192	m3607	m3718	m3739	m3716
23 m0399	m0772	m3716		m0906	m3718
24 m0409	m0790	m3718		m3194	
25 m0906	m0793	m0772		m3293	
26 m3607	m0800	m0790		m3296	
27 m3716	m0882			m3387	
28 m3718				m3399	
29				m3526	
30				m0408	
31				m2860	

TABLE 7

Markers used at BLRS to screen for heterozygosity in the parents of the double haploid candidates.		
1	OPSSR1	
2	OPSSR2	
3	OPSSR3	
4	OPSSR4	
5	OPSSR5	
6	OPSSR6	
7	OPSSR7	
8	OPSSR8	
9	OPSSR9	
10	OPSSR10	
11	OPSSR11	
12	OPSSR12	
13	OPSSR13	
14	OPSSR14	
15	VS1	

TABLE 8

List of the double haploid candidate palms used in the homozygosity screening and their respective female parent palms.		
	Double Haploid Candidate	Female Parent
1	0619/142; 05069109; 0002	A1124/39-15
2	0640/68; 05121236; 0004	BL10882/34-18
3	0642/235; 05059119; 0003	BL701/13-09

TABLE 8-continued

List of the double haploid candidate palms used in the homozygosity screening and their respective female parent palms.		
	Double Haploid Candidate	Female Parent
4	0644/47; 06011226; 0001	BL1231/06-11
5	0644/219; 05049082; 0003	BL013/12-06
6	0622/193; 04129038; 0001	BL1129/09-11
7	0626/N; 04129038; 0032	BL1129/09-11
8	0626/N; 04129038; 0005	BL1129/09-11
9	0626/N; 04129038; 0025	BL1129/09-11
10	0626/N; 04129038; 0039	BL1129/09-11
11	0626/N; 04129038; 0009	BL1129/09-11
12	0626/N; 04129038; 0016	BL1129/09-11
13	0626/N; 04069109; 0015	A1124/39-15
14	0706/397; 06039153; 0002	BL1232/47-05
15	0706/367; 06029106; 0001	BL1223/03-23
16	0706/368; 06029106; 0002	BL1223/03-23
17	0622/216; 04069109; 0006	A1124/39-15
18	0626/N; 04069109; 0024	A1124/39-15
19	0626/N; 04069109; 0026	A1124/39-15
20	B67; 0409034MC; 15	287/10626/07-21
21	B65; 0409034MC; 35	287/10626/07-21
22	64-0409021MC-34	287/10625/09-15
23	64-0410040MC-1	286/10622/09-06
24	64-0410040MC-20	286/10622/09-06
25	64-0410040MC-16	286/10622/09-06
26	65-0409021MC-2	287/10625/09-15

TABLE 9

Microsatellite markers identified as heterozygous in the maternal parent and homozygous in the double haploid candidate 5; ID - 0644/219; 05049082; 0003.	
1	m0195
2	m0425
3	m0788
4	m0825
5	m0894
6	m0905
7	m0801
8	m2577
9	m2628
10	m3160
11	m3360
12	m3543
13	m0146
14	m0588
15	m1773
16	m3311
17	m3544
18	m3557
19	VS1
20	m3737
21	m0779
22	m0878
23	m3739
24	mEgUWA44
25	mEgUWA50
26	mEgUWA07
27	m2518
28	m3826
29	m3535
30	m3310
31	m3693
32	m3705
33	m0059
34	OPSSR30
35	OPSSR32

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<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
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<400> SEQUENCE: 41

tagccatgcc gccaccactt 20

<210> SEQ ID NO 42

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 42

cttaccgccg ctcctctcct 20

<210> SEQ ID NO 43

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
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<400> SEQUENCE: 43

ccttatatcg cacgggttcc 20

<210> SEQ ID NO 44

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
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<400> SEQUENCE: 44

gcaagatgca atggagtcca 20

<210> SEQ ID NO 45

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<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 45

gcaaaattca aagaaaactt a 21

<210> SEQ ID NO 46

<211> LENGTH: 20

<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 46

cgttcatccc accacctttc 20

<210> SEQ ID NO 47
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 47

gaatgtggct gtaaatgctg agtg 24

<210> SEQ ID NO 48
<211> LENGTH: 22
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 48

acattccctc tattattctc ac 22

<210> SEQ ID NO 49
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 49

aagccaactt cacagatatg ttgat 25

<210> SEQ ID NO 50
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 50

agtgaggtat ggttgattag ga 22

<210> SEQ ID NO 51
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 51

ctccgatggt caagtcaga 19

<210> SEQ ID NO 52

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<211> LENGTH: 20
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 52

gccggtcaag tcaattagac 20

<210> SEQ ID NO 53
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Synthetic oligonucleotide primer

<400> SEQUENCE: 53

tgcttcttgt ccttgataca 20

<210> SEQ ID NO 54
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<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 54

caccacatga agcaagcagt 20

<210> SEQ ID NO 55
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<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 55

ttttattttc cctctctttt ga 22

<210> SEQ ID NO 56
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 56

catatggcgc acaggcac 18

<210> SEQ ID NO 57
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 57

agttggtttg ctgatttg 18

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<210> SEQ ID NO 58
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 58

gctgaagatg aaattgatgt a 21

<210> SEQ ID NO 59
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 59

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<210> SEQ ID NO 60
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 60

ggtgcaagag aggaggaatg 20

<210> SEQ ID NO 61
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 61

gtttggcttt ggacatg 17

<210> SEQ ID NO 62
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 62

tgttttgttt cgtgcatgtg 20

<210> SEQ ID NO 63
<211> LENGTH: 19
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<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 63

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cggttttgtc gcattctatg 19

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Synthetic oligonucleotide primer

<400> SEQUENCE: 64

caatcattgg cgagaga 17

<210> SEQ ID NO 65
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 65

gagcatgacg caaacaagg 20

<210> SEQ ID NO 66
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 66

tccaagtagc aaatgatgac 20

<210> SEQ ID NO 67
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 67

gaaggggcat tggattt 17

<210> SEQ ID NO 68
<211> LENGTH: 20
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 68

aacactccag aagccaggtc 20

<210> SEQ ID NO 69
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

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<400> SEQUENCE: 69

gatcccaatg gtaaagact

19

<210> SEQ ID NO 70

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 70

tgtggtttga ggcattcttct

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<210> SEQ ID NO 71

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 71

tagccgcact cccacgaagc

20

<210> SEQ ID NO 72

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 72

tcaaagagcc gcacaacaag

20

<210> SEQ ID NO 73

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 73

ggggatgagt ttgtttgttc

20

<210> SEQ ID NO 74

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 74

tctaagtctc ccaaggtaca

20

<210> SEQ ID NO 75

<211> LENGTH: 18

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:

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Synthetic oligonucleotide primer

<400> SEQUENCE: 75

agctctcatg caagtaac

18

<210> SEQ ID NO 76

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 76

tcctcactgc tcctctaac

20

<210> SEQ ID NO 77

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 77

agggaggcga acgagaaaca

20

<210> SEQ ID NO 78

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 78

ctacggactc acacctatat

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<210> SEQ ID NO 79

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 79

gtgagcgatt gaggggtgtg

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<210> SEQ ID NO 80

<211> LENGTH: 17

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 80

agggcaagtc atgtttc

17

<210> SEQ ID NO 81

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 81

gaagcctgag accgcataga 20

<210> SEQ ID NO 82
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 82

tttcttatgg caatcacacg 20

<210> SEQ ID NO 83
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 83

gtttatcatt ttggggtcag 20

<210> SEQ ID NO 84
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 84

catgcacgta aagaaagtgt 20

<210> SEQ ID NO 85
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 85

aatccaagtg gcctacag 18

<210> SEQ ID NO 86
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 86

tgtaggtggt ggtagg 17

<210> SEQ ID NO 87
<211> LENGTH: 19

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 87

agcaagacac catgtagtc 19

<210> SEQ ID NO 88
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 88

aaaagccgat agtgggaaca 20

<210> SEQ ID NO 89
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 89

gtccatgtgc ataagagag 19

<210> SEQ ID NO 90
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 90

agccaatgaa ggataaagg 19

<210> SEQ ID NO 91
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 91

caattccagc gtcactatag 20

<210> SEQ ID NO 92
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 92

gggctttcat tttccactat 20

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<210> SEQ ID NO 93
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 93

gacagctcgt gatgtaga 18

<210> SEQ ID NO 94
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 94

acttgtaaac cctcttctca 20

<210> SEQ ID NO 95
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 95

ccttcaagca aagatacc 18

<210> SEQ ID NO 96
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 96

ccactgcttc aaatttacta g 21

<210> SEQ ID NO 97
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 97

gggagaggaa aaaatagag 19

<210> SEQ ID NO 98
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 98

agcagggcaa gagcaatact 20

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<210> SEQ ID NO 99
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 99
gcctatcccc tgaactatct 20

<210> SEQ ID NO 100
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 100
catcagagcc ttcaaactac 20

<210> SEQ ID NO 101
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 101
attcattgcc attccettca 20

<210> SEQ ID NO 102
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 102
attgcagaga tgatgagaag 20

<210> SEQ ID NO 103
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 103
tctcccaaat cactagac 18

<210> SEQ ID NO 104
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 104

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acgttttggc aactctc 17

<210> SEQ ID NO 105
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 105

tccactctgg caactcc 17

<210> SEQ ID NO 106
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 106

tttagaggac aaggagataa g 21

<210> SEQ ID NO 107
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 107

agcaaaatgg caaaggagag 20

<210> SEQ ID NO 108
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 108

gtagcttgaa cctgaaa 17

<210> SEQ ID NO 109
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 109

aagccaccag gatcatc 17

<210> SEQ ID NO 110
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

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<400> SEQUENCE: 110

ttacttgcta agctctctag c

21

<210> SEQ ID NO 111

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 111

tctatatattg gttggcttga

20

<210> SEQ ID NO 112

<211> LENGTH: 17

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 112

tgctacgtgc tgaata

17

<210> SEQ ID NO 113

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 113

cctccacttc tcttcatctt

20

<210> SEQ ID NO 114

<211> LENGTH: 17

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 114

gatgttgccg ctgtttg

17

<210> SEQ ID NO 115

<211> LENGTH: 17

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 115

atgctccacc aagttta

17

<210> SEQ ID NO 116

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

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<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 116

aagcaatata ggttcagttc

20

<210> SEQ ID NO 117

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 117

gctcggtttt gtttaggtga

20

<210> SEQ ID NO 118

<211> LENGTH: 17

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 118

cagcacacaa atgacat

17

<210> SEQ ID NO 119

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 119

cctattcctt acctttctgt

20

<210> SEQ ID NO 120

<211> LENGTH: 17

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 120

ccttgcatc cactatt

17

<210> SEQ ID NO 121

<211> LENGTH: 17

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 121

cctccttgg aattatg

17

<210> SEQ ID NO 122

<211> LENGTH: 20

<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 122

attggagagc acttgatag 20

<210> SEQ ID NO 123
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide
primer

<400> SEQUENCE: 123

agccagatgg aaatacac 18

<210> SEQ ID NO 124
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 124

tagttttccc atcacagagt 20

<210> SEQ ID NO 125
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 125

gtgcagatgc agattatag 20

<210> SEQ ID NO 126
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 126

acaataacct gagacaacaa gaaac 25

<210> SEQ ID NO 127
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 127

gcaggcctga aatcccaaat 20

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<210> SEQ ID NO 128
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 128

cgaccctcag tcaatcagta ag 22

<210> SEQ ID NO 129
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 129

aaacaagtaa tgtgcataac ctttc 25

<210> SEQ ID NO 130
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 130

accccggtcc aaataaaatc 20

<210> SEQ ID NO 131
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 131

gtccctgtgg ctgctgtttc 20

<210> SEQ ID NO 132
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 132

acttcattg tctcattatt ct 22

<210> SEQ ID NO 133
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 133

ggccccgatc ccgtctactg 20

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<210> SEQ ID NO 134
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<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 134

tccctgcagt gtccctcttt 20

<210> SEQ ID NO 135
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 135

tcctgagctg ggggtggtc 18

<210> SEQ ID NO 136
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 136

cttgggggct tcgctatc 18

<210> SEQ ID NO 137
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 137

caatccatta gcgtgccctt ct 22

<210> SEQ ID NO 138
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 138

cgaaatgcc ttccctttaca cta 23

<210> SEQ ID NO 139
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 139

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ttcttgggggt ctgctacgg 20

<210> SEQ ID NO 140
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 140

caaaccgcag caagtcaga 19

<210> SEQ ID NO 141
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 141

ctgacagtgc agaaaatggt atagt 25

<210> SEQ ID NO 142
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 142

gctgcgaggc cactgatac 19

<210> SEQ ID NO 143
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 143

aagccgcatg gacaactcta gtaa 24

<210> SEQ ID NO 144
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 144

gttttgtttg gtatgcttgt 20

<210> SEQ ID NO 145
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

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<400> SEQUENCE: 145

atgagcctaa caaagcacat tctaa

25

<210> SEQ ID NO 146

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 146

tattgatagc atttgggatt ag

22

<210> SEQ ID NO 147

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 147

aaatggggaa ggcaatagtg

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<210> SEQ ID NO 148

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 148

tttgggagca agcattatca

20

<210> SEQ ID NO 149

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 149

ccacgtctac gaaatgataa

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<210> SEQ ID NO 150

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 150

cctaccacaa ccccagtctc

20

<210> SEQ ID NO 151

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

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<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 151

attgcgtctc tttccattga

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<210> SEQ ID NO 152

<211> LENGTH: 21

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 152

gcaataacaag agcaccctaaa t

21

<210> SEQ ID NO 153

<211> LENGTH: 19

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 153

tggtgcttct ttgattttc

19

<210> SEQ ID NO 154

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 154

ttcaggtcca ctttcattta

20

<210> SEQ ID NO 155

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 155

acagatcatg cttgctcaca

20

<210> SEQ ID NO 156

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 156

tttggtagtc gggcgtttta

20

<210> SEQ ID NO 157

<211> LENGTH: 19

<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 157

tccatcacag gaggtatag 19

<210> SEQ ID NO 158
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 158

ggctgacatg caacactaac 20

<210> SEQ ID NO 159
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 159

gtcgtcaggg aacaacagt 19

<210> SEQ ID NO 160
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 160

cgtcaccttt caggatatg 19

<210> SEQ ID NO 161
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 161

gcaacatggt tgatgcatta atagtc 26

<210> SEQ ID NO 162
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 162

tgccctgaaa cccttga 17

<210> SEQ ID NO 163

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<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
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<400> SEQUENCE: 163
tacctattac agcgagagtg 20

<210> SEQ ID NO 164
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 164
ggtttaggta ttggaactga tagac 25

<210> SEQ ID NO 165
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 165
aagcctcaaa agaagacc 18

<210> SEQ ID NO 166
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 166
gcccacaaaa agaaagtagt 20

<210> SEQ ID NO 167
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 167
ccagaatcat cagactcgga cag 23

<210> SEQ ID NO 168
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
 Synthetic oligonucleotide primer

<400> SEQUENCE: 168
actttgtgc ttggtgactt a 21

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<210> SEQ ID NO 169
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 169

cctgcttggc gagatga 17

<210> SEQ ID NO 170
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 170

ggcttggtcc acgatctt 18

<210> SEQ ID NO 171
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 171

ttcaacatac cgtctgta 18

<210> SEQ ID NO 172
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 172

actccctatg gaccttagtc 20

<210> SEQ ID NO 173
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 173

cgactgctga tgggaagag 20

<210> SEQ ID NO 174
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 174

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atgggttcac aatgagatc 19

<210> SEQ ID NO 175
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 175

ggggccttgat tgagtatttc ca 22

<210> SEQ ID NO 176
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 176

tataagggcg aggtatt 17

<210> SEQ ID NO 177
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 177

ttcggatgatg aagattgaag 20

<210> SEQ ID NO 178
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 178

ggagggcagg aacaaaaagt 20

<210> SEQ ID NO 179
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 179

cgggtgtccct caggatgta 19

<210> SEQ ID NO 180
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

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<400> SEQUENCE: 180

ccaaatgcac cctaaga

17

<210> SEQ ID NO 181

<211> LENGTH: 18

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 181

catggctttg ctcagtca

18

<210> SEQ ID NO 182

<211> LENGTH: 17

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 182

tgtcagaccc accatta

17

<210> SEQ ID NO 183

<211> LENGTH: 18

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 183

gacacgtggg atctagac

18

<210> SEQ ID NO 184

<211> LENGTH: 23

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 184

atgctgagag gtggaaaata gag

23

<210> SEQ ID NO 185

<211> LENGTH: 19

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 185

ctcttgcat ttcagatac

19

<210> SEQ ID NO 186

<211> LENGTH: 19

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:

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Synthetic oligonucleotide primer

<400> SEQUENCE: 186

caagctaaaa cccctaatac

19

<210> SEQ ID NO 187

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 187

agtggcagtg gaaaaacagt

20

<210> SEQ ID NO 188

<211> LENGTH: 18

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 188

gctcaacctc atccacac

18

<210> SEQ ID NO 189

<211> LENGTH: 17

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 189

gttcttggcc gctatat

17

<210> SEQ ID NO 190

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 190

gtttcattac ttggcttctg

20

<210> SEQ ID NO 191

<211> LENGTH: 17

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 191

ggcaccaaac acagtaa

17

<210> SEQ ID NO 192

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 192

gcgtccaaaa cataaatcac 20

<210> SEQ ID NO 193
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 193

cctccctgag actgagaag 19

<210> SEQ ID NO 194
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 194

ttcagcagca ggaaacatc 19

<210> SEQ ID NO 195
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 195

tgacataacc agcaacagag 20

<210> SEQ ID NO 196
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 196

agcctgaatt gcctctc 17

<210> SEQ ID NO 197
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 197

ttgtcccctc tgttcactca 20

<210> SEQ ID NO 198
<211> LENGTH: 20

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 198

gagatgctga caatggtaga 20

<210> SEQ ID NO 199
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 199

atctgcaagg catattc 17

<210> SEQ ID NO 200
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 200

actcccctct ttgacat 17

<210> SEQ ID NO 201
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 201

aaggatgggc tttgtagt 18

<210> SEQ ID NO 202
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 202

cgaccgtgtc aagagtg 17

<210> SEQ ID NO 203
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 203

ggtgtgtgct atggaagatc atagt 25

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<210> SEQ ID NO 204
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 204

agaaccaccg gagttac 17

<210> SEQ ID NO 205
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 205

gtcattgccca cctctaact 19

<210> SEQ ID NO 206
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 206

tggetgttta atctgtctg 19

<210> SEQ ID NO 207
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 207

actcatttca atctcagtgt c 21

<210> SEQ ID NO 208
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 208

atttcagggtt cgcttca 17

<210> SEQ ID NO 209
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 209

cttcctcaag ctcaaacaat 20

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<210> SEQ ID NO 210
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 210

catccccattt ccctctt 17

<210> SEQ ID NO 211
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 211

cacatcctag catcattg 18

<210> SEQ ID NO 212
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 212

tcattttcta attccaaaca ag 22

<210> SEQ ID NO 213
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 213

ttttctccat agtccgttac 20

<210> SEQ ID NO 214
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Synthetic oligonucleotide primer

<400> SEQUENCE: 214

cacctttcct ttttgtc 17

<210> SEQ ID NO 215
<211> LENGTH: 20
<212> TYPE: DNA
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1-30. (canceled)

31. A method for selecting haploid or doubled haploid oil palm plants useful for seed production, multiplication and crop improvement, the method comprising: (a) providing a population of palm plants;

- (b) choosing from the population a subset of individual plants with atypical phenotype;
- (c) assessing the DNA content of plants in the subset;
- (d) classifying plants in the subset as haploid or diploid according to the results of step (c).

32. A method according to claim **31** which further comprises:

- (e) assessing the heterozygosity of diploid plants in the subset;
- (f) discarding from the subset those diploid plants found to be heterozygous;
- (g) classifying the remaining diploid plants as doubled haploids.

33. A method according to claim **32** in which step (c) is performed before step (e).

34. The method according to claim **32** in which plants classified as diploids in step (e) are further assessed for heterozygosity using multiple molecular markers, those found to be heterozygous being discarded and the remainder being classified as doubled haploids.

35. The method according to claim **32** wherein the heterozygosity screen step (c) uses molecular or biochemical markers.

36. The method according to claim **34**, wherein the heterozygosity screen step (c) uses multiple co-dominant molecular markers, for example between 2 and 40 microsatellite markers or Sequenced Characterised Polymorphic Regions (SCARs) markers or Single Nucleotide Polymorphism (SNP) markers.

37. The method according to claim **31**, wherein the atypical phenotype is an atypical growth morphology or growth pattern.

38. The method according to claim **37** wherein the atypical growth morphology is one or more of reduced radicle growth, altered radicle:plumule length ratio, changed radicle:plumule angle, altered colour of radicle or plumule, altered seed shape, size or density and altered radicle width:length ratio.

39. The method according to claim **37** in which the population of palms comprises nursery or field planted palms wherein the atypical growth morphology or growth pattern is one or more of slower vegetative growth, reduced ratio of leaflet width to length, reduced frond internode distance, angle of frond to plant axis, leaf colour, and precocious flowering.

40. The method according to claim **37** wherein the atypical phenotype is germination of two or more embryos from a single seed.

41. The method according to claim **31** in which the atypical phenotype by which plants are selected is chosen from atypical phenotypes shown from previous tests to correlate with haploid or dihaploid character.

42. The method according to claim **34** wherein the further assessment of heterozygosity using multiple DNA markers comprises using between 50 and 200, for example between 70 and 120 microsatellite markers.

43. The method according to claim **41** in which the plant is a germinated seed or seedling.

44. The method according to claim **34** wherein the step of further assessing the homozygosity of the chosen plants using multiple molecular markers uses pooled samples.

45. The method of claim **31** wherein a chosen plant is classified as lacking heterozygosity if it shows only one allele per locus for each molecular marker used.

46. The method according to claim **31** wherein the population comprises at least 1,000,000 plants.

47. The method of claim **45** wherein the population of plants comprises between 5,000,000 and 20,000,000 individuals.

48. A method according to claim **31** in which one or more plants classified as haploids or doubled haploids are subsequently used in breeding, multiplication or seed production.

49. Progeny plants from somatic or reproductive cells of a plant selected by a method according to claim **31**.

50. Clones, pollen or ovules of a plant selected by a method according to claim **31** or of a plant according to claim **49**.

51. A method for producing a homozygous doubled haploid oil palm plant, the method comprising:

- (a) selecting a haploid plant using a method according to claim **31**; (b) obtaining a doubled haploid plant through spontaneous chromosome doubling; or by doubling the chromosome number by application of an external stimulus to the haploid plant; or by application of an

external stimulus to a cell or cells isolated from the haploid plant, followed by regeneration of a plant using tissue culture; or by setting or cloning or pollinating the haploid plant

52. A method according to claim **48** which comprises crossing two distinct doubled haploids obtainable by the method of claim **34**, or progeny of such doubled haploids.

53. A method for identifying doubled haploid plants in a population of progeny of a maternal parent comprising:

- (a) identifying at least 20 heterozygous unlinked loci in the maternal parent, using co-dominant molecular markers such as microsatellites or SNP-based markers;
- (b) performing a preliminary screen of the population using 1-5 of the identified markers; discarding heterozygotes; retaining the remainder as candidate doubled haploids
- (c) applying flow cytometry or other method to measure DNA in the retained candidates; discarding haploids; retaining diploids as potential doubled haploids;
- (d) applying at least a further 15 of the remaining markers to the retained candidates, and classifying individuals that are diploid and homozygous for all applied markers as doubled haploids.

54. Progeny plants from somatic or reproductive cells of a doubled haploid plant identified by the method of claim **53**.

55. A haploid oil palm plant.

56. A doubled haploid oil palm plant.

57. A population of genetically uniform F1 hybrid oil palm plants or seeds, obtainable from crossing two plants claimed in claim **56**.

58. Harvested and extracted products, including oil and kernels, from a plant claimed in claim **56**

59. Harvested and extracted products, including oil and kernels, from a plant claimed in claim **57**.

60. A method of obtaining palm oil comprising extraction from fruit of an F1 hybrid oil palm created from doubled haploid parents.

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