

OBTAINING PLANTS OF ATYPICAL PLOIDY OR ZYGOSITY

The present invention provides plants of atypical ploidy or zygosity, and methods of
5 obtaining them.

The genetic information in organisms is coded by genes (DNA sequences). These are located on chromosomes. In higher organisms, including plants and animals, chromosomes often occur in pairs. Such organisms are termed diploid. In diploid
10 organisms, generally, the genes carried on each member of a pair of chromosomes are similar but not identical. Each member of such a corresponding pair of genes is termed an allele. The genotype of a diploid organism is made up of the various allelic combinations at genetic loci on pairs of chromosomes. A diploid organism will have a characteristic number (n) of pairs of chromosomes, usually between about 5 and about
15 100. The two alleles at any genetic locus in a chromosome pair may be identical or different. An organism in which all the chromosomes have exactly matched identical alleles is termed homozygous: if the chromosome pairs are not identical the organism is termed heterozygous. Heterozygosity is common: complete homozygosity is quite atypical. In sexual reproduction each parent provides to progeny a single set of n
20 chromosomes: each progeny therefore has a set of $2n$ paired chromosomes carrying one set of alleles from each parent. In heterozygous parents each offspring will be different, according to which combination of alleles have been transmitted; this is why offspring generally differ from each other, and from their parents. Under natural conditions, the variation so generated provides offspring with different combinations of genes, which
25 compete in the environment, the fittest being selected.

This variation is not always helpful to farmers. Once a good combination of genes has been found, a plant breeder may wish to preserve it, multiply it and release it as a specific variety. This can be done, for example, by crossing two homozygous parents. If a parent
30 is homozygous (i.e. a pure line that carries only identical alleles at each genetic locus), it

can only give a single genetic inheritance to its offspring. Crossing two different homozygous parents will give a population of heterozygous F_1 hybrids that are genetically completely uniform - though as these F_1 hybrids are heterozygous, they will not themselves breed true.

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Modern agricultural practices are geared towards crop varieties that are uniform and stably reproducible and which have good agronomic traits: for example, responding to standard fertiliser and pesticide treatments and producing a uniform harvest at the most suitable time. One way of providing uniform seed of high quality is to produce F_1 hybrids, which is achieved by crossing together two complementary homozygous parents. Plants from such seed can exhibit 'hybrid vigour' (heterosis), a typical quality of crosses between dissimilar genetic stock. Accordingly, there is a need for homozygous parents for such hybrids.

Historically, maize (corn) was the first major crop in which genetically uniform F_1 hybrids were marketed. In maize, the necessary homozygous parent lines may be obtained by inbreeding: selfing and selection, discarding off-types, over several inbreeding generations (typically at least 6). This is necessary to produce a largely homozygous line that can be used as a parent. Maize is an annual crop: even so, developing a new F_1 hybrid from one or more new homozygous parents can take a decade or more. Where the crop to be bred is not an annual, but has a longer life cycle, e.g. of several years, developing new F_1 varieties from homozygous parents produced by repeated rounds of selfing is impracticably slow (it might take half a century). This has delayed the genetic improvement of many important plantation crop varieties, including for example oil palm and many tree crops (including cocoa, coffee and date palm).

Another possible source of homozygous parents for crop development is haploid plants. Haploid organisms are those which contain a single set of chromosomes (rather than a set of paired chromosomes). Haploid plants are often not fertile: but they can become diploid (either spontaneously or after treatment with certain agents, such as colchicine)

and thereby regain fertility. The resulting doubled haploids are, atypically, completely homozygous, as their diploid status is achieved by duplication of the single set of chromosomes from the haploid progenitor: doubled haploids make ideal parents for F₁ hybrids.

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Haploid plants occur relatively rarely in nature, at a frequency dependent on the species involved. In some species, haploids have never been reported. This was the case in oil palm until recently. Recognizing the potential for haploids for use in oil palm breeding, work has been carried out for decades seeking to generate haploids artificially by
10 standard *in vitro* methods of androgenesis or gynogenesis (Maluszynski et al. 2003). Although this has been unsuccessful, it has continued, because of the need for such haploids and the lack of an obvious alternative route to produce them.

Such an alternative route to haploids has recently been published. In patent application
15 PCT WO 2008/114000 A1 oil palm haploids and doubled haploids are provided by testing abnormal oil palm seedlings for ploidy and homozygosity. This publication has shown for the first time that haploids may occur among oil palm at low frequency, and provides a method of isolating them.

20 The present invention provides a general method of selecting propagatable plant material of atypical ploidy or zygoty, which comprises sampling the endosperm of individual seeds or seedlings without damaging the embryo, determining the ploidy of the sample, and selecting individuals in which the ploidy of the endosperm is abnormal. The method is generally applicable to plants and crops, including for example small grain cereals (e.g.
25 wheat, barley, rice, etc.), large grain cereals (e.g. maize) and other monocotyledonous plants and crops (e.g. forage grasses) and is particularly useful for crops having a longer life-cycle, for example woody crops, including plantation crops such as cocoa, coconut and palms, particularly oil palm.

The invention is based on the relation between the ploidy of the endosperm and of its associated embryo in a seed. In typical diploid plants, the embryo is diploid and the endosperm is triploid. We find that when the endosperm of a diploid species is not triploid (as it normally is), in the majority of cases (in the case of oil palm, around 70% of cases) the embryo is also of abnormal ploidy or zygosity. It may be haploid, or triploid or of higher ploidy, or it may be mixoploid, comprising cells of more than one ploidy. It may be a doubled haploid (resulting, perhaps, from early spontaneous doubling of a haploid prior to sampling). All such abnormal material is of potential use in plant breeding and crop improvement. Doubled haploids (spontaneous or induced) may be used directly as parents of genetically uniform and vigorous F₁ hybrids. Haploids may convert into doubled haploids, either spontaneously or after treatment. The same is true of mixoploids. Triploids and plants of higher ploidy may be useful for varietal production in species that are not required to fruit (e.g. rubber) or in seedless fruit crops (e.g. banana). Polyploid plants generally have larger cells than diploids, and accordingly plants from which they are formed are often larger and may give higher yields than normal.

Our invention is not confined to use with diploid plants. In normal seedlings, there is a general relation between the ploidy of the endosperm and that of the tissue of the parent plant: namely that the ploidy of the endosperm is 1.5 times that of the parent. Thus diploid (e.g. barley) embryos are associated with triploid endosperm; tetraploid (e.g. durum wheat) embryos are associated with hexaploid endosperms and hexaploid (e.g. bread wheat) embryos are associated with nonagon endosperms in their seed. Accordingly, by 'endosperm of abnormal ploidy' we mean endosperm of which the ploidy is not 1.5 times that of the parent.

Material selected by the process of our invention can be treated in various ways, in order to confirm its properties of abnormal ploidy or zygosity and to put these to use. Typically individuals will be grown on. Further tests may be done to confirm ploidy status or monitor changes in it, for example by flow cytometry of samples from the embryo or

tissue developing from it. Homozygosity may be checked to see if the individual is a doubled haploid. Individuals of other abnormal ploidies may spontaneously develop into doubled haploids: or may be encouraged to do so by appropriate treatments, for example with agents such as colchicine. Doubled haploids may be used in breeding and
5 production of superior plant varieties: in particular, different doubled haploids may be crossed to produce genetically uniform F₁ hybrids, which may also have superior properties.

Our invention is useful in the process claimed in PCT WO 2008/114000 A1, as an
10 additional or alternative step or to augment haploid/doubled haploid production. It also extends the range of material to which the process of WO 2008/114000 may be applied. That process is typically, though not exclusively, applied to germinating or germinated seed of atypical phenotype. The present invention may be used with any individual plant entity that contains both endosperm and an embryo (or material formed from the growth
15 of the embryo), this ranges from developing seed to mature seed and to seedlings. The individual seed or seedling may or may not have an atypical phenotype, and it may or may not be germinating or have germinated. Indeed, it may be a seed that has failed to germinate or a developing seed that has not reached maturity. The method is applicable to seedlings, un-germinated and non-germinating seeds and immature and developing
20 seed.

Accordingly, in a second aspect, our invention further comprises a method of obtaining propagatable plant material of atypical ploidy or zygoty, which comprises exposing seeds to germinating conditions for a time sufficient to cause most to germinate; in those
25 that fail to germinate, sampling the endosperm without damaging the embryo, and testing ploidy of the sample; selecting sampled seeds in which the endosperm is of abnormal ploidy and further propagating them in order to produce viable plants of atypical ploidy or zygoty.

The method is particularly applicable to palms, for example oil palm. Two main oil palm plant species are grown commercially: *Elaeis oleifera* Kunth and *Elaeis guineensis* Jacq. 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
5 West Africa to the Pacific region in the second half of the nineteenth century and it has been widely cultivated since the second half of the last century throughout that and other tropical regions. Two countries in which it is currently widely grown are Malaysia and Indonesia.

- 10 An advantage of this aspect of our invention is that it provides a significant use for material that would otherwise be discarded as useless. A further advantage is that the population of un-germinated seed is significantly richer in material of abnormal ploidy (or zygosity) than the seed population as a whole. Such material, it seems, often is reluctant to germinate. The germination stage accordingly acts as a coarse pre-screen for
15 individuals of abnormal ploidy or zygosity, and cuts down the number of unfruitful tests that need to be carried out.

Once material has been identified as of interest by means of the ploidy test on the endosperm, efforts are made to propagate it, either by *in vivo* or *in vitro* methods.

- 20 Although the seed has been selected for its failure to germinate, exposure to germinating conditions for a further period may be enough to cause it to germinate. Germinating conditions may be changed, or intensified, for example by raising or lowering the temperature; the seed may be treated with germination aids, for example plant hormones such as gibberellins or auxins, or chemicals such as hydrogen cyanamide. If such
25 methods fail (or are not tried) another possibility is that of embryo rescue, in which the embryo is excised from the seed and cultured *in vitro*.

Pre- germination *in vivo* methods may include the following two procedures:

1.

soaking un-germinated seed for various lengths of time (e.g. overnight) in a solution of gibberellic acid (e.g. 500 ppm);

(a) rinsing treated seed with water;

5 (b) incubating seeds at ambient temperature.

2.

(a) soaking un-germinated seed for various lengths of time (e.g. three days) in water;

10 (b) heating seed (e.g. for up to 60 days at 38–40°C);

(c) incubating seed at ambient temperature.

3.

(a) soaking un-germinated seed in 0,5% hydrogen cyanamide (CH_2N_2) overnight;

15 (b) rinsing seed with water;

(c) soaking in water for up to 5 days;

(d) drying

20 *In vitro* methods include:

(a) surface sterilization of un-germinated seed;

(b) embryo rescue.

This typically involves aseptic excision of embryos from un-germinated seed, followed by *in vitro* culture of embryos on media with or without plant hormones or

25 heat treatments or both. Methods in embryo rescue and culture are similar across species. Typical procedures for barley haploid production are illustrated by Hayes et al. (2003) using the '*Hordeum bulbosum* technique'.

Once a growing plant has been established, further tests may be carried out to determine
30 ploidy and other properties, for example zygoty. Conveniently the plant may be tested

first for abnormal ploidy. If abnormal ploidy is confirmed, the plant will generally be retained. If it is found to be diploid, it may be tested for zygosity. Material may be homozygous, hemizygous or heterozygous. Heterozygous material of normal ploidy is of no special interest, and may be discarded. Materials that are both completely
5 homozygous and diploid are doubled haploids and may be used directly in breeding, for example by crossing with other doubled haploids to produce genetically uniform F₁ hybrids (e.g. as is done with hybrid peppers): or (depending on the crop) may be released as a true-breeding cultivar (e.g. cereals such as wheat and barley) Materials of abnormal ploidy (haploids, mixoploids, triploids, etc.) may be grown on. Haploids and mixoploids
10 may form doubled haploids spontaneously, or be encouraged to do so by treatment with agents such as colchicine.

The homozygosity of diploid embryos and seedlings may be assessed using one or more genetic, biochemical or phenotypic markers. Multiple genetic markers may conveniently
15 be used to confirm the homozygosity of haploids and doubled haploid plants detected by the process of the invention.

In practising the second aspect of our invention, un-germinated seeds (seeds showing no visible signs of germination) may be collected after a standard germination process. This
20 may conveniently be the germination stage for seeds that are to be planted for commercial use. Endosperm from the un-germinated seed is then sampled, conveniently (in the case of oil palm) by drilling out some of the kernel (the endosperm). This may be done by drilling a hole through the shell of the un-germinated seed, discarding all drilled shell materials (which are of maternal origin), using the pre-drilled hole in the shell as an
25 entry point for taking a core of endosperm (kernel) material from the seed. In this way a small sample of the kernel may be removed without damage to the embryo.

The ploidy of the endosperm is then conveniently determined by subjecting a sample to flow cytometry. By "flow cytometry", we mean a method for determining ploidy of cells
30 suspended in a stream of liquid (analyte). Flow cytometry is used for assessing the

genome content of plant or animal cells. It can be used to show whether the cells are haploid, diploid, triploid, or of higher ploidy levels; and can identify cells from mixoploid tissues.

- 5 Following ploidy determination, if the seed is still considered of interest, attempts may be made to propagate it. Un-germinated seed can be induced to germinate *in vivo* by various treatments (used singly or in combination) that break dormancy: such as temperature changes, drying and wetting, air pressure changes, irradiation, scarifying the seed surface and treatment with plant growth substances such as gibberellic acid or other chemicals.
- 10 Embryos from un-germinated seed may be induced to grow by excision from the seed and culture *in vitro* with or without dormancy breaking treatments. When the seed has been successfully germinated, it may be convenient to apply further ploidy tests to samples of the tissue generated, for example using flow cytometry. Such tests may indicate, for example, that ploidy has changed, e.g. from haploid to doubled haploid.
- 15 the process of germination or growing on, spontaneous chromosome doubling from haploid to doubled haploid may occur. Confirmation that diploid material is a double haploid can be achieved by genotyping diploid embryos to determine homozygosity. This may be performed by progressive screening with multiple polymorphic genetic markers. Homozygosity at a range of loci across the genome is taken to confirm the individual as a
- 20 doubled haploid, especially if the markers used are polymorphic in the parental lines. A range of genetic markers may be used; including simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs). Full details of how to carry out such genotyping for oil palm are given in WO 2008/114000.
- 25 A doubled haploid plant may be obtained from a haploid or mixoploid in a variety of ways. These include: spontaneous chromosome doubling; doubling the chromosome number by application of an external doubling agent either *in vivo* or *in vitro*; by application of such an agent to plant tissue isolated from the haploid plant, followed by regeneration of a plant using tissue culture; by embryogenesis of haploid tissue culture
- 30 with or without doubling agents; by selfing the haploid plant by exploiting occasional

spontaneously doubled sectors in male and female reproductive cells; by applying a doubling agent to male and female reproductive cells, tissues or inflorescences.

Induced chromosome doubling can be achieved by various methods, published or adapted from other crops. These include:

- 5 (a) Treatment of seed or seedlings *in vivo* with a doubling agent.
- (b) Treatment of various plant parts, *in vivo* e.g. apical meristem, male and female inflorescences, with a doubling agent.
- (c) Treatment of embryos or plantlets *in vitro* with a doubling agent.
- (d) Treatment of secondary embryos induced *in vitro* with a doubling agent.

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A preferred doubling agent is colchicine, which is the most frequently used doubling agent in plants: others that may be useful are trifluralin, oryzalin and pronamide or analogues thereof. These agents may be used with wetting agents, with dimethylsulphoxide (DMSO) and with other enhancers (e.g. plant hormones that

15 stimulate cell division).

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The use of molecular markers, preferably co-dominant molecular markers, allows the identification of hemizygous haploids, homozygous diploids and mixoploid individuals. Haploids and doubled haploids have only one allele at all loci within their nuclear

20 genomes. Therefore, any individual exhibiting two alleles for any locus can be discarded as a potential haploid or doubled haploid plant. In practice, it may sometimes be preferable to divide this step into two stages: Stage 1 - a low-cost pre-screen to discard large numbers of false candidates using a set of highly polymorphic markers; and Stage 2 - a high-resolution genome characterization using markers with wide genome coverage to

25 confirm haploid or doubled haploid status. These stages may be carried out, for example, as described in WO 2008/114000.

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One way of carrying out the invention is as follows. A population of oil palm seed is provided. This may be the product of self pollination, out-crossing (deliberate or natural)

30 or crossing with related or alien species. The endosperm of individual seed is sampled to

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determine its ploidy. If the endosperm is not normal (not triploid in diploid oil palm), the seed is grown on (if necessary or preferred, by *in vitro* culture of the embryo). The ploidy of the resulting embryo-derived tissue is then determined, conveniently by comparative flow cytometry. If it is haploid or mixoploid, chromosome doubling may be induced. The homozygosity of the material may be determined using genetic markers: homozygosity in diploid material indicates a doubled haploid. These tests may be repeated to check if the ploidy of tested material has changed.

- 10 In a further aspect, the invention provides a method for producing a genetically uniform diploid F_1 hybrid, e.g. of oil palm, the method comprising crossing two separate homozygous doubled haploid plants obtained according to the first aspect of the invention.

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BRIEF DESCRIPTION OF FIGURES

The following Figures illustrate the invention.

- 20 In the figures:

Figure 1 shows flow cytometry histograms of haploid (A), diploid (B) and (normal) triploid (C) oil palm endosperms.

Figure 2 shows flow cytometry histograms of haploid (A) and diploid (B) oil palm plant tissue (non-endosperm).

- 25 Figure 3 show photomicrographs of representative chromosome complements of haploid ($n=16$) (A) and diploid ($2n=32$) (B) oil palm plants.

Figure 4 shows images of typical haploid and diploid oil palm seedlings of similar age.

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EXAMPLES

The following examples illustrate the invention. The oil palm germplasm (*Elaeis guineensis* Jacq) used in the following experiments was obtained in Indonesia (Sumatra) where most of the experiments were conducted.

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1. Selection of un-germinated seed

1.1. The mesocarp was mechanically removed from the seeds of harvested oil palm fruits 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
10 seeds were then stored for one to three months in an air-conditioned room (25 °C) in plastic bags or trays (it is possible to store seeds for up to one year in this way).

1.2. The seeds were soaked for three days to increase their moisture content, to between 15 and 20% by weight, and then heat-treated in plastic bags or trays for 40 to 60 days at 38 – 40 °C to break dormancy.

15 1.3. After heating, the seeds were soaked for five days to raise their moisture content to over 22% and then dried at ambient temperature for approximately four hours.

1.4. The seeds were transferred to a germination room where they were maintained under ambient temperatures. Germination usually starts after 7 to 10 days and continues for two to three months.

20 1.5. Seeds failing to germinate after three months were selected.

2. Endosperm sampling

2.1. A hole was drilled through the shell of selected seed using a 3mm drill bit. All shell material was removed from the hole and discarded.

25 2.2. An endosperm sample of approximately 0.35 g was then taken by entering the pre-drilled hole through the shell and removing a core (approximately 2 cm long) of the endosperm by drilling with a 3 mm drill bit.

2.3. The endosperm sample was then prepared for flow cytometry.

30 3. Flow cytometry of endosperm sample

3.1. Flow cytometry analyses of cell nuclei from the endosperm.

3.2. Approximately 0.04 g of the drilled-out sample was placed in a Petri dish and 1.5 ml of Cystain buffer (stored at 4 °C) containing DAPI, 0.1% DTT and 1% PVP-40 (to reduce background 'noise') added.

5 3.3. The sample was then macerated in the dark with a razor blade using a chopping action for about 30 seconds). DAPI is a fluorescent dye that selectively binds to DNA forming a complex with double-stranded DNA. The product fluoresces at 456 nm. DAPI has specific DNA-binding properties, with preference for adenine-thymine (AT)-rich sequences. The amount of fluorescence is proportional to the DNA content
10 (ploidy).

3.4. Samples were incubated on ice for 2 hr, macerated a second time and passed through a Partec 'Celltric' filter (30 µm mesh). An additional 0.5 ml of Cystain was then added to the filtrate.

This method produces thousands of nuclei from the endosperm sample.

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Controls are required of known ploidy (DNA content) as a reference – for oil palm, tissue from normal diploid oil palm was used to standardize the histograms generated. The fluorescence of the stained nuclei, passing through a focused light beam from a high-pressure mercury lamp, was measured by a photo-multiplier to yield integral and peak
20 signals for processing by a computer. By running the samples with the appropriate filter settings for excitation and emission, DNA content histograms were produced. Typical results for haploid, diploid and triploid endosperm are shown in Figure 1.

Material

25 The DNA buffer (stored at 4 °C) was based on: Arumuganathan (1991), and comprised:

5 mM Hepes

10 mM Magnesium sulphate heptahydrate

50 mM Potassium chloride

0.2 % Triton X-100

30 2% DTT (Dithiothreitol)

2 mg/litre DAPI

20 pH 8

Flow cytometer: CyFlow Space (Partec GmbH, Otto Hahnstrasse 32, D-4400 Münster,
5 Germany) with a high pressure mercury lamp, OSRAM HBO 100 long life.

Objective: 40 x N.A. 0.8 air (Partec)

Filter combination with DAPI: Heat protection filter KG-1

Excitation-filters: UG-1 and BG-38.

Dichroic mirrors: TK 420 and TK 560.

10 Emission-filter: GG 435

CyFlo©space Basic Unit AC 100/240V/60Hz. 350 VA max

CyFlo©space Basic Unit 560 mm x 650 mm x 300 mm (W x D x H)

Software: Flomax version 2.4 d (Partec).

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4. Breaking embryo/seed dormancy

Embryos of un-germinated seed can be induced to germinate by various *in vivo* or *in vitro* treatments. In oil palm, vital staining (using fluoresceine diacetate, FDA) of embryos from un-germinated seed showed that 30% were viable. In the other 70% of un-germinated
20 seed the embryo was missing, infected or dead. The two methods described below (4.1.1. and 4.1.2) were each able to produce 30% germination of un-germinated seed batches and thus provide methods of germinating the viable embryos of these un-germinated seed.

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4.1.1. First *in vivo* method:

(a) un-germinated seed is soaked for various lengths of time (e.g. overnight) in a solution of gibberellic acid (e.g. 500 ppm);

(b) washing treated seed with water;

30 (c) incubating seeds in the dark at ambient temperature until germination;

(d) root material is sampled for ploidy determination (flow cytometry) and seedlings are potted on.

4.1.2. (Second *in vivo* method)

- 5 (a) un-germinated seed are soaked in water for various lengths of time (e.g. three days);
(b) seed are heat treated (e.g. for up to 60 days at 38-40°C);
(c) seed are incubated in the dark at ambient temperature;
(d) root material is sampled for ploidy determination (flow cytometry) and the
10 seedlings are potted on.

4.1.3. (Third *in vivo* method)

- (a) un-germinated seed are soaked in 0.5% hydrogen cyanamide (CH₂N₂) overnight;
(b) seeds are rinsed thoroughly in running water;
15 (c) seeds are soaked in water for 5 days;
(d) seeds are dried;
(e) seeds are re-soaked;
(f) seeds are incubated in the dark at ambient temperature;
(g) root material is sampled for ploidy determination (flow cytometry) and the
20 seedlings are potted on.

4.2. *In vitro* methods include:

- (a) surface sterilization of un-germinated seed;
(b) aseptic excision of embryos from un-germinated seed;
25 (c) *in vitro* culture of embryos on media with or without plant hormones;
(d) culture of embryos with or without heat treatments.

5. Flow cytometry of selected embryos, plantlets and plants

- (a) Cell nuclei are obtained from embryos, plantlets and plant parts. The method is non-
30 lethal and typically uses root or leaf materials of about 2 cm².

The procedure then follows that given in 3 above (Flow cytometry of endosperm sample). Representative DNA content histograms of diploid and haploid cells obtained by flow cytometry are shown in Figure 2.

Genetic screening for homozygosity

- 5 The protocol applied to assess homozygosity of selected embryos, plantlets, seedlings and plants comprises the following stages:
- 6.1. DNA extraction
 - 6.2. Amplification of SSR (microsatellite) markers by PCR
 - 6.3. Separation of PCR products
 - 10 6.4. Scoring of results to discard individuals with one or more heterozygous loci.

Homozygosity screening is carried out as a two-stage procedure:

Stage 1: this deploys 15 SSR markers known to be highly polymorphic in oil palm germplasm of all kinds (Table 1). They are used progressively, i.e. Marker 1 is used first
15 and all samples showing heterozygosity for this marker are discarded. Only those showing homozygosity are carried forward for a second round of analysis with Marker 2 and so on until all 15 SSR markers have been used. Materials that show homozygosity for all 14 markers of Stage 1 are then taken forward to Stage 2.

Stage 2: this exploits 20 additional SSR markers which are polymorphic in the parents of
20 the material under test and have wide genome coverage. This therefore involves a pre-screen of genetically mapped markers in the various parental lines of the potential doubled haploids and may differ depending on the pedigree.

The procedure is similar to that disclosed in WO 2008/114000, from which further details may be obtained.

25

Each stage is described below:

6.1. DNA extraction

Around 0.5 cm of the root or leaf (around 50 mg) is removed from the seedling and used
30 to extract DNA using the Qiagen 96 DNeasy extraction kit according to the

manufacturer's instructions as described below. Other systems for DNA extraction could alternatively be used.

A. PREPARATION

- 5 1. For new kits, add 100% ethanol to AP3/E buffer and AW buffer.
2. Set water bath to 65 °C.
3. Preheat AE and AP1 buffer to 65 °C.
4. If AP1 buffer has a cloudy appearance, heat to 65 °C and shake until the solution becomes clear.

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B. PROTOCOL

1. Add 50 mg plant material into each tube in two collection microtube racks. Retain the clear cover.
2. Add one tungsten carbide bead into each microtube.
- 15 3. Prepare the lysis solution: (400 µl AP1+ 1 µl RNase + 1 µl Reagent DX)/reaction plus 15% of each component.
4. Disrupt the sample using MM 300, 30 Hz for 1.5 minutes.
5. Pulse centrifuge to 3,000 rpm.
6. Remove and discard caps, add 130 µl AP2 buffer into each collection microtube.
- 20 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.
8. Incubate the racks for 10 min at -20 °C.
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
- 25 strips and use the lowest pipette speed. Recover the tungsten beads.
10. Add 1.5 volume (typically 600 µl) of AP3/E buffer.
11. Close the microtubes with new caps and mix vigorously.
12. Pulse centrifuge (3,000 rpm) to collect solution).
13. Place 96 well plates on top of S-Blocks provided.
- 30 14. Transfer 1 ml of sample into each well of the 96 well plate.

15. Seal with Airpore Tape sheet and centrifuge for 4 min at 6,000 rpm.
16. Add 800 µl of Buffer AW to each sample.
17. Centrifuge for 15 min at 6,000 rpm.
18. Add 100 µl of buffer AE to each sample and seal with new AirPore sheets.
- 5 19. Incubate for 1 min at room temperature (15-25 °C).
20. Centrifuge for 2 min at 6,000 rpm.

6.2. Amplification of SSR markers by PCR

Primers

- 10 A set of SSR markers distributed among all 16 oil palm chromosomes was used:

Table 1. Forward and reverse primer sequences of 15 highly polymorphic markers used in homozygosity testing.

Marker	Forward primer	Reverse primer
1	GAGATTACAAAGTCCAAACC (SEQ ID No 1)	TCAAAATTAAGAAAGTATGC (SEQ ID No 16)
2	ACGCATGCAGCTAGCTTTTC (SEQ ID No 2)	CGCGTGAAAGATATGAATCAAC (SEQ ID No 17)
3	CACGCACGCAGTTTATTCTT (SEQ ID No 3)	GGATGTATGCTTTACCTCCGAAT (SEQ ID No 18)
4	CCCCTTTTGCTTCCCTATTT (SEQ ID No 4)	CTCCTTTTCCCCATCACAGA (SEQ ID No 19)
5	GACACAAGCAAAAACAAAAGCA (SEQ ID No 5)	ATTCTGAAAGGAGGGGAAA (SEQ ID No 20)
6	ATATGTGTGGGTGTGCGTGT (SEQ ID No 6)	TGCCTCTGGTTGTTAGTCTGG (SEQ ID No 21)
7	TCTCTCTCTCTCTCTATGTGTGT GT (SEQ ID No 7)	TGGCAATCAGCACACATTCT (SEQ ID No 22)
8	GCAGCTCTTCCACACCTCT	TGTGGTCTCCTGAGGAAGATG

	(SEQ ID No 8)	(SEQ ID No 23)
9	TTTTCCCCATCACAGAATTG (SEQ ID No 9)	CCCCTTTTGCTTCCCTATTT (SEQ ID No 24)
10	TAGCCGCACTCCCACGAAGC (SEQ ID No 10)	CCAGAATCATCAGACTCGGACAG (SEQ ID No 25)
11	AGCTCTCATGCAAGTAAC (SEQ ID No 11)	TTCAACATACCGTCTGTA (SEQ ID No 26)
12	CCTTCAAGCAAAGATACC (SEQ ID No 12)	GGCACCAAACACAGTAA (SEQ ID No 27)
13	GTAGCTTGAACCTGAAA (SEQ ID No 13)	AGAACCACCGGAGTTAC (SEQ ID No 28)
14	GCTCGTTTTTTGTTTAGGTGA (SEQ ID No 14)	TTTTCTCCATAGTCCGTTAC (SEQ ID No 29)
15	CCTCGGGTTATCCTTTTTTACC (SEQ ID No 15)	TGGCTGGCTTCGGTCTTAG (SEQ ID No 30)

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

Reaction Mixtures

- 5 In all cases, 10 µl a PCR reaction mixture contained the following reagents; 1.0 µl of 10x PCR buffer (Bioline), 0.3 µl MgCl₂ (10 mM), 0.4 µl dNTPs (10 mM of each), 0.2 µl of each primer pair (10 µM), 1-5 ng of DNA (extracted as above) and 1 U of *Taq* DNA polymerase (5 U µl⁻¹ Bioline).

10 PCR conditions

The following conditions were used for the Polymerase Chain Reaction for all SSR 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.

15 6.3. Separation of and analysis of PCR products

PCR products were sent to a service company for separation and analysis

6.4. Scoring of results to discard individuals with one or more heterozygous loci

5 PCR products generated by each SSR-genotype combination were evaluated for the presence of one or two distinct profiles after analysis. Any genotype that yielded two products for any of the SSR loci was deemed to be heterozygous and so discarded as a possible candidate haploid or doubled haploid plant.

Chromosome doubling to produce doubled haploids

10 Various methods for chromosome doubling have been published and can be applied to oil palm. A convenient method that may be used routinely is to treat seedlings *in vivo* with colchicine as described by Mienanti et al. (2009).

REFERENCES

15 The following publications are hereby incorporated by reference.

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- Mienanti D, Sitorus AC, Forster BP, Nelson S, Caligari PDS (2009). Chromosome
5 doubling of oil palm (*Elaeis guineensis* jacq.) haploids. Proceedings of PIPOC
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Claims

1. A method of selecting propagable plant material of atypical ploidy or zygoty, applicable
5 to plants that produce seed in which the endosperm is normally triploid, which comprises
sampling the endosperm of individual seeds or seedlings without damaging the embryo,
determining the ploidy of the sample, and selecting individuals in which the endosperm is not
triploid.
2. A method as claimed in claim 1 in which at least some selected individuals are further
10 propagated.
3. A method as claimed in either of claims 1 and 2 in which at least some selected
individuals are tested for ploidy of non-endosperm material.
4. A method as claimed in any of claims 1-3 in which at least some selected individuals are
tested for homozygosity.
- 15 5. A method as claimed in any of claims 1-4 in which the plant material is
monocotyledonous.
6. A method as claimed in claim 5 in which the plant material is oil palm.
7. A method as claimed in any of claims 1- 6 which comprises exposing seeds to
germinating conditions for a time sufficient to cause most to germinate; in those that fail to
20 germinate, sampling the endosperm without damaging the embryo, and testing ploidy of the
sample; selecting sampled seeds in which the endosperm is not triploid and further
propagating them in order to produce viable plants of atypical ploidy or zygoty.
8. A method as claimed in claim 7 in which the seeds are monocotyledons; or of a tropical
woody crop, in particular oil palm.
- 25 9. A method as claimed in either of claims 7 or 8 in which selected seeds are further
propagated *in vivo*.
10. A method as claimed in either of claims 7 or 8 in which selected seeds are further
propagated *in vitro*, e.g., by embryo rescue.
11. A method as claimed in any of claims 7 - 10 in which propagated tissue is tested for
30 ploidy.

12. A method as claimed in any of claims 7 - 11 in which propagated tissue is tested for zygosity.
13. A method as claimed in any of claims 7-12 in which the plants obtained by the method are haploids or doubled haploids.
- 5 14. Plants of abnormal ploidy obtained by the method of any of claims 1-13.
15. Doubled haploids obtained by the method of any of claims 1-13.
16. A method of obtaining genetically uniform F1 hybrids of tropical woody crops which comprises crossing distinct doubled haploids obtained using a process claimed in any of claims 1-13.
- 10 17. Genetically uniform F1 hybrids of tropical woody crops obtainable by the method claimed in claim 15.