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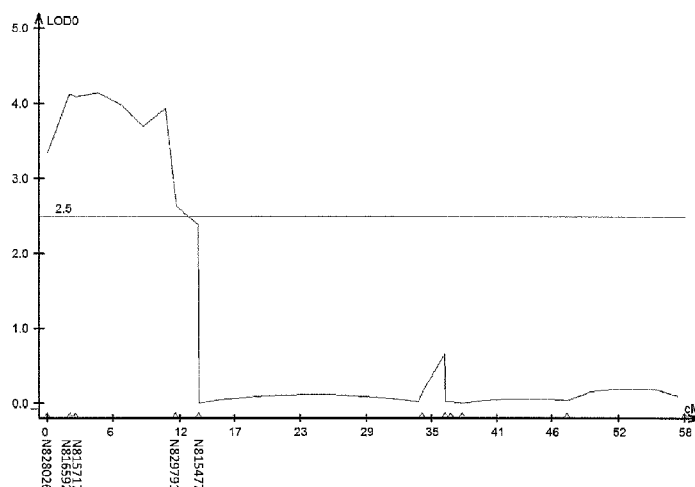
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(54) Title: LEAF-BLADE-LENGTH-RELATED MARKER OF PLANT OF THE GENUS SACCHARUM AND THE USE THEREOF

[Fig. 4]



(57) Abstract: According to the present invention, a novel sugarcane-leaf-blade-length-related marker linked to a sugarcane quantitative trait such as an increase in leaf-blade-length is provided. Such sugarcane-leaf-blade-length-related marker is a sugarcane-leaf-blade-length-related marker, which comprises a continuous nucleic acid region existing in a region sandwiched between the nucleotide sequence shown in SEQ ID NO: 1 and the nucleotide sequence shown in SEQ ID NO: 5 of a sugarcane chromosome.



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Description

Title of Invention: LEAF-BLADE-LENGTH-RELATED MARKER OF PLANT OF THE GENUS SACCHARUM AND THE USE THEREOF

Technical Field

[0001] The present invention relates to a leaf-blade-length-related marker whereby a plant line of the genus *Saccharum* characterized by an increase in leaf-blade-length can be selected, and a method for use thereof.

Background Art

[0002] Sugarcane has been cultivated as a raw material for sugar, liquor, and the like for edible use. In addition, sugarcane has been used as, for example, a raw material for biofuel in a variety of industrial fields. Under such circumstances, there is a need to develop novel sugarcane varieties having desirable characteristics (e.g., sugar content, enhanced vegetative capacity, sprouting capacity, disease resistance, insect resistance, cold resistance, an increase in leaf-blade-length, an increase in leaf area, and an increase in stem length).

In general, the following three ways may be used for identification of a plant variety/line: "characteristics comparison" for comparison of characteristics data, "comparison during cultivation" for comparison of plants cultivated under the same conditions, and "DNA assay" for DNA analysis. There are many problems in line identification with characteristics comparison or comparison during cultivation, including reduction of precision due to differences in cultivation conditions, lengthy duration of field research that requires a number of steps, and the like. In particular, since sugarcane plants are much larger than other graminaceous crops such as rice and maize, it has been difficult to conduct line identification based on field research. In addition, in order to identify a line having morphologically distinct characteristics in terms of leaf-blade-length, leaf area, stalk length, and the like, it is necessary to collect such characteristic data after long-term cultivation of sugarcane. In addition, even after long-term cultivation of sugarcane, it is difficult to identify such line with high accuracy because morphological characteristics are environmentally susceptible.

Further, for creation of a novel sugarcane variety, first, tens of thousands of seedlings are created via crossing, followed by seedling selection and stepwise selection of excellent lines. Eventually, 2 or 3 types of novel varieties having desired characteristics can be obtained. As described above, for creation of a novel sugarcane variety, it is necessary to cultivate and evaluate an enormous number of lines, and it is also necessary to prepare a large-scale field and make highly time-consuming efforts.

Therefore, it has been required to develop a method for identifying a sugarcane line having desired characteristics with the use of markers present in the sugarcane genome. In particular, upon creation of a novel sugarcane variety, if excellent markers could be used to examine a variety of characteristics, the above problems particular to sugarcane would be resolved, and the markers would be able to serve as very effective tools. However, since sugarcane plants have a large number of chromosomes (approximately 100 to 130) due to higher polyploidy, the development of marker technology has been slow. In the case of sugarcane, although the USDA reported genotyping with the use of SSR markers (Non-Patent Document 1), the precision of genotyping is low because of the small numbers of markers and polymorphisms in each marker. In addition, the above genotyping is available only for American/Australian varieties, and therefore it cannot be used for identification of the major varieties cultivated in Japan, Taiwan, India, and other countries or lines that serve as useful genetic resources.

In addition, Non-Patent Document 2 suggests the possibility that a sugarcane genetic map can be created by increasing the number of markers, comparing individual markers in terms of a characteristic relationship, and verifying the results. However, in Non-Patent Document 2, an insufficient number of markers are disclosed and markers linked to desired characteristics have not been found.

Citation List

Non Patent Literature

[0003] NPL 1: Maydica 48(2003)319-329 "Molecular genotyping of sugarcane clones with microsatellite DNA markers"

NPL 2: Nathalie Piperidis et al., Molecular Breeding, 2008, Vol. 21, 233-247

Summary of Invention

Technical Problem

[0004] In view of the above, an object of the present invention is to provide a marker related to leaf-blade-length, which is a quantitative trait of sugarcane plants.

Solution to Problem

[0005] In order to achieve the object, the present inventors conducted intensive studies. The present inventors prepared many sugarcane plant markers and carried out linkage analysis of quantitative traits along with sugarcane plant markers for hybrid progeny lines. Accordingly, the present inventors found markers linked to quantitative traits such as an increase in leaf-blade-length. This has led to the completion of the present invention.

The present invention encompasses the following.

(1) A sugarcane-leaf-blade-length-related marker, which comprises a continuous

nucleic acid region existing in a region sandwiched between the nucleotide sequence shown in SEQ ID NO: 1 and the nucleotide sequence shown in SEQ ID NO: 5 of a sugarcane chromosome.

(2) The sugarcane-leaf-blade-length-related marker according to (1), wherein the continuous nucleic acid region comprises any nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 1 to 5.

(3) The sugarcane-leaf-blade-length-related marker according to (1), wherein the continuous nucleic acid region is located at a position in a region sandwiched between the nucleotide sequence shown in SEQ ID NO: 3 and the nucleotide sequence shown in SEQ ID NO: 4 of a sugarcane chromosome.

(4) A method for producing a sugarcane line having an increased leaf-blade-length comprising: a step of extracting a chromosome of a progeny plant obtained from parent plants, at least one of which is a sugarcane plant; and a step of determining the presence or absence of the sugarcane-leaf-blade-length-related marker according to any one of (1) to (3) in the obtained sugarcane chromosome.

(5) The method for producing a sugarcane line according to (4), wherein a DNA chip provided with probes each serving as the sugarcane-leaf-blade-length-related marker is used in the determination step.

(6) The method for producing a sugarcane line according to (4), wherein the progeny plant is in the form of seeds or a young seedling and the chromosome is extracted from the seeds or the young seedling.

Advantageous Effects of Invention

[0006] According to the present invention, a novel sugarcane-leaf-blade-length-related marker linked to a sugarcane quantitative trait such as an increase in leaf-blade-length can be provided. With the use of the sugarcane-leaf-blade-length-related marker of the present invention, the leaf-blade-length of a line obtained by crossing sugarcane lines can be identified. Thus, a sugarcane line characterized by an increase in leaf-blade-length can be identified at a very low cost.

Brief Description of Drawings

[0007] [fig.1]Fig. 1 schematically shows the process of production of a DNA microarray used for acquisition of sugarcane chromosome markers.

[fig.2]Fig. 2 schematically shows a step of signal detection with the use of a DNA microarray.

[fig.3]Fig. 3 is a characteristic chart showing leaf-blade-length data for sugarcane variety/line groups used in the Examples.

[fig.4]Fig. 4 is a characteristic chart showing QTL analysis results regarding leaf-blade-length.

[fig.5]Fig. 5 is a characteristic chart showing N815717 signal levels for individual lines.

Description of Embodiments

[0008] The sugarcane-leaf-blade-length-related marker and the method for using the same according to the present invention are described below. In particular, a method for producing a sugarcane line using a sugarcane-leaf-blade-length-related marker is described.

<<Sugarcane-leaf-blade-length-related markers>>

The sugarcane-leaf-blade-length-related marker of the present invention corresponds to a specific region present on a sugarcane chromosome and is linked to causative genes (i.e., gene group) for a trait that causes an increase in sugarcane leaf-blade-length. Thus it can be used to identify a trait characterized by an increase in sugarcane leaf-blade-length. Specifically, it is possible to determine that a progeny line obtained using a known sugarcane variety/line is a line having a trait characterized by an increase in leaf-blade-length by confirming the presence of a sugarcane-leaf-blade-length-related marker in such progeny line.

The term "sugarcane" used herein refers to a plant belonging to the genus *Saccharum* of the family Poaceae. In addition, the term "sugarcane" includes both so-called noble cane (scientific name: *Saccharum officinarum*) and wild cane (scientific name: *Saccharum spontaneum*). The term "known sugarcane variety/line" is not particularly limited. It includes any variety/line capable of being used in Japan and any variety/line used outside Japan. Examples of sugarcane varieties cultivated in Japan include, but are not limited to, Ni1, NiN2, NiF3, NiF4, NiF5, Ni6, NiN7, NiF8, Ni9, NiTn10, Ni11, Ni12, Ni14, Ni15, Ni16, Ni17, NiTn19, NiTn20, Ni22, and Ni23. Examples of main sugarcane varieties used in Japan described herein include, but are not limited to, NiF8, Ni9, NiTn10, and Ni15. In addition, examples of main sugarcane varieties that have been introduced into Japan include, but are not limited to, F177, NCo310, and F172.

In addition, a progeny line may be a line obtained by crossing a mother plant and a father plant of the same species, each of which is a sugarcane variety/line, or it may be a hybrid line obtained from parent plants when one thereof is a sugarcane variety/line and the other is a closely related variety/line (*Erianthus arundinaceus*). In addition, a progeny line may be obtained via so-called backcrossing.

The sugarcane-leaf-blade-length-related marker of the present invention has been newly identified by QTL (Quantitative Trait Loci) analysis using a genetic linkage map containing 3004 markers originally obtained from sugarcane chromosomes and sugarcane leaf-blade-length data. In addition, many genes are presumably associated with sugarcane leaf-blade-length, which is a quantitative trait characterized by a

continuous distribution of leaf-blade-length values. For QTL analysis, the QTL Cartographer gene analysis software (Wang S., C. J. Basten, and Z.-B. Zeng (2010); Windows QTL Cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh, NC) is used, and the analysis is carried out by the composite interval mapping (CIM) method.

Specifically, a peak with an LOD score equivalent to or exceeding a given threshold (e.g., 2.5) has been found in the above genetic linkage map by QTL analysis described above. That is, an approximately 13.7-cM (centimorgan) region having such peak has been specified. The term "morgan (M)" used herein refers to a unit representing the relative distance between genes on a chromosome, and it is expressed by the percentage of the crossover rate. In a case of a sugarcane chromosome, 1 cM corresponds to approximately 2000 kb. In addition, it is suggested that causative genes (i.e., gene group) for a trait that causes an increase in leaf-blade-length could be present at the peak position or in the vicinity thereof.

The 13.7-cM region having the above peak is a region that comprises 5 types of markers listed in table 1 below in the order shown in table 1.

[0009] [Table 1]

Linkage group	Marker name	Nucleotide sequence information	Signal threshold	SEQ ID NO
10	N828026	GTATCGCTGAGCGTGGTGCTATTTTACTCTCA ACAGGACCTTAAAAAACGAATTAAATATAA	2,000	SEQ ID NO: 1
	N816592	ACGTGTATATATGAACCACAACACTCCATTCTTT ACTGTCAACAATCTGTGTTGCCAACTACTACAA TATAA	1,000	SEQ ID NO: 2
	N815717	CTGCGATTGAGCGTGGGGCAGCAGCAACATGC GTATAAACTACTACTCC	2,000	SEQ ID NO: 3
	N829791	GGGAAGCCATGGCGCTTGTTCTCCTCCTCTGG ATGGAGCATCGCGCTTC	1,500	SEQ ID NO: 4
	N815477	ATGCTCAACAGAATAGATGGTGTAATCTTGCTGC ACCGTAATACTTTCATTGACATAATTTTCCTT TTG	3,000	SEQ ID NO: 5

In addition, in table 1, "Linkage group" represents the number given to each group among a plurality of linkage groups specified by QTL analysis. In table 1, "Marker name" represents the name given to each marker originally obtained in the present invention. In table 1, "Signal threshold" represents a threshold used for determination of the presence or absence of a marker.

In addition, the peak is present in a region sandwiched between a marker (N815717) having the nucleotide sequence shown in SEQ ID NO: 3 and a marker (N829791) having the nucleotide sequence shown in SEQ ID NO: 4.

A continuous nucleic acid region existing in a region containing 5 types of markers shown in table 1 can be used as a sugarcane-leaf-blade-length-related marker. The term "nucleic acid region" used herein refers to a region having a nucleotide sequence having 95% or less, preferably 90% or less, more preferably 80% or less, and most preferably 70% or less identity to a different region present on a sugarcane chromosome. If the identity of a nucleic acid region serving as a sugarcane-leaf-blade-length-related marker to a different region falls within the above range, the nucleic acid region can be specifically detected according to a standard method. The identity level described herein can be calculated using default parameters and BLAST or a similar algorithm.

In addition, the base length of a nucleic acid region serving as a sugarcane-leaf-blade-length-related marker can be at least 8 bases, preferably 15 bases or more, more preferably 20 bases or more, and most preferably 30 bases. If the base length of a nucleic acid region serving as a sugarcane-leaf-blade-length-related marker falls within the above range, the nucleic acid region can be specifically detected according to a standard method.

In particular, among the 5 types of markers, a sugarcane-leaf-blade-length-related marker is preferably designated as existing in the region sandwiched between the nucleotide sequence shown in SEQ ID NO: 3 and the nucleotide sequence shown in SEQ ID NO: 4. This is because the above peak is present in the region sandwiched between the nucleotide sequence shown in SEQ ID NO: 3 and the nucleotide sequence shown in SEQ ID NO: 4.

In addition, a nucleic acid region containing a single marker selected from among the 5 types of markers can be used as a sugarcane-leaf-blade-length-related marker. For example, it is preferable to use, as a sugarcane-leaf-blade-length-related marker, a nucleic acid region containing a marker (N815717) comprising the nucleotide sequence shown in SEQ ID NO: 3 located closest to the peak position, a nucleic acid region containing a marker (N816592) comprising the nucleotide sequence shown in SEQ ID NO: 2 located close to the peak position, or a nucleic acid region containing a marker (N829791) comprising the nucleotide sequence shown in SEQ ID NO: 4. In such case, the nucleotide sequence of a nucleic acid region containing the marker can be specified by inverse PCR using primers designed based on the nucleotide sequence of such marker.

Further, as a sugarcane-leaf-blade-length-related marker, any of the above 5 types of markers can be directly used. Specifically, one or more type(s) of markers selected

from among the 5 types of such markers can be directly used as a sugarcane-leaf-blade-length-related marker. For example, it is preferable to use, as a sugarcane-leaf-blade-length-related marker, a marker (N815717) consisting of the nucleotide sequence shown in SEQ ID NO: 3 located closest to the peak position, a marker (N816592) comprising the nucleotide sequence shown in SEQ ID NO: 2 located close to the peak position, or a marker (N829791) comprising the nucleotide sequence shown in SEQ ID NO: 4.

<<Sugarcane marker identification>>

As described above, sugarcane-leaf-blade-length-related markers were identified from among 3004 markers originally obtained from sugarcane chromosomes in the present invention. These 3004 markers are described below. Upon identification of these markers, a DNA microarray can be used according to the method disclosed in JP Patent Application No. 2009-283430.

Specifically, 3004 markers originally obtained from sugarcane chromosomes are used with a DNA microarray having probes designed by the method disclosed in JP Patent Application No. 2009-283430. The method for designing probes as shown in fig. 1 is described below. First, genomic DNA is extracted from sugarcane (step 1a). Next, the extracted genomic DNA is digested with a single or a plurality of restriction enzyme(s) (step 1b). In addition, in the example shown in fig. 1, 2 types of restriction enzymes illustrated as restriction enzymes A and B are used (in the order of A first and then B) to digest genomic DNA. The restriction enzymes used herein are not particularly limited. However, examples of restriction enzymes that can be used include PstI, EcoRI, HindIII, BstNI, HpaII, and HaeIII. In particular, restriction enzymes can be adequately selected in consideration of the frequency of appearance of recognition sequences such that a genomic DNA fragment having a base length of 20 to 10000 can be obtained when genomic DNA is completely digested. In addition, when a plurality of restriction enzymes are used, it is preferable for a genomic DNA fragment obtained after the use of all restriction enzymes to have a base length of 200 to 6000. Further, when a plurality of restriction enzymes are used, the order in which restriction enzymes are subjected to treatment is not particularly limited. In addition, a plurality of restriction enzymes may be used in an identical reaction system if they are treated under identical conditions (e.g., solution composition and temperature). Specifically, in the example shown in fig. 1, genomic DNA is digested using restriction enzymes A and B in such order. However, genomic DNA may be digested by simultaneously using restriction enzymes A and B in an identical reaction system. Alternatively, genomic DNA may be digested using restriction enzymes B and A in such order. Further, 3 or more restriction enzymes may be used.

Next, adapters are bound to a genomic DNA fragment subjected to restriction enzyme

treatment (step 1c). The adapter used herein is not particularly limited as long as it can be bound to both ends of a genomic DNA fragment obtained via the above restriction enzyme treatment. For example, it is possible to use, as an adapter, an adapter having a single strand complementary to a protruding end (sticky end) formed at each end of genomic DNA via restriction enzyme treatment and a primer binding sequence to which a primer used upon amplification treatment as described in detail below can hybridize. In addition, it is also possible to use, as an adapter, an adapter having a single strand complementary to the above protruding end (sticky end) and a restriction enzyme recognition site that is incorporated into a vector upon cloning.

In addition, when genomic DNA is digested using a plurality of restriction enzymes, a plurality of adapters corresponding to the relevant restriction enzymes can be prepared and used. Specifically, it is possible to use a plurality of adapters having single strands complementary to different protruding ends formed upon digestion of genomic DNA with a plurality of restriction enzymes. Here, a plurality of adapters corresponding to a plurality of restriction enzymes each may have a common primer binding sequence such that a common primer can hybridize to each such adapter. Alternatively, they may have different primer binding sequences such that different primers can separately hybridize thereto.

Further, when genomic DNA is digested using a plurality of restriction enzymes, it is possible to use, as an adaptor, adapter(s) corresponding to one or some restriction enzyme(s) selected from among a plurality of the used restriction enzymes.

Next, a genomic DNA fragment to both ends of which adapters have been added is amplified (step 1d). When an adapter having a primer binding sequence is used, the genomic DNA fragment can be amplified using a primer that can hybridize to the primer binding sequence. Alternatively, a genomic DNA fragment to which an adapter has been added is cloned into a vector using the adapter sequence. The genomic DNA fragment can be amplified using primers that can hybridize to specific regions of the vector. In addition, as an example, PCR can be used for a genomic DNA fragment amplification reaction using primers.

In addition, when genomic DNA is digested using a plurality of restriction enzymes and a plurality of adapters corresponding to the relevant restriction enzymes are ligated to genomic DNA fragments, the adapters are ligated to all genomic DNA fragments obtained via treatment with a plurality of restriction enzymes. In this case, all the obtained genomic DNA fragments can be amplified by carrying out a nucleic acid amplification reaction using primer binding sequences contained in adapters.

Alternatively, genomic DNA is digested using a plurality of restriction enzymes, followed by ligation of adapter(s) corresponding to one or more restriction enzyme(s) selected from among a plurality of the used restriction enzymes to genomic DNA

fragments. In such case, among the obtained genomic DNA fragments, a genomic DNA fragment to both ends of which the selected restriction enzyme recognition sequences have been bound can be exclusively amplified.

Next, the nucleotide sequence of the amplified genomic DNA fragment is determined (step 1e). Then, at least one region, which has a base length shorter than the base length of the genomic DNA fragment and corresponds to at least partial region of the genomic DNA fragment, is specified. Sugarcane probes are designed using at least one of the thus specified regions (step 1f). A method for determining the nucleotide sequence of a genomic DNA fragment is not particularly limited. A conventionally known method using a DNA sequencer applied to the Sanger method or the like can be used. For example, a region to be designed herein has a 20- to 100-base length, preferably a 30- to 90-base length, and more preferably a 50- to 75-base length as described above.

As described above, a DNA microarray can be produced by designing many probes using genomic DNA extracted from sugarcane and synthesizing an oligonucleotide having a desired nucleotide sequence on a support based on the nucleotide sequence of the designed probe. With the use of a DNA microarray prepared as described above, 3004 markers, including the above 5 types of sugarcane-leaf-blade-length-related markers shown in SEQ ID NOS: 1 to 5, can be identified.

More specifically, the present inventors obtained signal data of known sugarcane varieties (NiF8 and Ni9) and a progeny line (line 191) obtained by crossing the varieties with the use of the DNA microarray described above. Then, genotype data were obtained based on the obtained signal data. Based on the obtained genotype data, chromosomal marker position information was obtained by calculation using the gene distance function (Kosambi) and the AntMap genetic map creation software (Iwata H, Ninomiya S (2006) AntMap: constructing genetic linkage maps using an ant colony optimization algorithm, *Breed Sci* 56: 371-378). Further, a genetic map datasheet was created based on the obtained marker position information using Mapmaker/EXP ver. 3.0 (A Whitehead Institute for Biomedical Research Technical Report, Third Edition, January, 1993) As a result, 3004 markers, including the aforementioned 5 types of sugarcane-leaf-blade-length-related markers shown in SEQ ID NOS: 1 to 5, were identified.

<<Use of sugarcane-leaf-blade-length-related markers>>

The use of sugarcane-leaf-blade-length-related markers makes it possible to determine whether a sugarcane progeny variety/line or the like, which has a phenotype exhibiting unknown leaf-blade-length, is a line having a phenotype showing an increase in leaf-blade-length. The expression "the use of sugarcane-leaf-blade-length-related markers" used herein indicates the use of a DNA microarray having probes corresponding to

sugarcane-leaf-blade-length-related markers in one embodiment. The expression "probes corresponding to sugarcane-leaf-blade-length-related markers" indicates oligonucleotides that can specifically hybridize under stringent conditions to sugarcane-leaf-blade-length-related markers defined as above. For instance, such oligonucleotides can be designed as partial or whole regions with base lengths of at least 10 continuous bases, 15 continuous bases, 20 continuous bases, 25 continuous bases, 30 continuous bases, 35 continuous bases, 40 continuous bases, 45 continuous bases, or 50 or more continuous bases of the nucleotide sequences or complementary strands thereof of sugarcane-leaf-blade-length-related markers defined as above. In addition, a DNA microarray having such probes may be any type of microarray, such as a microarray having a planar substrate comprising glass, silicone, or the like, a bead array comprising microbeads as carriers, or a three-dimensional microarray having an inner wall comprising hollow fibers to which probes are fixed.

The use of a DNA microarray prepared as described above makes it possible to determine whether a sugarcane line such as a progeny line or the like, which has a phenotype exhibiting unknown leaf-blade-length, is a line having a phenotype showing an increase in leaf-blade-length. In addition, in the case of a method other than the above method involving the use of a DNA microarray, it is also possible to determine whether a sugarcane line, which has a phenotype exhibiting unknown leaf-blade-length, is a line having a trait characterized by an increase in leaf-blade-length by detecting the above sugarcane-leaf-blade-length-related markers by a conventionally known method.

The method involving the use of a DNA microarray is described in more detail. As shown in fig. 2, first, genomic DNA is extracted from a sugarcane sample. In this case, a sugarcane sample is a sugarcane line such as a sugarcane progeny line, which has a phenotype exhibiting unknown leaf-blade-length, and thus which can be used as a subject to be determined whether to have a trait characterized by an increase in leaf-blade-length or not. Next, a plurality of genomic DNA fragments are prepared by digesting the extracted genomic DNA with restriction enzymes used for preparing the DNA microarray. Then, the obtained genomic DNA fragments are ligated to adapters used for preparation of the DNA microarray. Subsequently, the genomic DNA fragments, to both ends of which adapters have been added, are amplified using primers employed for preparation of the DNA microarray. Accordingly, sugarcane-sample-derived genomic DNA fragments corresponding to the genomic DNA fragments amplified in step 1d upon preparation of the DNA microarray can be amplified.

In this step, among the genomic DNA fragments to which adapters have been added, specific genomic DNA fragments may be selectively amplified. For instance, in a case

in which a plurality of adapters corresponding to a plurality of restriction enzymes are used, genomic DNA fragments to which specific adapters have been added can be selectively amplified. In addition, when genomic DNA is digested with a plurality of restriction enzymes, genomic DNA fragments to which adapters have been added can be selectively amplified by adding adapters only to genomic DNA fragments that have protruding ends corresponding to specific restriction enzymes among the obtained genomic DNA fragments. Thus, DNA fragment concentration can be increased by selectively amplifying specific genomic DNA fragments.

Thereafter, amplified genomic DNA fragments are labeled. Any conventionally known substance may be used as a labeling substance. Examples of a labeling substance that can be used include fluorescent molecules, dye molecules, and radioactive molecules. In addition, this step can be omitted using a labeled nucleotide in the step of amplifying genomic DNA fragments. This is because when genomic DNA fragments are amplified using a labeled nucleotide in the amplification step, amplified DNA fragments can be labeled.

Next, labeled genomic DNA fragments are allowed to come into contact with the DNA microarray under certain conditions such that probes fixed to the DNA microarray hybridize to the labeled genomic DNA fragments. At such time, preferably, highly stringent conditions are provided for hybridization. Under highly stringent conditions, it becomes possible to determine with high accuracy whether or not sugarcane-leaf-blade-length-related markers are present in a sugarcane sample. In addition, stringent conditions can be adjusted based on reaction temperature and salt concentration. That is, an increase in temperature or a decrease in salt concentration results in more stringent conditions. For example, when a probe having a length of 50 to 75 bases is used, the following more stringent conditions can be provided as hybridization conditions: 40 degrees C to 44 degrees C; 0.21SDS; and 6 x SSC.

In addition, hybridization between labeled genomic DNA fragments and probes can be confirmed by detecting a labeling substance. Specifically, after the above hybridization reaction of labeled genomic DNA fragments and probes, unreacted genomic DNA fragments and the like are washed, and the labeling substance bound to each genomic DNA fragment specifically hybridizing to a probe is observed. For instance, in a case in which the labeling substance is a fluorescent material, the fluorescence wavelength is detected. In a case in which the labeling substance is a dye molecule, the dye wavelength is detected. More specifically, apparatuses such as fluorescent detectors and image analyzers used for conventional DNA microarray analysis can be used. As described above, it is possible to determine whether or not a sugarcane sample has the above sugarcane-leaf-blade-length-related marker(s) with the use of a DNA microarray. In particular, according to the method described above, it is not necessary to

cultivate a sugarcane sample to such an extent that the actual leaf-blade-length thereof becomes measurable. For instance, seeds of a progeny line or a young seedling obtained as a result of germination of such seeds can be used. Therefore, the area of a field used for cultivation of a sugarcane sample and other factors such as cost of cultivation can be significantly reduced with the use of the sugarcane-leaf-blade-length-related marker(s).

In particular, when a novel sugarcane variety is created, it is preferable to produce several tens of thousands of hybrid varieties via crossing and then to identify a novel sugarcane variety using sugarcane-leaf-blade-length-related markers prior to or instead of seedling selection. The use of such sugarcane-leaf-blade-length-related marker(s) makes it possible to significantly reduce the number of excellent lines that need to be cultivated in an actual field. This allows drastic reduction of time-consuming efforts and the cost required to create a novel sugarcane variety.

Examples

[0010] The present invention is hereafter described in greater detail with reference to the following examples, although the technical scope of the present invention is not limited thereto.

1. Production of DNA microarray probes

(1) Materials

The following varieties were used: sugarcane varieties: NiF8, Ni9, US56-15-8, POJ2878, Q165, R570, Co290 and B3439; closely-related sugarcane wild-type varieties: Glagah Kloet, Chunee, Natal Uba, and Robustum 9; and Erianthus varieties: IJ76-349 and JW630.

(2) Restriction enzyme treatment

Genomic DNA was extracted from each of the above sugarcane varieties, closely-related sugarcane wild-type varieties, and Erianthus varieties using DNeasy Plant Mini Kits (Qiagen). Genomic DNAs (750 ng each) were treated with a PstI restriction enzyme (NEB; 25 units) at 37 degrees C for 2 hours. A BstNI restriction enzyme (NEB; 25 units) was added thereto, followed by treatment at 60 degrees C for 2 hours.

(3) Adapter ligation

PstI sequence adapters (5'-CACGATGGATCCAGTGCA-3' (SEQ ID NO: 6) and 5'-CTGGATCCATCGTGCA-3' (SEQ ID NO: 7)) and T4 DNA Ligase (NEB; 800 units) were added to the genomic DNA fragments treated in (2) (120 ng each), and the obtained mixtures were subjected to treatment at 16 degrees C for a full day. Thus, the adapters were selectively added to genomic DNA fragments having PstI recognition sequences at both ends thereof among the genomic DNA fragments treated in (2).

(4) PCR amplification

A PstI sequence adapter recognition primer (5'-GATGGATCCAGTGCAG-3' (SEQ ID NO: 8)) and Taq polymerase (TAKARA; PrimeSTAR; 1.25 units) were added to the genomic DNA fragment (15 ng) having the adaptors obtained in (3). Then, the genomic DNA fragment was amplified by PCR (treatment at 30 cycles of 98 degrees C for 10 seconds, 55 degrees C for 15 seconds, 72 degrees C for 1 minute for 30 cycles, and then at 72 degrees C for 3 minutes, followed by storage at 4 degrees C).

(5) Genome sequence acquisition

The nucleotide sequence of the genomic DNA fragment subjected to PCR amplification in (4) was determined by the Sanger method. In addition, information on a nucleotide sequence sandwiched between PstI recognition sequences was obtained based on the total sorghum genome sequence information contained in the genome database (Gramene: <http://www.gramene.org/>).

(6) Probe design and DNA microarray production

50- to 75-bp probes were designed based on the genome sequence information in (5). Based on the nucleotide sequence information of the designed probes, a DNA microarray having the probes was produced.

2. Acquisition of signal data using a DNA microarray

(1) Materials

Sugarcane varieties/lines (NiF8 and Ni9) and the progeny line (line 191) were used.

(2) Restriction enzyme treatment

Genomic DNAs were extracted from NiF8, Ni9, and the progeny line (line 191) using DNeasy Plant Mini Kits (Qiagen). Genomic DNAs (750 ng each) were treated with a PstI restriction enzyme (NEB; 25 units) at 37 degrees C for 2 hours. Then, a BstNI restriction enzyme (NEB; 25 units) was added thereto, followed by treatment at 60 degrees C for 2 hours.

(3) Adapter ligation

PstI sequence adapters (5'-CACGATGGATCCAGTGCA-3' (SEQ ID NO: 6) and 5'-CTGGATCCATCGTGCA-3' (SEQ ID NO: 7)) and T4 DNA Ligase (NEB; 800 units) were added to the genomic DNA fragments treated in (2) (120 ng each), and the obtained mixtures were treated at 16 degrees C for a full day. Thus, the adaptors were selectively added to a genomic DNA fragment having PstI recognition sequences at both ends thereof among the genomic DNA fragments treated in (2).

(4) PCR amplification

A PstI sequence adapter recognition primer (5'-GATGGATCCAGTGCAG-3' (SEQ ID NO: 8)) and Taq polymerase (TAKARA; PrimeSTAR; 1.25 units) were added to the genomic DNA fragment (15 ng) having the adapters obtained in (3). Then, the genomic DNA fragment was amplified by PCR (treatment at 98 degrees C for 10 seconds, 55 degrees C for 15 seconds, 72 degrees C for 1 minute for 30 cycles, and

then at 72 degrees C for 3 minutes, followed by storage at 4 degrees C).

(5) Labeling

The PCR amplification fragment obtained in (4) above was purified with a column (Qiagen). Cy3-labeled 9mers (TriLink; 1 O.D.) was added thereto. The resultant was treated at 98 degrees C for 10 minutes and allowed to stand still on ice for 10 minutes. Then, Klenow (NEB; 100 units) was added thereto, followed by treatment at 37 degrees C for 2 hours. Thereafter, a labeled sample was prepared by ethanol precipitation.

(6) Hybridization/signal detection

The labeled sample obtained in (5) was subjected to hybridization using the DNA microarray prepared in 1 above in accordance with the NimbleGen Array User's Guide. Signals from the label were detected.

3. Identification of QTL for sugarcane leaf-blade-length and development of markers

(1) Creation of genetic map datasheet

Genotype data of possible 3004 markers were obtained based on the signal data detected in 2 above of the NiF8 and Ni9 sugarcane varieties and the progeny line (line 191). Based on the obtained genotype data, chromosomal marker position information was obtained by calculation using the gene distance function (Kosambi) and the AntMap genetic map creation software (Iwata H, Ninomiya S (2006) AntMap: constructing genetic linkage maps using an ant colony optimization algorithm, *Breed Sci* 56: 371-378). Further, a genetic map datasheet was created based on the obtained marker position information using Mapmaker/EXP ver. 3.0 (A Whitehead Institute for Biomedical Research Technical Report, Third Edition, January, 1993).

(2) Acquisition of leaf-blade-length data

The tested sugarcane varieties (NiF8 and Ni9) and the progeny line (191 linws) were subjected to cultivation with 2 replicates (13 individuals per replicate). In each replicate of cultivation, 5 individuals were subjected to measurement of the length between the dewlap and the leaf blade tip. The mean values obtained from the results for separate replicates of cultivation were used as leaf-blade-length data. The calculated leaf-blade-length values are shown in fig. 3.

(3) Quantitative trait (quantitative trait loci: QTL) analysis

Based on the genetic map datasheet obtained in (1) above and the leaf-blade-length data obtained in (2) above, QTL analysis was carried out by the composite interval mapping (CIM) method using the QTL Cartographer gene analysis software (Wang S., C. J. Basten, and Z.-B. Zeng (2010), Windows QTL Cartographer 2.5, Department of Statistics, North Carolina State University, Raleigh, NC). Upon analysis, the LOD threshold was determined to be 2.5. As a result, as shown in fig. 4, a peak exceeding the LOD threshold was observed in the following range for the NiF8 sugarcane

variety: the range between markers N828026 to N815477 present in the 10th linkage group. It was possible to specify the obtained peak as shown in table 2, suggesting the presence of causative genes (i.e., gene group) each having the function of causing an increase in leaf-blade-length at the relevant peak position.

[0011] [Table 2]

Linkage group	Position (cM)	Range (cM)	Adjacent marker	LOD value	Effect (cm)
10	4.6	13.7	N828026-N815477	4.1	1.94

As shown in fig. 4, markers located in the vicinity of the peak are inherited in linkage with causative genes (i.e., gene group) each having the function of causing an increase in leaf-blade-length. This shows that the markers can be used as sugarcane-leaf-blade-length-related markers. Specifically, as shown in fig. 4, it has been revealed that markers N828026, N816592, N815717, N829791, and N815477 can be used as sugarcane-leaf-blade-length-related markers. In addition, as examples of signals detected in 2 (6) above, table 3 shows signal levels of N828026, N816592, N815717, N829791, and N815477 for NiF8 and Ni9 and their 18 progeny lines (F1_1 to F1_18). In particular, the signal levels of N815717 are shown in fig. 5.

[0012] [Table 3]

Linkage group	Marker name	Line name									
		NiF8	Ni9	F1_1	F1_2	F1_3	F1_4	F1_5	F1_6	F1_7	F1_8
10	N828026	3,429	597	496	440	3,737	3,961	395	3,451	3,066	480
	N816592	1,842	525	497	477	1,438	1,185	423	1,349	1,508	575
	N815717	3,137	445	420	471	3,699	3,423	490	3,856	4,260	383
	N829791	2,674	666	957	759	3,369	3,238	651	2,870	2,492	925
	N815477	6,504	397	1,259	335	4,547	5,172	1,078	4,468	5,134	1,136

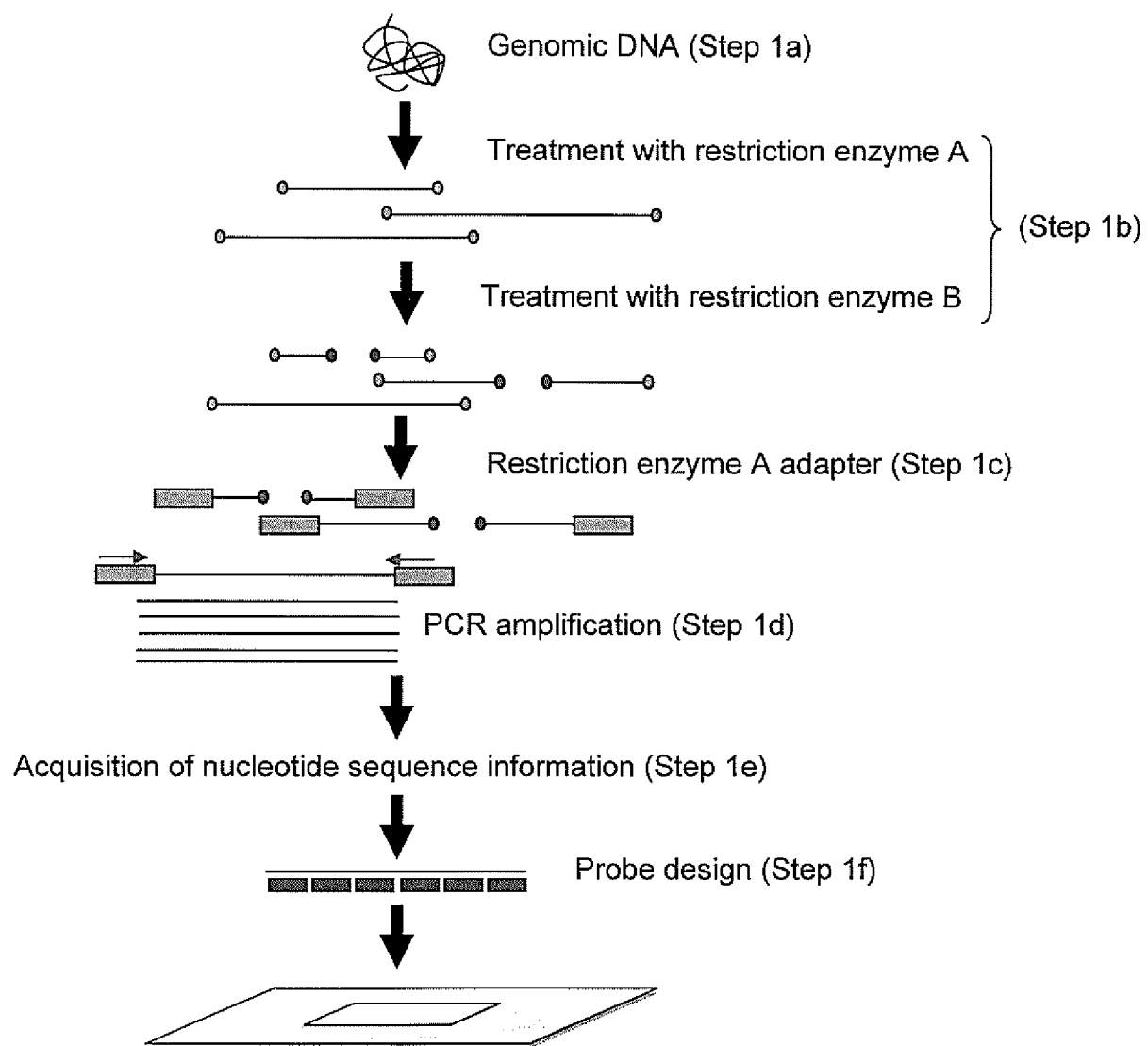
Linkage group	Marker name	Line name									
		F1_9	F1_10	F1_11	F1_12	F1_13	F1_14	F1_15	F1_16	F1_17	F1_18
10	N828026	4,469	5,433	447	3,616	3,804	3,858	518	377	416	515
	N816592	2,579	1,705	424	1,971	1,578	2,172	412	418	489	448
	N815717	3,547	3,787	369	3,302	3,408	4,003	429	422	382	372
	N829791	3,017	2,781	706	3,219	3,532	3,182	1,233	1,248	943	995
	N815477	6,154	6,224	1,184	6,494	6,136	5,945	418	803	436	1,048

Signal levels of N828026, N816592, N815717, N829791, and N815477, were found to be very high for NiF8 and the progeny lines such as F1_3 and F1_4 with relatively long leaf-blade-lengths. These results also revealed that markers N828026, N816592, N815717, N829791, and N815477 can be used as sugarcane-leaf-blade-length-related markers.

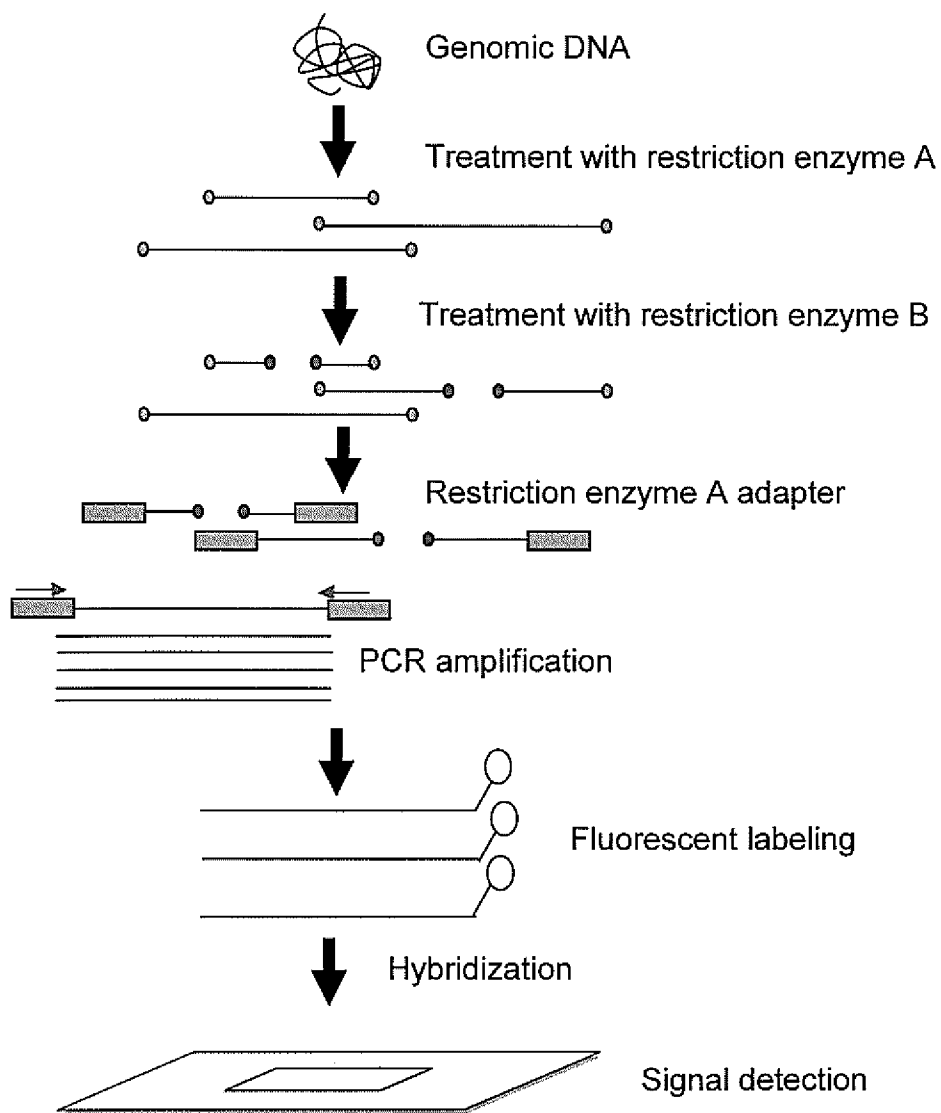
Claims

- [Claim 1] A sugarcane-leaf-blade-length-related marker, which comprises a continuous nucleic acid region existing in a region sandwiched between the nucleotide sequence shown in SEQ ID NO: 1 and the nucleotide sequence shown in SEQ ID NO: 5 of a sugarcane chromosome.
- [Claim 2] The sugarcane-leaf-blade-length-related marker according to claim 1, wherein the continuous nucleic acid region comprises any nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 1 to 5.
- [Claim 3] The sugarcane-leaf-blade-length-related marker according to claim 1, wherein the continuous nucleic acid region is located at a position in a region sandwiched between the nucleotide sequence shown in SEQ ID NO: 3 and the nucleotide sequence shown in SEQ ID NO: 4 of a sugarcane chromosome.
- [Claim 4] A method for producing a sugarcane line having an increased leaf-blade-length comprising: a step of extracting a chromosome of a progeny plant obtained from parent plants, at least one of which is a sugarcane plant; and a step of determining the presence or absence of the sugarcane-leaf-blade-length-related marker according to any one of claims 1 to 3 in the obtained sugarcane chromosome.
- [Claim 5] The method for producing a sugarcane line according to claim 4, wherein a DNA chip provided with probes each serving as the sugarcane-leaf-blade-length-related marker is used in the determination step.
- [Claim 6] The method for producing a sugarcane line according to claim 4, wherein the progeny plant is in the form of seeds or a young seedling and the chromosome is extracted from the seeds or the young seedling.

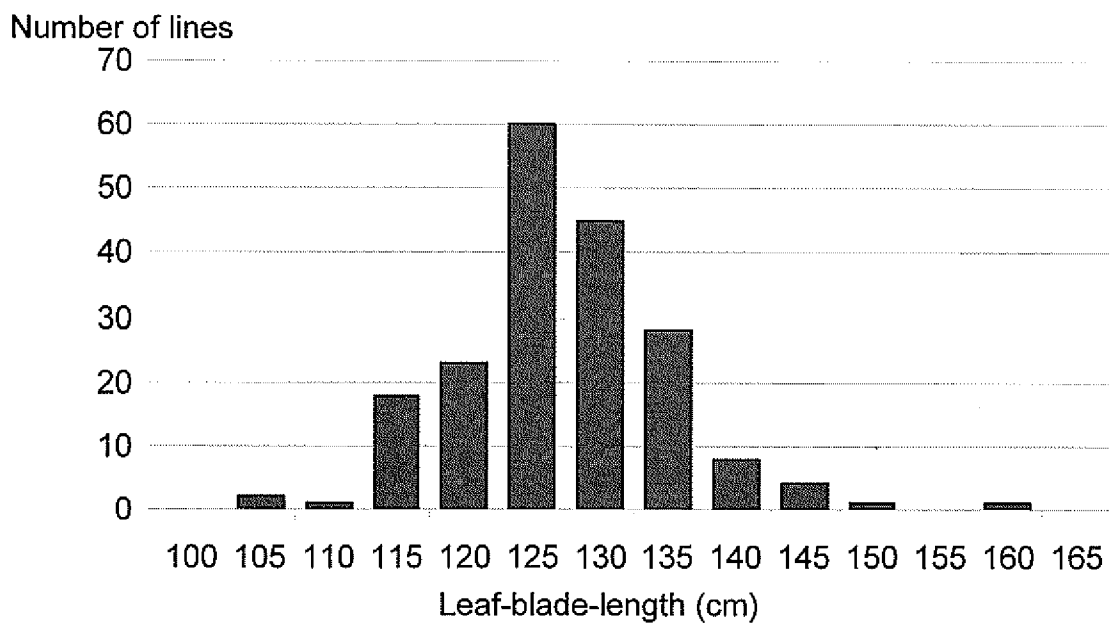
[Fig. 1]



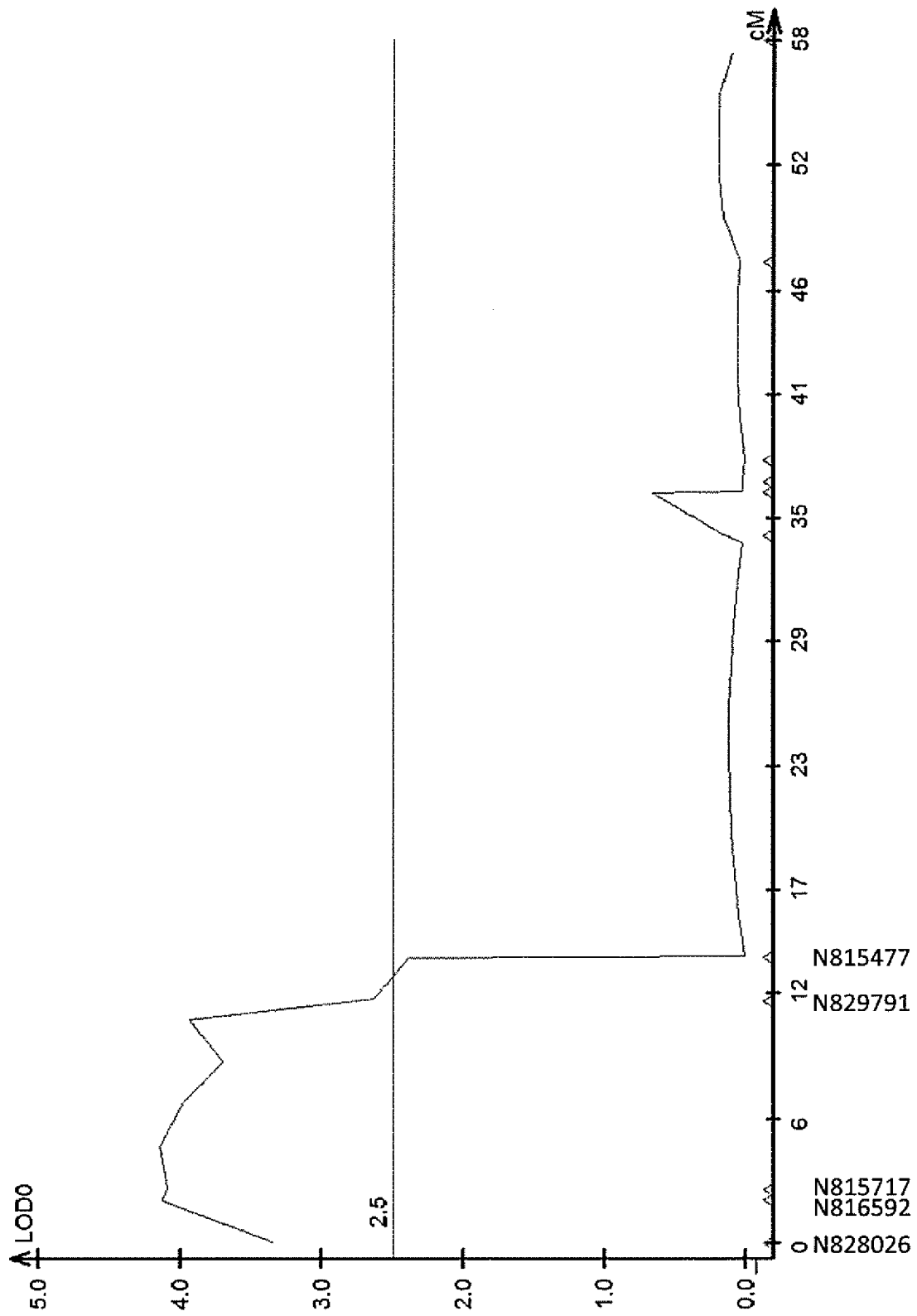
[Fig. 2]



[Fig. 3]



[Fig. 4]



[Fig. 5]

