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Genome constitution of *Narcissus* variety, 'Tête-à-Tête', analysed through GISH and NBS profiling

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Abstract The *Narcissus* variety, 'Tête-à-Tête', has been the most popular variety since 1949, and a well known allotriploid ($2n = 3x = 24 + B$) of spontaneous origin. Because the identity of one of the parents of this variety was uncertain, the genome constitution of 'Tête-à-Tête' was investigated by using genomic in situ hybridization (GISH) and NBS profiling. Both of these techniques confirmed that two different species of *Narcissus*, viz., *N. tazetta* ($2n = 2x = 20$) and *N. cyclamineus* ($2n = 2x = 14$) are the parents. GISH clearly identified 10 chromosomes of *N. tazetta* and 14 chromosomes of *N. cyclamineus*, the former has contributed one and the latter has contributed two genomes. One B chromosome was identically labelled as those of *N. cyclamineus* indicating the affinity of the special chromosome to this species. Due to its male and female sterility 'Tête-à-Tête' is unsuitable as a parent for utilizing in further breeding, it might be possible to re-synthesize a 'Tête-à-Tête' like variety using the now known parents and the original pathway.

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Introduction

There are thousands of cultivars of *Narcissus* registered during the past century. Among these some are more famous than others for their horticultural success and/or popularity. But one cultivar that is the most outstanding among them is 'Tête-à-Tête', that was developed during 1949 (Throckmorton 1980). It is an early spring variety with wide adaptation and can be grown as a garden or pot-plant. It is well established that 'Tête-à-Tête' is a triploid ($2n = 3x = 24 + 1B$ chromosome) and has a complicated parentage involving two different species belonging to two different subgenera of *Narcissus,* viz., *Narcissus* and *Hermione*. One of the parents of 'Tête-à-Tête' is 'Cyclataz' that was derived from an interspecific cross which involved *N. cyclamineus,* a diploid ($2n = 2x = 14$) that belongs to the subgenus *Narcissus* (Brandham and Kirton 1987), and *N.* tazetta, also a diploid with a different basic chromosome number ($2n = 2x = 20, 22$) that belongs to the subgenus, *Hermione.* As a first step, by crossing diploid *N. cyclamineus* x diploid *N. tazetta,* (cv. 'Soleil d 'Or'), the variety 'Cyclataz' ($2n = 2x = 17$) was produced. Probably, as a result of a cross between 'Cyclataz' and an unknown genotype of diploid *N. cyclamineus*, the triploid 'Tête-à-Tête' has

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originated (Brandham and Kirton 1987). The assumption in this case is that the parent 'Cyclataz' has contributed a numerically unreduced ($2n = 17$) gamete and the unknown diploid *N. cyclamineus* has contributed a haploid gamete ($n = 7$) which resulted in triploid 'Tête-à-Tête' with $2n = 3x = 24 + 1B$.

The origin of 'Tête-à-Tête' is a typical example in which the polyploid cultivars have originated through the functioning of *2n* gametes that occur spontaneously in interspecific hybrids of *Narcissus*. In fact the historical sequence of the origin and evolution of polyploid cultivars of *Narcissus* has been well documented (Brandham 1986; Throckmarton 1980). Because the *2n* gametes have played a prominent role in the origin of polyploids of *Narcissus*, (i.e. sexual polyploidization) it is of interest to shed more light on the genome composition, and intergenomic recombination, if any, in such polyploid cultivars. This information might be relevant for a better understanding of the breeding of polyploid *Narcissus* cultivars.

Because 'Tête-à-Tête' is an allotriploid with a complicated chromosome constitution, it is totally sterile and cannot be used as a parent in breeding. However, an alternative approach could be to use the original parental species and/or cv. 'Cyclataz' to re-synthesize a 'Tête-à-Tête' like variety. Since the exact identity of one of the parents of 'Tête-à-Tête' is either uncertain or unknown, it was essential to determine the genome constitution of this variety through molecular cytogenetic methods. For this purpose we have used genomic in situ hybridization (GISH) and NBS profiling (nucleotide binding sites) techniques.

GISH has been successfully used in several other ornamental plant species hybrids and their progenies for the purpose of identifying the parental genomes, as for example in *Crocus* (Ørgaard et al. 1995), *Aloe x Gasteria* hybrids (Takahashi et al. 1997), *Alstroemeria* (Kamstra et al. 1999; Kuipers et al. 1997), *Lilium* (Karlov et al. 1999; Barba-Gonzalez 2005), *Tulipa* (Marasek et al. 2006), *Narcissus* (Diaz Lifante et al. 2009). In some of these cases, in addition to identifying parental genomes, the extent of intergenomic recombination and introgression of alien chromosome segments have also been identified in the progenies. Some of the examples are: *Aloe x Gasteria*, *Alstroemeria aurea* × *A. pelegrina, Lilium longiflorum* × Asiatic hybrids, *Tulipa gesneriana* × *T. fosteriana*.

NBS profiling is an approach based on amplification from conserved Nucleotide–binding-site-leucine-rich-repeat (NBS-LRR) motifs, and has been used as molecular marker technology for R-gene isolation and molecular mapping (Van der Linden et al. 2004; Calenge et al. 2005). It is a versatile multi-locus marker system which shows Mendelian segregation of unique R-genes and RGAs that therefore was used to assess the genetic relationships between parents and progeny in Narcissus.

In the present article, we demonstrated the genome composition of 'Tête-à-Tête' disclosed through GISH and NBS profiling techniques, and discuss its relevance to polyploidy in Narcissus.

Materials and methods

Plant material

The bulbs of 'Tête-à-Tête' to get roots for mitotic chromosome preparations were supplied by PPO (Applied Plant Research), which is a research institute of Wageningen University & Research Center (Wageningen UR). The bulbs were rooted in greenhouse under standard growing condition applicable for Tête-à-Tête cultivation (18–20°C). The plants of *N. tazetta,* (cv. 'Soleil d 'Or'), *N. cyclamineus*, 'Tête-à-Tête', and all other genotypes that were used for collecting the young leaves for DNA isolation were supplied by the Plant breeding Department, Wageningen UR. Some species, cultivars related to the origin of 'Tête-à-Tête' or breeding lines from the related genotypes and their parents were selected for NBS profiling analysis (Table 1). The young leaves were collected and frozen rapidly in liquid nitrogen and stored at −80° C for further use. DNA isolation was performed as described by Fulton et al. (1995) with some slight modifications.

Cytological technique

Chromosome preparation

Root tips were collected early in the morning, incubated in 0.7 mM cycloheximide solution for 4–6 h and then fixed in ethanol-acetic acid (3:1)

Table 1 List of Narcissus genotypes used for NBS profiling analysis (according to the RHS The International Daffodil Register & Classified List)

Number	Name	Horticultural classification	Genotype
03331	Snipe	6 W-W	M.P. Wilner × *N. cyclamineus*
10001	*N. tazetta* Ziva	8 W-W	Cultivar
03318	Meeting	4 Y-Y	Gold Castle sport
03314	Jenny	6 W-W	Mitylene × *N. cyclamineus*
03313	High society	2 W-WGP	May Queen × ?
04070-1	Breeding line	–	Snipe × *N. cyclamineus*
04070-2	Breeding line	–	Snipe × *N. cyclamineus*
10006	*N. tazetta* Soleil d 'Or	8 Y-O	Cultivar
04298-1	Breeding line	–	Red Aria × *N. cyclamineus*
04419-1	Breeding line	–	Meeting × *N. cyclamineus*
04511	*N. papyraceus*	8 W-W	Species
04517	Tête-à-Tête	12 Y-Y	Cyclataz open pollinated
03327	Red aria	2 O-R	Kindled × Feeling Lucky
03340	Wedding Bells	2 W-W	Gracious × Zero
05002-1	Breeding line	–	Monal × Tête-à-Tête
05002-2	Breeding line	–	Monal × Tête-à-Tête
05250-1	Breeding line	–	Monal × *N. cyclamineus*
05250-2	Breeding line	–	Monal × *N. cyclamineus*
04144-1	Breeding line	–	Wedding Bells × *N.cyclamineus*
04144-2	Breeding line	–	Wedding Bells × *N.cyclamineus*
04427-1	Breeding line	–	High Society × *N. cyclamineus*
03043-1	*N. cyclamineus*	–	Species
03043-2	*N. cyclamineus*	–	Species
08308	Topolino	1 W-Y	Cultivar
03353	Monal	2 Y-R	Armada × Paricutin

solution for 12–24 h and stored at 4°C until use. The root tips were washed in distilled water and incubated in a pectolytic enzyme mixture containing 0.2% (w/v) pectolyase Y23, 0.2% (w/v) cytohelicase and 0.2% (w/v) cellulase RS in 10 mM citrate buffer (pH 4.5) at 37°C for about 1 h. Squash preparations were made in a drop of 45% acetic acid and frozen in liquid nitrogen. The cover slips were removed by using a razor blade. The slides were then dehydrated in absolute ethanol and air dried. The best slides were selected under a phase contrast microscope (Leica Dialux 20 EB) and stored at −20°C until use.

Genomic in situ hybridization and detection

Total genomic DNA of *N. tazetta* and *N. cyclamineus* was labelled using a standard nick translation protocol (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions. The double targets were carried out in this experiment. *N. tazetta*, cv 'Soleil d 'or' DNA was labelled with biotin-11-dUTP and *N. cyclamineus* DNA with digoxigenin-11-dUTP. Total genomic DNA of both *N. cyclamineus*, and *N. tazetta*, cv 'Soleil d 'or' was sonicated to fragment sizes of 1–10 kb as probe, and *N. tazetta* 'Soleil d 'or' autoclaved to fragment sizes of 200–400 bp as blocking.

DNA denaturation and in situ hybridization steps were performed according to Hasterok et al. (2001) and Marasek et al. (2010). Digoxigenin-labelled DNA was detected with antidigoxigenin-FITC (sheep) (Boehringer, Mannheim, Germany) and amplified with anti-sheep-FITC (rabbit) (Vector Laboratories). Biotin labelled DNA was detected with CY-3 conjugated streptavidin and amplified with biotinylated goat-antistreptavidin (Vector laboratories). The chromosomes were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) in Vectashield (Vector

Laboratories). Preparations were analysed using Zeiss Axiophot epifluorescence microscope and photographed by Canon digital camera attached to an Axiophot microscope with an appropriate filter and then processed using software (Axio Vision 4.2).

NBS profiling

NBS profiling was designed to identify and isolate R-gene or RGAs (Van der Linden et al. 2004; Calenge et al. 2005). The NBS profiling was conducted followed the protocol developed by Van der Linden et al. (2004) with some optimization to suit the species used. The total genomic DNA (300 ng each genotype) was digested overnight at 37°C with the restriction enzyme *Mse*I or *Rsa*I in the appropriate reaction buffer. An enzyme specific adapter was ligated to the ends of the restriction fragments. This adapter was based on the one described in Fischer et al. (1995), but the 3′ end of the short strand was blocked for extension with Taq DNA polymerase by the presence of an amino group. The 5′ end was phosphorylated to facilitate ligation to blunt-end fragments. For *MseI* ligations, the short strand of the adapter was extended to match the *MseI* restriction fragments. Adapter ligation was performed using T4 ligase (5 U/µl). The reaction was terminated by heat inactivation at 65°C for 15 min.

Amplification of NBS-specific fragments involved a two-step PCR procedure. The first step was a linear (asymmetric) PCR with 5 µl diluted product of restriction ligation, 2 µl of specific degenerated primer (10 pmol/µl), 2 µl adapter primer (10 pmol/îl), 1 µl dNTPs (5 Mm), 2.5 µl Hotstart PCR buffer, 0.08 (5 U/µl) of Hotstart polymerase in the reaction volume of 25 µl. The program consisted of 30 cycles of 30 s at 95°C, 1 min 40 s at 55–60°C annealing, and 2 min at 72°C. Annealing temperature was 55°C for NBS1, NBS 5, NBS6, and NBS-glpl primers, and 60°C for NBS2 and NBS3 primers. The asymmetric PCR was followed by an exponential PCR with the NBS primer and a labelled adapter primer. The reaction was performed in 10 µl assay containing 0.6 µl labelled 1 pmol/µl IRD 700 labelled adapter primer, 0.3 µl of specific degenerated primer, 0.4 dNTPs (5 mM), 1 µl 10× Dreamtaq PCR buffer, 0.04 (5 U/µl) of Dreamtaq polymerase. The cycling program was similar to that of the linear PCR.

The labelled PCR products were separated on a LiCor DNA Analyzer (LI-COR Inc, Lincoln, USA). Bands were visually inspected and scored as present/absent. Genotypes that were considered as putative parents of 'Tête-à-Tête' and other breeding lines were assessed based on parentage exclusion i.e. all bands present in an offspring have be present in at least one of the putative parents.

Results

Genome composition

At mitotic metaphase stages there were clearly 25 chromosomes in each cell of 'Tête-à-Tête'. GISH clearly revealed 10 red and 15 green coloured chromosomes in each case (Fig. 1). The conclusion was that 10 red chromosomes represented the haploid complement of *N. tazetta* ('Soleil d 'Or') and the remaining 15 were derived from *N. cyclamineus*. The

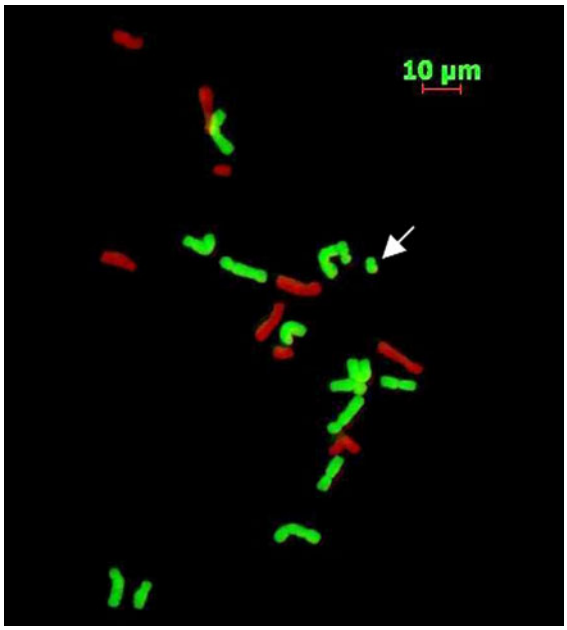

Fig. 1 Narcissus variety 'Tête-à-Tête' chromosomes at mitotic metaphase stage ($2n = 3x = 24 + 1B$) probed with labelled nuclear DNA of *N. tazetta*, (cv. 'Soleil d 'or') with digoxigenin-11-dUTP and detected by CY-3 conjugated streptavidin (*red fluorescence*), and *N. cyclamineus* with digoxigenin-11-dUTP and detected by anti-Dig FITC (*green fluorescence*). *Bars* = 5 µm. *Arrow* indicates the putative B-chromosome

presence of six large acrocentric and four smaller chromosomes of *N. tazetta* confirmed the earlier description of the karyotype of this species (Fernandes 1966). Of the 15 chromosomes representing *N. cyclamineus*, 10 acrocentric chromosomes were slightly larger than the other five and one of the latter was the smallest as compared with others (Fig. 1). This probably was the B chromosome recorded by Brandham and Kirton (1987). Thus the 14 green chromosomes clearly represented the diploid complement of *N. cyclamineus*. Except for the small size, there was no other criterion to call this smallest chromosome a B chromosome. However, because the small B chromosome was of the same colour as those of *N. cyclamineus* it was concluded that it was probably not derived from *N. tazetta*.

NBS profiling

With different combination of NBS primers and restriction enzyme, NBS profiling resulted in a total of 358 polymorphic bands (Table 2) in 'Tête-à-Tête', all which could be traced back to either *N. tazetta*, (cv. 'Soleil d 'Or') and/or *N. cyclamineus*. The bands in 'Tête-à-Tête' have a similarity of 68.5% with *N. cyclamineus* and 55.3% with *N. tazetta*. An example with the combination of primer NBS-glpl6 and restriction enzyme *Rsa*I is shown in Fig. 2.

Similarly, some other related species/cultivars and some breeding lines of *Narcissus* and their parents (Table 1) were also analyzed by NBS profiling. Comparisons of NBS profiling results among

Table 2 Number of polymorphic bands detected with different primer/enzyme combination in 'Tête-à-Tête' and similarity with *N. cyclamineus* and *N. tazetta 'Soleil d'Or'*

Primer	Enzyme		Similarity			
			With cyclamineus		With tazetta	
	*Rsa*I	*Mse*I	*Rsa*I	*Mse*I	*Rsa*I	*Mse*I
NBS1	27	43	85.2%	67.4%	48.1%	55.8%
NBS2	32	18	78.1%	61.1%	46.9%	61.1%
NBS3	66	38	66.7%	56.5%	53.0%	60.5%
NBS5A	–	17	–	70.6%	–	76.5%
NBS6	–	32	–	65.6%	–	53.1%
Glpl6	85	–	68.2%	–	42.4%	–
Total			68.5%		55.3%	

Primer/enzyme combination did not work

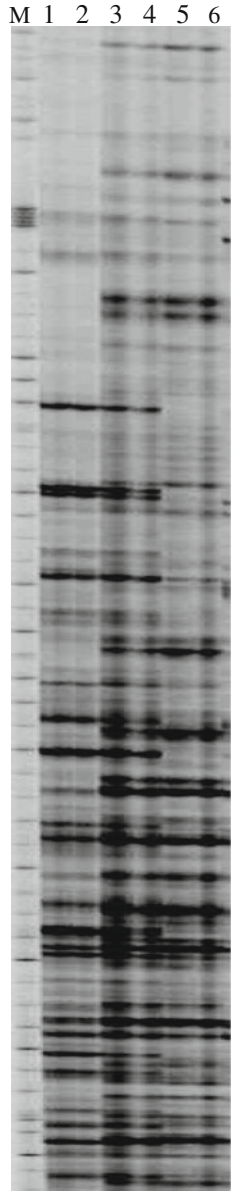

Fig. 2 NBS profile of *N. tazetta* (cv. 'Soleil d 'Or') (lane 1,2), 'Tête-à-Tête' (*lane 2,3*), and *N. cyclamineus* (*lane 5,6*) with primer NBS-glpl6 and restriction enzyme *Rsa*I. The fragments found in the profile of 'Tête-à-Tête' were all contained by *N. tazetta* or/and *N. cyclamineus*

different species, breeding lines and cultivars showed that the technique can be used to exclude putative parental genotypes from parentage. For example, the bands in breeding lines: 04427, 04070-1, 04070-2, 04144-1, 04144-2, 04298-1, 04419-1, 05250-1, 05250-2 were all present in either their male or female parents confirming their putative parentage. The phenotypic characteristics (e.g. leaf, flowering performances) of these lines, showed them to be real hybrids. However, the band pattern in 05002-1 and 05002-2 was exactly the same as that of their female

parent 'Monal', indicating that they are not hybrids but probably have resulted from selfing. The phenotypic characteristics of the latter lines also led to the same conclusion.

NBS profiling is an approach to study R-genes and R-gene analogs (RGAs). Our results showed that this technique is a powerful method for assessment of the genetic relationships between parents and progeny in Narcissus, especially for distant hybrids like 'Tête-à-Tête'. The more distant the parents' relationship is, the lower of similarity of polymorphic bands among parents, which give higher possibility to identify the parentage of hybrids or cultivars.

Discussion

Both GISH and NBS profiling confirm the presence of the genomes of two species, i.e., *N. tazetta* and *N. cyclamineus* in 'Tête-à-Tête'. Especially, GISH confirms that it is an allotriploid with one genome of *N. tazetta* and two genomes of *N. cyclamineus.* While confirming the earlier assumption that it is an allotriploid (Brandham and Kirton 1987), the present investigation shows that the hitherto unknown diploid parent of 'Tête-à-Tête' is likely *N. cyclamineus* although the involvement of another species from the same section cannot be ruled out completely. Obviously, the cultivar 'Cyclataz' ($2n = 17$) has contributed the *2n* gamete, plus the fusion with a haploid gamete of *N. cyclamineus* ($n = 7$) has given rise to the triploid with $2n = 3x = 24$ chromosomes. The occurrence of one B chromosome in this case has been supposed to be inherited from any one of the three immediate ancestors (Brandham and Kirton 1987). Because the smallest chromosome of the complement (Fig. 1, arrow) is labelled similar to *N. cyclamineus* chromosomes, the putative B chromosome is probably not a derivative of *N. tazetta*. In fact, the occurrence of B chromosomes in the species *N. cyclamineus* has been documented (Brandham and Kirton 1987). Moreover, these authors did not record any B chromosomes in the parent 'Cyclataz'. The most likely explanation is that the putative B chromosome has been added by the unknown diploid *N. cyclamineus*. The cultivar 'Jumblie', that was a sister seedling of Narcissus 'Tête-à-Tête', has a similar B chromosome, inferring that the crossing combination of unreduced gametes of (*N. tazetta* × *N. cyclamineus)* × haploid gamete *of N. cyclamineus* probably is an interesting example to study the formation of B chromosomes.

As was pointed out earlier, 'Tête-à-Tête' is a product of sexual polyploidization in which one of the parents, 'Cyclataz', has contributed the *2n* gamete. Normally, when *2n* gametes are produced by a distant species hybrid such as 'Cyclataz', one should expect such *2n* gamete to have originated either through first division restitution (FDR) or indeterminate meiotic restitution (IMR) (Lim et al. 2001; Ramanna and Jacobsen 2003). During meiosis, if homoeologous chromosomes fail to pair, first division restitution can occur and produce FDR 2*n* gametes; if bivalents disjoin and univalents divide simultaneously at anaphase I, indeterminate meiotic restitution can occur and produce IMR 2*n* gametes. FDR gametes may occur either with or without crossing-over between homoeologous chromosomes. Because all the three genomes are totally intact in 'Tête-à-Tête' (i.e., without any intergenomic crossovers), it is evident that 'Cyclataz' has contributed the 2*n* gamete, which has originated through FDR without any crossover. Similar phenomena have been recorded in *Alstroemeria aurea* × *A. inodora* (Kamstra et al. 1999), *Lilium longiflorum* × Asiatic hybrids (Lim et al. 2001) and Oriental × Asiatic hybrids of *Lilium* (Barba-Gonzalez et al. 2005). On the other hand, in some of the genotypes of *Lilium* species hybrids considerable amount of intergenomic recombination has been proved to occur during the origin of FDR gametes (Khan et al. 2009). FDR 2n gametes have the unique property to transfer heterozygosity, epitasis and parental gene combinations intact of distant taxa in sexual polyploids. (Kumar et al. 1984; Lim et al. 2001). Because thousands of polyploid cultivars of *Narcissus* have originated spontaneously through sexual polyploidization (Brandham 1986; Brandham and Kirton 1987), this crop might be of considerable interest for investigating the role of intergenomic recombination in the evolution and the extent of introgression in this crop.

It has been well established that in several ornamental crops, polyploid cultivars have gradually replaced the diploid forms during the past century (Van Tuyl et al. 2002; Ramanna and Jacobsen 2003). Perhaps the best example and a well documented case of such crops is *Narcissus* in which polyploid cultivars have originated spontaneously in the breeders nurseries (Brandham 1986). As a result of

unconscious selection by horticultural breeders, predominantly tetraploid cultivars of *Narcissus* have emerged as by far the best performers in a certain group with a basic chromosome number of $x = 7$ (Brandham et al. 1995). Although 'Tête-à-Tête' is an allotriploid with a complement with two different basic chromosome number, it might be attractive to re-synthesize a similar cultivar by using the now known parents or other species/cultivars from the same section. The expectation in this case would be that the FDR *2n* gametes from the parent 'Cyclataz' may also potentially include gametes with intergenomic recombination. In this case certain amount of genetic variation might be expected in the progenies. Furthermore, it might be worthwhile to increase the ploidy level of a 'Tête-à-Tête' like variety to tetraploid level if this is indeed the optima for *Narcissus* cultivars as has been claimed.

In conclusion, it might be pointed out that the results of GISH and NBS profiling complement each other to establish the parentage of 'Tête-à-Tête'. By testing several genotypes involving the two parents, viz. *N. tazetta* and *N. cyclamineus* (Table 1), it has been possible that NBS profiling alone can determine parentage by exclusion quite reliably. This can be of great help for confirming parentages of *Narcissus* cultivars and confirming hybrids resulting from difficult interspecific crosses on a large scale. Nevertheless GISH, in addition to determining the parentage, can also establish whether or not any inter-genomic recombination has occurred during the origin of a cultivar.

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