

The Use of Chromosomal Markers Linked with Nucleoli Organisers for F₁ Hybrid Verification in *Lilium*

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Abstract

In this study, chromosomal markers based on the presence and position of secondary constrictions, connected with the nucleoli organising regions (NORs), were described with the goal to use them for verification of the hybrid status of F₁ lily plants obtained from crosses 'Marco Polo' × *Lilium henryi* and 'Expression' × *L. henryi*. Two methods of differentiation of secondary constrictions were used – silver staining, which detects some nucleolus-linked proteins and can be used to determine expression of 18S-5.8S-25S rRNA genes and in situ hybridisation with a 25S rDNA probe, which reveals the chromosomal localisation of these genes regardless of their transcriptional status.

These two techniques allowed identifying 4 chromosomes in *L. henryi* and 5 chromosomes in 'Expression' out of the complement of 24 chromosomes. In 'Marco Polo' 5 chromosomes were recognisable after silver staining and 6 ones using in situ hybridisation with 25S rDNA probe. The two techniques also revealed some polymorphism regarding both the number of hybridisation sites and Ag-positive bands observed in 'Marco Polo' and 'Expression'. For hybrid verification only chromosomes carrying non-polymorphic landmarks were chosen: chromosomes D and K for cultivars and chromosomes F for *L. henryi*.

INTRODUCTION

Secondary constrictions have been used as markers in *Lilium* for both identification of individual chromosomes and hybrid status verification (Fernandez et al., 1996; Obata et al., 2000; Marasek and Orlikowska, 2001). However, there may be a difficulty to discriminate between primary and secondary constrictions when using standard cytological stainings, e.g. with acetocarmine or Schiff's reagent. Also, due to a very low NOR activity, secondary constrictions are in many cases shrank and then it can be difficult to spot them at *Lilium* chromosomes (Sato et al., 1980). Therefore, additional techniques that allow more specific visualisation of those regions on chromosomes, which are linked to rDNA presence and activity, are needed. A very simple, technique allowing to demonstrate clearly nucleoli in interphase and NORs at chromosomes, is silver staining – a cytochemical technique, which utilises the fact that silver ions bind preferentially to some of the nucleolus-linked proteins. The method can be supplemented by fluorescence in situ hybridisation (FISH) with relevant rDNA probe, which enables detection of all loci of genes encoding for 18S, 5.8S and 25S ribosomal RNAs regardless their transcriptional status.

The purpose of this study was to find out a method of discrimination of secondary constrictions on lily chromosomes with the goal to verify the hybrid status of F₁ lily plants obtained from interspecific crosses.

MATERIALS AND METHODS

Plant Material

The following genotypes were investigated: parental forms 'Expression' and

'Marco Polo' (cultivars belonging to the horticultural group "Oriental hybrids", which contains genomes of species of Section *Archelirion*), *Lilium henryi* Baker and putative hybrid plants obtained from the crosses of 'Marco Polo' × *L. henryi* and 'Expression' × *L. henryi*. The bulbs of *L. henryi* originated from the germplasm collection administrated by the Research Institute of Pomology and Floriculture, Skierniewice, Poland. The bulbs of all cultivars were obtained commercially. Putative hybrids were obtained as a result of ovule in vitro culture.

Slide Preparation

Root tips were treated with 0.1% colchicine for 4 hours, fixed in 3:1 ethanol-glacial acetic acid for 4 hours and then digested in a mixture of enzymes consisting of 20% pectinase (Sigma) and 4% cellulase (Sigma) for 5 hours at 37°C and squashed in a drop of 45% acetic acid.

Silver Staining

The staining was performed according to the modified method of Hizume et al. (1980). The preparations were stained in a humid chamber with 50% aqueous solution of AgNO₃ at 60°C for 1 h, and then washed in bi-distilled water, air-dried and mounted in DPX.

Fluorescence in Situ Hybridisation with the 25S rDNA Probe

Fluorescence in situ hybridisation (FISH) procedure was adopted from Hasterok et al. (2001). The ribosomal probe was generated by nick translation of a 2.3 kb sub-clone of the 25S rDNA coding region from *A. thaliana* (Unfried and Gruendler, 1990) with digoxigenin-11-dUTP (Roche). Chromosome preparations and pre-denatured (75°C for 10 min) probe were subject to combined denaturation at 70-72°C for 5 min and then hybridised overnight at 37°C in a moist chamber. After hybridisation, the slides were stringently washed in 20% formamide in 0.1xSSC at 42°C. The probe was immunodetected by FITC-conjugated anti-digoxigenin antibodies (Roche). The chromosomes were mounted and counterstained in Vectashield antifade buffer (Vector Laboratories) containing 2.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma).

Slide Analysis

Chromosomes were identified according to Stewart (1947), on the basis of the arm lengths, the position of the primary constrictions and the presence, and position of the secondary constrictions (Marasek and Orlikowska, 2003). The FISH preparations were photographed onto Fujicolour 400 ISO colour negative film with a 35 mm camera attached to Nikon Optiphot-2 epifluorescence microscope equipped with the appropriate set of filters. The slides, which were used for silver staining were photographed using a Nikon Microphot-FXA microscope on Fujicolour 100 ISO negative film. For each plant obtained as a result of distant crosses, at least 5 metaphase plates were analyzed on which marker chromosomes were searched for.

RESULTS

The chromosomes of parental genotypes, which have Ag-positive bands after silver staining and the signals of hybridisation with the 25S rDNA probe are shown on Fig. 1 and 2. The number and the localisation of NORs revealed, using both methods, were the same for 'Expression' and *L. henryi*. In 'Expression' (Fig. 1b and 2b) both methods revealed the presence of 5 NORs, which were localised in the short arm of one of the chromosomes A and on chromosomes D and K. NORs on chromosomes D were present in the short arm close to primary constriction, and intercalary in the long arm on chromosomes K. In *L. henryi*, (Fig. 1c and 2c) the presence of four NORs was revealed. They were localised in the short arms of chromosomes A close to the centromere and intercalary in the long arm of F chromosomes. In 'Marco Polo', as a result of silver staining 5 Ag-NORs were observed. They were localised on only one of homologous

chromosomes A, B, D in their short arms and intercalary in the long arms on a pair of K chromosomes (Fig. 1a). FISH with 25S rDNA probe revealed the presence of additional rDNA sites in the short arms of both chromosomes D (Fig. 2a).

The comparison of the parental karyotypes enabled selection of marker chromosomes, which were then used for verification of hybrids. Only chromosomes having NORs on both homologous chromosomes could be the markers, therefore chromosomes D and K were chosen as characteristic for the maternal forms – ‘Marco Polo’ and ‘Expression’. For *L. henryi*, chromosomes F were selected as diagnostic. The chromosomes possessing marker Ag-NOR sites for verification of ‘Marco Polo’ × *L. henryi* and ‘Expression’ × *L. henryi* hybrids are shown on Fig. 3 and 4. The presence of marker chromosomes characteristic for both parental forms was the confirmation of hybrid status of the seedlings tested.

DISCUSSION

An analysis of chromosomes is often used for hybrid verification at the early stage of their development. In the simplest case, the verification is possible on the base of the number of chromosomes (Giddings and Rees, 1992; Rokka et al., 1998), the size of chromosomes (Metwally et al., 1996; Kisaka et al., 1998) and/or the presence of the marker chromosomes having secondary constrictions (Kruse, 1972; Wolters et al., 1994). In *Lilium*, due to high similarity in size of chromosomes between species and cultivars, only those having secondary constrictions have been more widely used for hybrids verification. However, a simple staining with acetocarmine or leucofuchsin as a dye may not reveal all constrictions especially in highly condensed somatic metaphase chromosomes and to distinguish between primary and secondary constrictions especially when these two structures are closely located at chromosomes. Both problems appeared in our earlier study of cultivars ‘Marco Polo’ and ‘Expression’. After Feulgen staining, secondary constrictions were invisible on chromosomes D. However, the presence of NORs was revealed, when silver staining and FISH with 25S rDNA were applied. Similarly, these techniques enabled the correct classification of K chromosomes having secondary constriction on the long arm, which was initially considered as the centromere.

In situ hybridisation with the 25S rDNA probe revealing loci of genes encoding for 18S-5.8S-25S rRNAs, seems to provide markers more reliable than silver staining does for hybrids verification as the results of FISH do not depend on NOR activity. The results obtained for ‘Marco Polo’, where only one locus at chromosomes D was transcriptionally active and therefore detectable after silver staining, can serve as a good visualisation of this problem. The suppression of NOR activity was also observed in some hybrids of *Ribes* (Keep, 1962) and *Hordeum* (Lacadena and Cermenó, 1985), which theoretically indicates that silver staining should not be considered as the only method for hybrid status verification. On the other hand, in the hybrids ‘Marco Polo’ × *L. henryi* and ‘Expression’ × *L. henryi*, being tested in this study, the number of NORs revealed after silver staining was exactly the same as the number of hybridisation sites after FISH with the 25S rDNA probe. Similarly, the suppression of nucleolar activity was not recorded in lily hybrid ‘Black Beauty’ (von Kalm and Smith, 1980). Therefore it can be concluded that silver staining can be used as an initial method for lily hybrid verification as a very rapid and robust NOR-targeted cytochemical staining. Nevertheless, due to a possible polymorphism in activity of some rDNA loci, it is recommended, however, to supplement this simple staining with some more sophisticated molecular cytogenetic techniques, like FISH with different ribosomal or total genomic DNA probes (Marasek et al., 2004 – in press).

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Figures

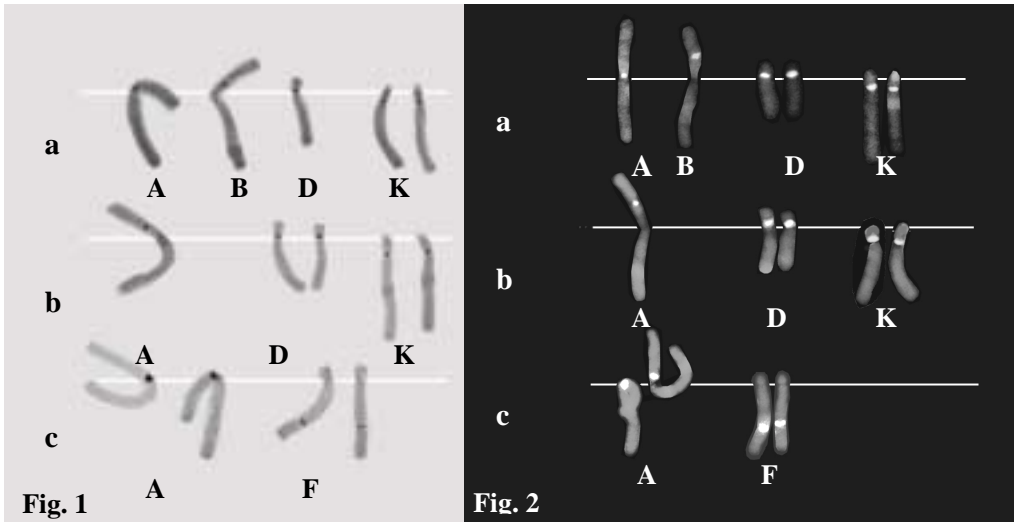


Fig. 1. Extracted chromosomes of 'Marco Polo' (a), 'Expression' (b) and *L. henryi* (c) carrying NORs revealed after silver staining.
 Fig. 2. The ribosomal DNA-bearing chromosomes of 'Marco Polo' (a), 'Expression' (b) and *L. henryi* (c) having NORs revealed after FISH with 25S rDNA probe.

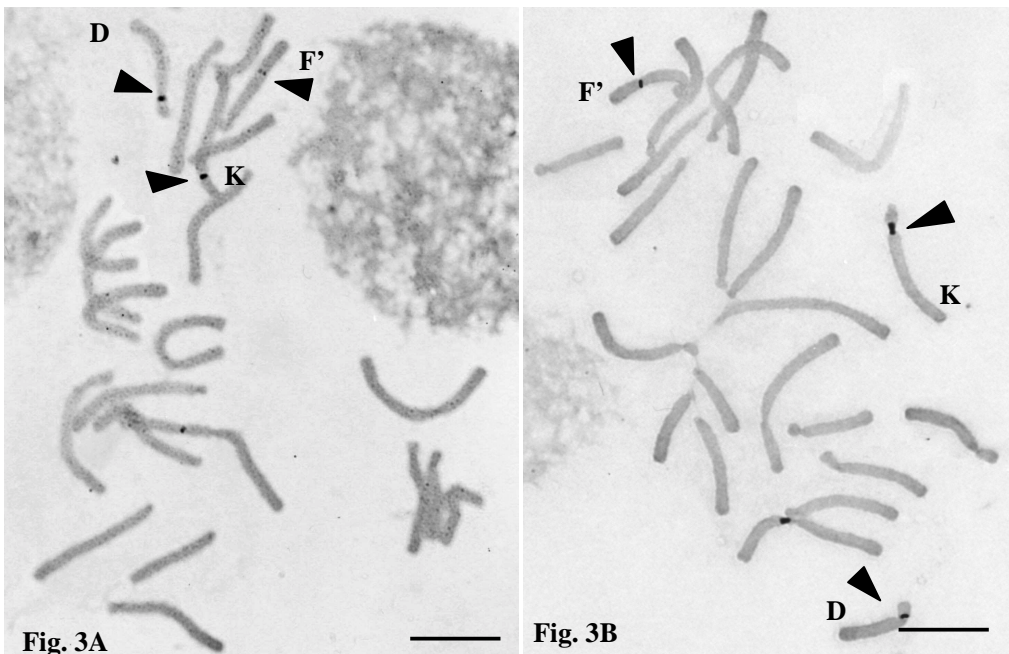


Fig. 3. Silver staining of mitotic metaphase chromosomes of 'Marco Polo' × *L. henryi* (A), and 'Expression' × *L. henryi* (B). The chromosomes which are essential for hybrid verification are marked by the asterisks. Scale bars: 10 μ m. Apostrophes show chromosomes of the paternal form.

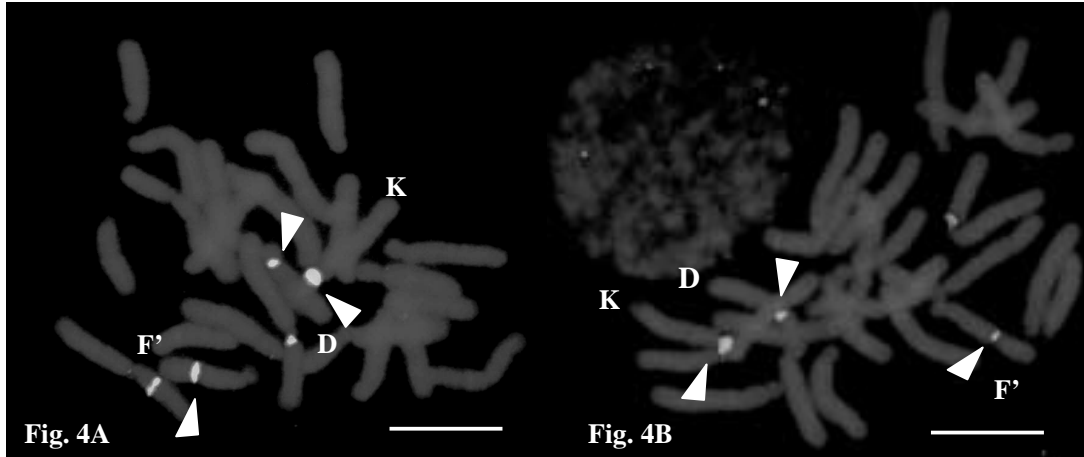


Fig. 4. FISH of 25S rDNA probe to mitotic metaphase chromosomes of 'Marco Polo' \times *L. henryi* (A), and 'Expression' \times *L. henryi* (B). Marker chromosomes important for hybrid verification are signified by the asterisks. Scale bars: 10 μ m. Apostrophes show chromosomes of the paternal form.