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# KARYOLOGY OF NINE LILY GENOTYPES

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Chromosome morphology was studied in lily genotypes *L. candidum*, *L.* × *formolongi*, *L. henryi* and *L. pumilum*, and cultivars 'Alma Ata,' 'Expression,' 'Marco Polo,' 'Muscadet' and 'Star Gazer' belonging to the horticultural group Oriental hybrids. All genotypes tested represented 2n = 2x = 24 chromosomes. Chromosomal markers were established after Feulgen and silver staining, from analysis of the chromosome length and position of the primary and secondary constrictions. For each chromosome the arm index was calculated. Based on these data, idiograms were drawn. For the genotypes analyzed the markers were the secondary constrictions, as confirmed by silver staining. Chromosome length can be used as a marker in only a few cases. From 4 to 10 chromosomes could be identified using secondary constrictions as markers, depending on the genotype. Markers are proposed for each possible species × cultivar and cultivar × species combination.

**Key words:** *Lilium* spp., chromosome morphology, hybrids.

## INTRODUCTION

Genotype characterization based on the morphological features of chromosomes is mainly used in studying the origin of species, chromosome aberrations and genotype identification. The latter is the subject of this study. For practical purposes it is important to discover chromosomes that can be used as markers. Depending on the purpose and the species investigated, the following morphological traits may be used for genotype identification: the total length of the chromosome complement, single chromosome length, the positions of the primary constrictions, the presence and positions of secondary constrictions and nucleolar organizing regions (NORs), and the lengths of satellites. These markers are established by analyzing mitotic metaphase chromosomes. In the case of insufficient morphological differentiation between chromosomes, techniques of longitudinal differentiation (banding) and localization of specific DNA fragments have been developed (Stace, 2000).

Comparison of the morphological details of chromosomes has enabled the identification of species, as in the genera *Amaryllis* (Narain and Khoshoo, 1968), *Alstroemeria* (Rustanius et al., 1991), *Clivia* (Ran et al., 1999) and *Brassica* (Cheng et al., 1995). Chromosome morphology has been used to verify hybrids, to eliminate plants of apomictic origin arising often as the result of distant hybridization (Keep, 1975; Georgi, 1985; Nassar et al., 1998). Significant differences in parental chromosome length, visible without measurements, enabled verification of hybrid *Oryza sativa* + *Hordeum vulgare* (Kisaka et al., 1998). In the genus *Anemone*, hybrids were identified on the basis of measurable differences in the total length of chromosome complements between parental forms (Heimburger, 1962). Differences in the length of chromosomes having secondary constrictions confirmed the hybrid status of plants obtained from crosses of *Hordeum vulgare* and *Triticum* species (Kruse, 1973), and differences in satellite length confirmed the hybrid status of *Lycopersicon esculentum* + *Solanum tuberosum* (Wolters et al., 1994). Hybrid plants resulting from distant crosses between *Lilium nobilissimum* × *L. regale* were identified based on

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differences in the presence and position of secondary constrictions on two pairs of the longest chromosomes (Obata et al., 2000). Hybrids obtained from pollination of *Allium cepa* by other species were easily identified due to the presence of acrocentric chromosomes characteristic for the paternal genotypes (Keller et al., 1996).

Our study of chromosome morphology in four species and five cultivars of lily was intended to find the rules for establishing markers that could most easily be used to conclusively verify seedlings arising from distant crosses. Previously we have obtained seedlings whose phenotypes at the time of blooming appeared identical to the maternal forms, seeming to indicate that they originated following an apomictic process.

Detailed studies of lily chromosomes have yielded idiograms for 42 species (37 karyotyped by Stewart, 1947; 2 karyotyped by Noda, 1973, 1978, after Smyth et al., 1991; 3 karyotyped by Lighty, 1960, after Smyth et al., 1991). We wanted to use that knowledge for verification of our distant hybrids, but it was not applicable to our material. Our study of chromosome morphology produced some results and conclusions relevant to this problem.

## MATERIALS AND METHODS

The following genotypes used in our breeding program were studied: *Lilium candidum* L. (European species), *L.* × *formolongi* (*L. formosanum* Wallace 'Wilson's Giant White' × *L. longiflorum* Thunberg – cross made by Wada in 1939, Japan), *L. henryi* Baker (Chinese species), *L. pumilum* De Candole (Siberian species), and cultivars 'Alma Ata,' 'Expression,' 'Marco Polo,' 'Muscadet' and 'Star Gazer.' Bulbs of *L. candidum*, *L. henryi* and *L. pumilum* were purchased from the germplasm collection administered by the Research Institute of Pomology and Floriculture. Their taxonomic verification was done according to UPOV (International Union for the Protection of New Varieties of Plant) guidelines for the conduct of tests for distinctness, homogeneity and stability in lily. Seeds of *L.* × *formolongi* were obtained from the Dai-Ichi Seed Co. (Holland). They were germinated and grown until mature bulbs were formed. Bulbs of cultivars were obtained commercially.

All genotypes tested except for *L.* × *formolongi* are propagated vegetatively. *L.* × *formolongi* is propagated by self-pollination, resulting in high homozygosity. The cultivars used in this study belong to the horticultural group Oriental hybrids. This group originated from hybridization within section *Archelirion* (*L. auratum*, *L. speciosum*, *L. japonicum*, *L. rubellum*, *L. nobilissimum*, *L. alexandre*) and also includes any of their crosses with *L. henryi* (Withers, 1967; Feldmaier and McRae, 1982).

Metaphase chromosomes in root meristem cells were examined. Root tips were obtained from roots 0.5–1.0 cm long, grown on bulb scales. Five bulbs for each genotype were scaled and incubated in a mixture of perlite and peat (1:1) in the dark at 13˚C.

### SLIDE PREPARATION

Root tips were treated with 0.1% (w/v) colchicine for 4 h in the dark at room temperature, then fixed in 3:1 ethanol-glacial acetic acid for 4 h and stored at -20˚C until required.

### FEULGEN STAINING

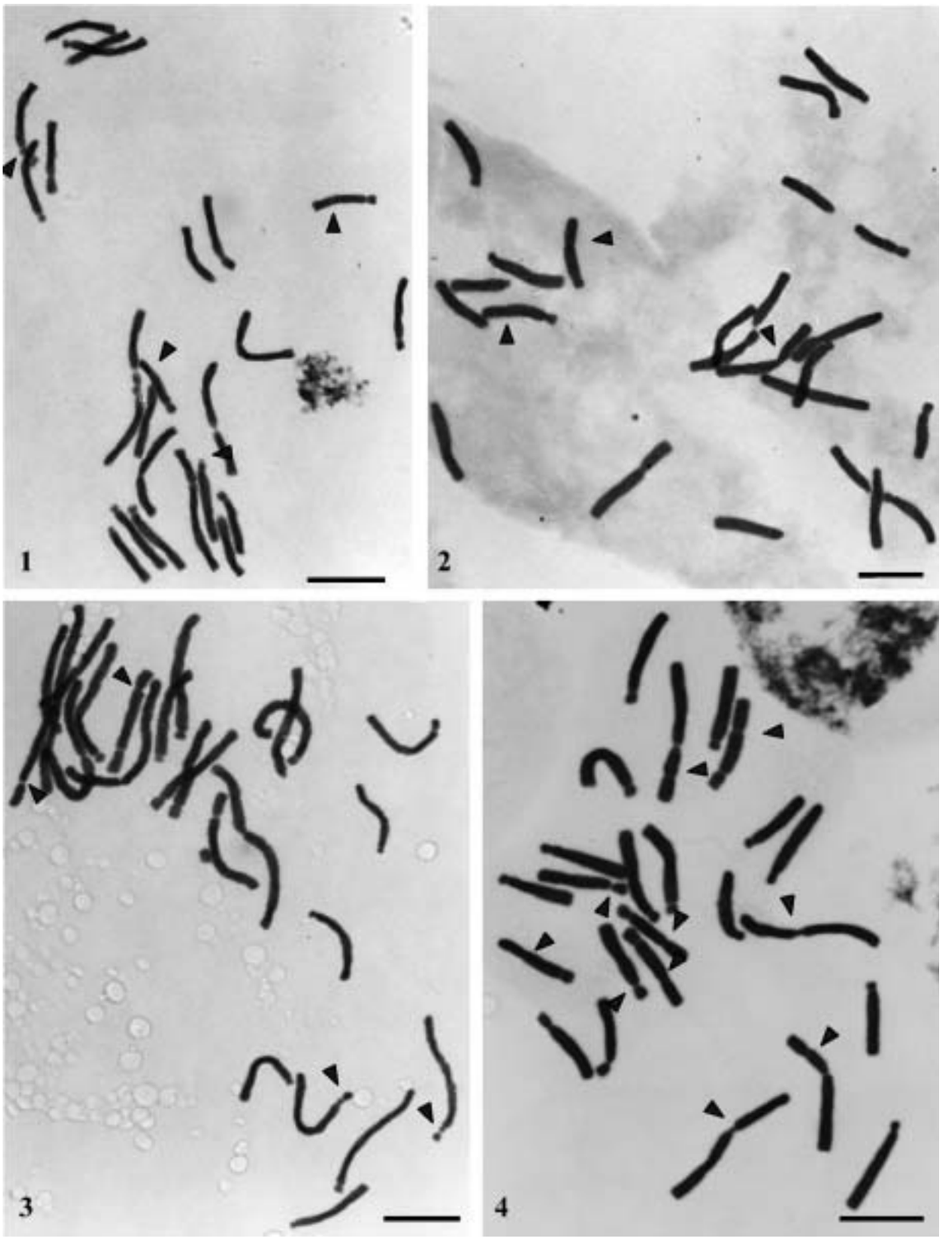
Fixed root tips were rinsed in bi-distilled water for 10 min, hydrolyzed in 1 N HCl for 18 min at 58˚C and stained in Schiff's reagent for 1 h in the dark and co-stained in 1% (w/v) acetocarmine for 10 min. Root meristems were squashed in a drop of 45% (v/v) acetic acid. After freezing, the coverslips were quickly removed and the slides were air-dried and embedded in DPX.

### SILVER STAINING

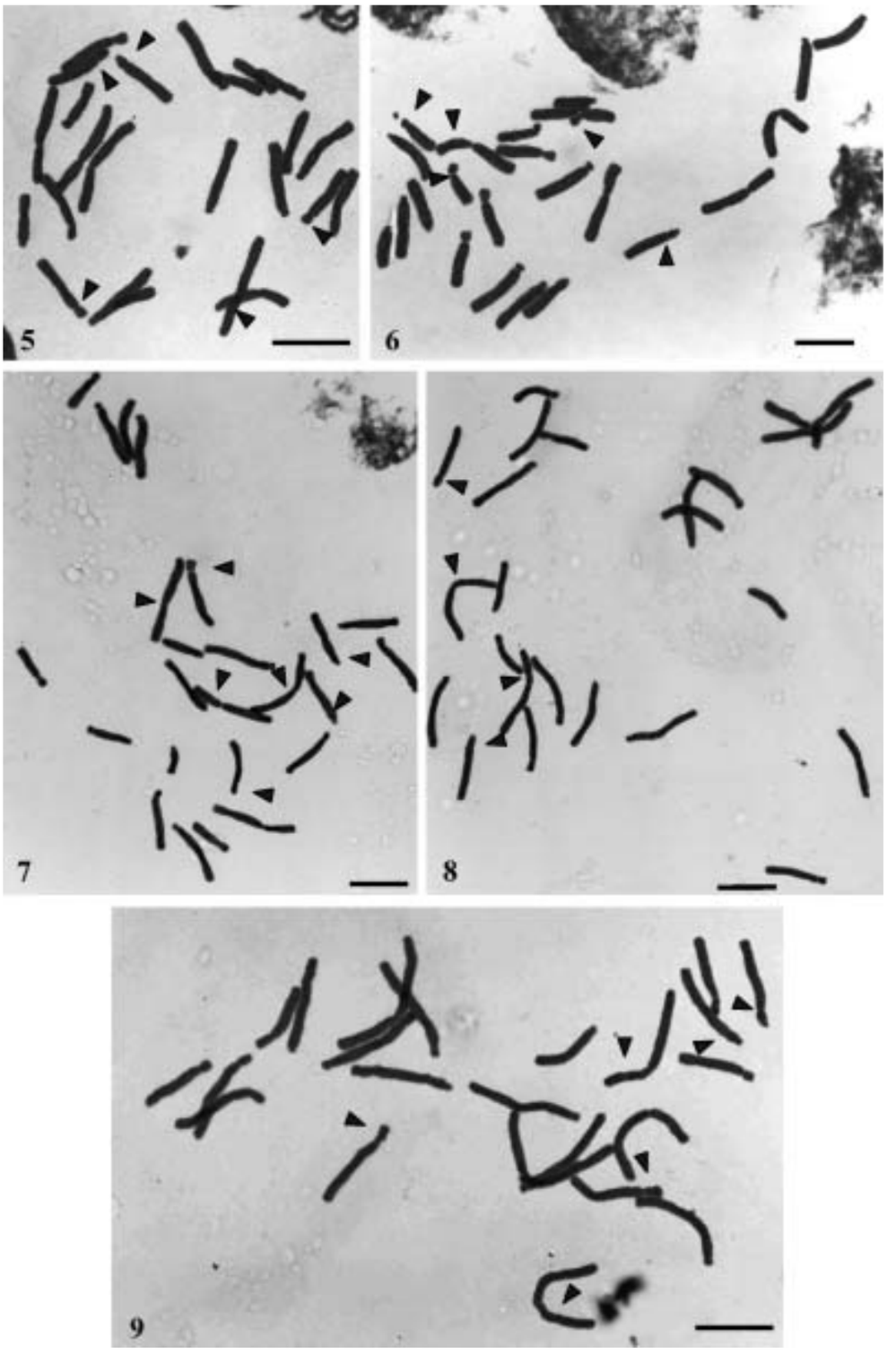
Staining was performed according to a modification of Hizume et. al. (1980). Root tips were rinsed in 0.01 M enzyme buffer (citric acid – sodium citrate, pH 4.8) for 20 min and digested in a mixture of enzymes consisting of 20% (v/v) pectinase (Sigma) and 4% (w/v) cellulase (Sigma) for 5 h at 37˚C. Root meristems were squashed in a drop of 45% acetic acid. After freezing, the coverslips were quickly removed and the slides were air-dried and stained in a humid chamber in 50% (w/v) silver nitrate at 60˚C for ~24 h. When the nucleoli organizers were stained black, the slides were rinsed in bi-distilled water, air-dried and embedded in DPX.

### SLIDE ANALYSIS, DATA COLLECTION AND PROCESSING

Selected chromosome spreads were photographed with a camera attached to a Nikon Microphot-FXA microscope on Fujicolor 100 ISO film. Whole chromosome and arm lengths were measured on photo-




**Figs. 1–4.** Feulgen-stained metaphase chromosomes of *Lilium*. **Fig. 1.** *L. candidum*. **Fig. 2.** *L. henryi*. **Fig. 3.** *L.* × *formolongi*. **Fig. 4.** *L. pumilum*. Secondary constrictions marked by arrowheads. Bar = 10 µm.




**Figs. 5–9.** Feulgen-stained metaphase chromosomes of *Lilium*. **Fig. 5.** 'Expression.' **Fig. 6.** 'Muscadet.' **Fig. 7.** 'Alma Ata.' **Fig. 8.** 'Star Gazer.' **Fig. 9**. 'Marco Polo.' Secondary constrictions marked by arrowheads. Bar = 10 µm.

graphs for 10 metaphase plates with similar chromosome condensation, obtained from different roots. The axes of the chromosomes were measured with the scale cut from the photograph (the scale was imprinted on the film with the Microphot-FXA microscope). Curves were measured by stages on small straight segments. The lengths of arms having a satellite were measured excluding the length of the secondary constriction. From this data, arm indices (long to short arm ratios) were calculated. The results of silver staining were used to distinguish closely located primary and secondary constrictions. The relative lengths of the chromosomes and chromosome arms were expressed as percentages of the total length of the diploid complement. Chromosomes were positioned on idiograms according to Stewart (1947) from left to right by decreasing length of the short arm, labelled A to L. When more chromosomes had the same short arm length, the arm index was used in decreasing order. Because some homologous chromosomes (especially in cultivars) differed in arm length and the presence of the secondary constrictions, each chromosome was characterized separately. In a homologous pair, the chromosome having a longer long arm was marked 1. Standard deviations were calculated for mean values.

## RESULTS

### POSITIONING OF CHROMOSOMES ON IDIOGRAMS

Figures 1–9 show examples of metaphase plates of the nine genotypes studied, and Figures 10–12 present the idiograms. Arranging the chromosomes by decreasing length of the short arms was complicated by the close proximity of the primary and secondary constrictions in some cases, as on chromosome A in *L. henryi*, *L. pumilum* and 'Alma Ata' (Figs. 10c, 11a,b) or D in *L. pumilum* (Fig. 11a), and the constriction type could be verified only after silver staining of the nucleolus organizing regions. Silver staining was also helpful in differentiating between K and C chromosomes. Chromosome classification based on short arm length could not guarantee that all chromosomes were correctly positioned, enabling comparisons between genotypes. For most chromosomes the short arms could not be the only criterion for positioning, especially when the standard deviations were taken into account. The final decisions on the positioning of chromosomes on the idiograms were made on the basis of short arm length, total chromosome length and arm ratio. The constructed idiograms were compared to Stewart's models for species.

### POLYMORPHISM OF THE HOMOLOGOUS CHROMOSOMES

Polymorphism was reflected in the presence/absence of secondary constrictions (satellites) and differences in the lengths of arms, mostly the long ones. Most of the length differences between homologous chromosomes visible on idiograms could not indicate polymorphism, because they remained within the standard deviations. Polymorphism related to the secondary constriction or confirmed by the standard deviation was starred on the idiograms. In species, polymorphism was limited to the presence of the secondary constrictions on I chromosomes in *L. candidum* (Fig. 10a). In cultivars, the differences among chromosomes referred to both arm length and the presence of secondary constrictions. The difference in the lengths of homologous chromosomes was significant in 'Alma Ata' (Fig. 11b). Polymorphism of secondary constrictions was recorded on A chromosomes in all cultivars except 'Alma Ata,' and on B chromosomes of 'Alma Ata,' 'Marco Polo' and 'Star Gazer' (Figs. 5–9, 11b, 12a–c).

Chromosomes of three genotypes – *Lilium candidum*, *L.* × *formolongi* and *L. henryi* – were initially analyzed on the basis of both absolute and relative values. We did not find significant differences that could change the chromosome position (data not shown). The general rule was that the standard deviations were smaller for means obtained from relative values. We therefore constructed the idiograms on the basis of absolute values.

### KARYOTYPE SIMILARITIES AND DIFFERENCES

All genotypes tested contained 2n = 24 chromosomes. The differences in the total length of all metaphase chromosomes were small in the analyzed genotypes, ranging in species from 277.69 ± 15.5 μm (*L. pumilum*) to 318.8 ± 37.51 μm (*L.* × *formolongi*) and in cultivars from 243.95 ± 18.57 μm ('Muscadet') to 297.01 ± 30.45 μm ('Star Gazer'). The differences in the lengths of matching chromosomes between genotypes were also small. For instance, A chromosome length varied from 17.08 ± 1.56 μm in *L. candidum* to 20.64 ± 2.06 μm in *L.* × *formolongi*, while in cultivars it ranged from 14.97 ± 1.24 μm in 'Muscadet' to 18.40 ± 1.98 μm in 'Star Gazer.' In all genotypes analyzed, differences in length between chromosomes in the cell were recorded. For example,

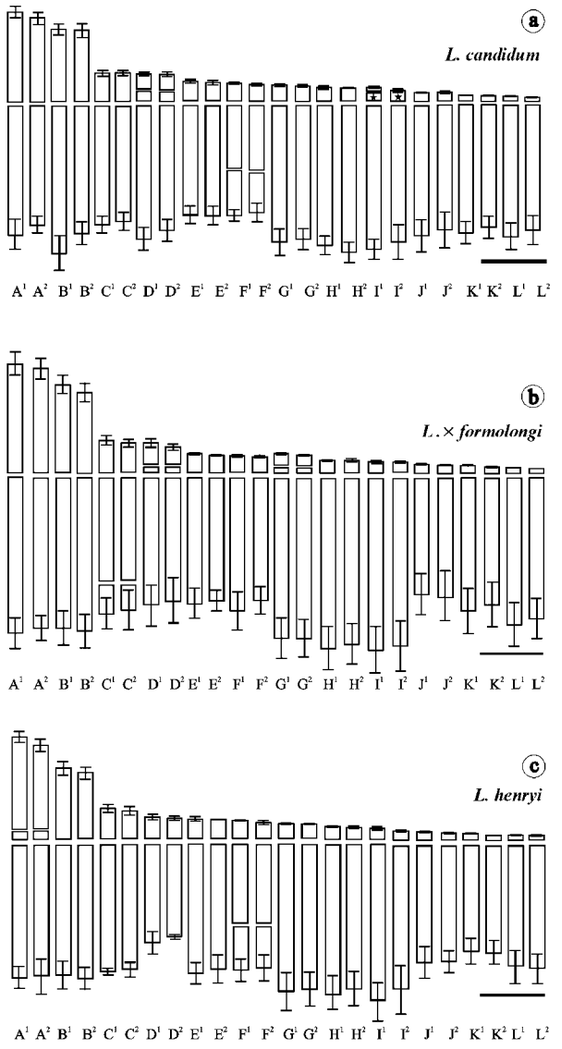



**Fig. 10.** Idiograms of (**a**) *L. candidum* (total length 285.30 ± 16.63 µm), (**b**) *L.* × *formolongi* (20.64 ± 2.06 µm), (**c**) *L. henryi* (284.96 ± 26.18 µm). Polymorphic chromosomes marked by star. Standard deviation marked on each chromosome. Bars = 5 µm.

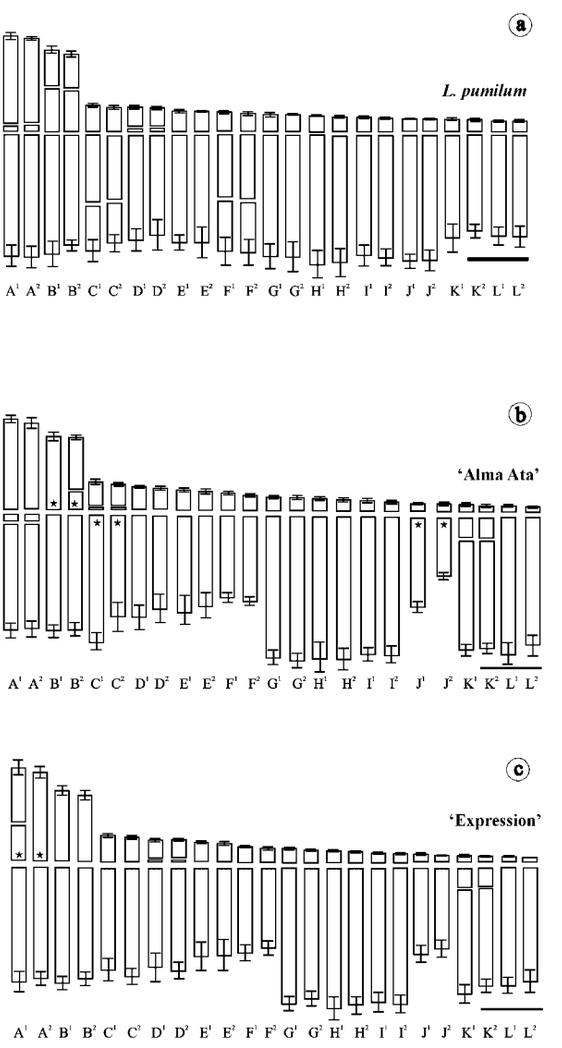



**Fig. 11.** Idiograms of (**a**) *L. pumilum* (277.69 ± 15.50 µm), (**b**) 'Alma Ata' (275.97 ± 9.99 µm), (**c**) 'Expression' (262.01 ± 15.63 µm). Polymorphic chromosomes marked by star. Standard deviation marked on each chromosome. Bars = 5 µm.

in *L. henryi* the difference between the longest and shortest chromosomes was 9.39 µm, in 'Alma Ata' it was 11.32 µm, and in 'Marco Polo' 9.13 µm.

The idiograms of the analyzed genotypes were very similar in shape. In each genotype the following types of chromosomes were observed: metacentric (all A and some B chromosomes), submetacentric (most B chromosomes), subtelocentric (chromosomes from C to F) and telocentric (G to L) (according to Levan et al., 1964). In all genotypes analyzed, the easiest to identify were the longest A and B chromosomes. In all idiograms, the group of chromosomes from G to I, having the longest long arm, and K and L chromosomes, having the shortest short arm, could be distinguished. In the species, two pairs of satellites were found in *L. henryi*, three pairs in *L.* × *formolongi*, five pairs in *L. pumilum*, and five satellites (2II + 1I) in *L. candidum*. In cultivars, seven satellites (3II + 1I) were found in 'Alma Ata,' six (2II + 2I) in 'Marco Polo,' five (2II + 1I) in 'Expression' and 'Muscadet,' and four (1II + 2I) in 'Star Gazer.' The presence of secondary constrictions on the I chromosome of *L. candidum*, the A chromosome in 'Alma

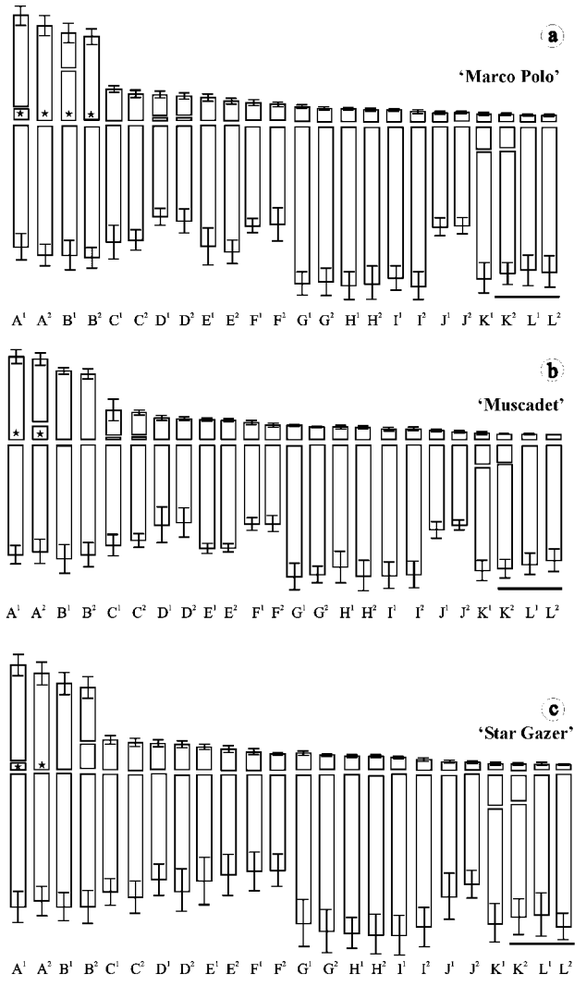



**Fig. 12.** Idiograms of (**a**) 'Marco Polo' (291.59 ± 23.06 μm), (**b**) 'Muscadet' (243.95 ± 18.57 μm), (**c**) 'Star Gazer' (297.01 ± 30.45 μm). Polymorphic chromosomes marked by star. Standard deviation is marked on each chromosome. Bars = 5 μm.

Ata' and the B chromosome in 'Star Gazer' was revealed by silver staining.

In all genotypes analyzed, the high similarity of G, H, I chromosomes made them difficult to differentiate. For example, in *L. henryi* the difference in short arm length between G and I chromosomes was 0.1 μm, and there were no differences in the length of their long arms. These chromosomes did not have any marker features. In species, the J, K and L chromosomes were also difficult to distinguish.

### CHOOSING MARKER CHROMOSOMES

Morphological comparisons enabled us to propose chromosomes characteristic for the genotypes of the parental pairs in crosses. We took into consideration only marker features present on both homologous chromosomes. For verification of hybrids from cultivar × species crosses we chose the F chromosomes as markers for *L. henryi*, *L. candidum* and *L. pumilum*, and the C chromosomes for *L. pumilum* and *L.* × *formolongi*, containing satellites on the long arms, because they did not have counterparts in the cultivar chromosomes. For species × cultivar crosses, the K chromosomes on cultivars, bearing a secondary constriction on the long arm, were chosen. With two exceptions – the J chromosomes in all cultivars and the F in 'Marco Polo' and 'Muscadet' – neither chromosome length nor arm length was proposed as a marker. The full list of markers proposed for verification of hybrids derived from crossing species with cultivars in both directions is presented in Table 1.

## DISCUSSION

Due to their large size, lily chromosomes are convenient for cytological study. Stewart (1947) described and presented chromosomal morphology of 37 lily species in idiograms. Stewart's work was the starting point for investigators, who have followed his rule of lily chromosome classification on the basis of decreasing short arm length (Uhring, 1968; Smyth and Kongsuwan, 1980; Smyth et al., 1989; Smyth et al., 1991; Lim et al., 2000). Unlike the cited authors, we encountered problems in classifying chromosomes. Our detailed analysis brought us to the conclusion that some lily chromosomes cannot be correctly classified on the basis of short arm length, nor on the basis of the other morphological features visualized after Feulgen and silver staining. In all genotypes studied, a group of G to I can be distinguished whose chromosomes are similar in length and arm indices and which do not possess any marker features. In *L. candidum*, chromosomes J, K and L are also indistinguishable. To increase the likelihood of error-free chromosome classification, other methods of chromosome differentiation should be used, such as staining of heterochromatin regions (C-banding) (Hoshi et al., 1998; Joachimiak et al., 1999), fluorochrome staining (F-banding) (Schweizer, 1980) or specific DNA probe hybridization (FISH) (Maluszynska, 1995; Hasterok et al., 2001). They have been applied to lily (Smyth and

TABLE 1. Marker chromosomes proposed for verification of *Lilium* hybrids resulting from species × cultivars and cultivars × species crosses

| Cultivar (C) | Cross | Species (S) | | | |
|---|---|---|---|---|---|
| | | *L. candidum* | *L. × formolongi* | *L. henryi* | *L. pumilum* |
| 'Alma Ata' | C × S | Sat. chromosomes D, F | Sat. chromosomes C, G | Sat. chromosomes A, F | Sat. chromosomes A, B, C, F |
| | S × C | Sat. chromosomes A, C, K<br>Short chromosome J | Sat. chromosomes A, K | Sat. chromosomes A, C, K<br>Short chromosome J | Sat. chromosomes A, K |
| 'Expression' | C × S | Sat. chromosomes D, F | Sat. chromosomes C, G | Sat. chromosome F | Sat. chromosomes A, C, F |
| | S × C | Sat. chromosomes D, K<br>Short chromosome J | Sat. chromosome K | Sat. chromosomes D, K<br>Short chromosomes J, D | Sat. chromosome K |
| 'Marco Polo' | C × S | Sat. chromosomes D, F | Sat. chromosomes C, G | Sat. chromosome F | Sat. chromosomes C, F |
| | S × C | Sat. chromosomes D, K<br>Short chromosome J | Sat. chromosome K | Sat. chromosomes D, K | Sat. chromosome K<br>Short chromosomes D, F, J |
| 'Muscadet' | C × S | Sat. chromosomes D, F | Sat. chromosomes C, G | Sat. chromosomes A, F | Sat. chromosomes B, C, F |
| | S × C | Sat. chromosomes C, K<br>Short chromosomes F, J | Sat. chromosome K | Sat. chromosomes C, K | Sat. chromosome K<br>Short chromosome J |
| 'Star Gazer' | C × S | Sat. chromosomes D, F | Sat. chromosomes C, D, G | Sat. chromosome F | Sat. chromosomes C, D, F |
| | S × C | Sat. chromosome K<br>Short chromosomes F, J | Sat. chromosome K | Sat. chromosome K | Sat. chromosome K |

Kongsuwan, 1980; Lim et al., 2001; Marasek and Orlikowska, 2001).

Converting absolute values to relative ones decreases the dispersion of values related to the differences in chromosome condensation between chromosomal plates. Such a characterization of lengths was reported for *Hypericum perforatum* (Brutovská et al., 2000), *Brassica campestris* and *Brassica alboglabra* (Cheng et al., 1995). In this study, idiograms were constructed for *L. candidum*, *L. henryi* and *L. pumilum* based on both absolute and relative (not shown) values. Only small differences in the values of standard deviations resulted, generally smaller for relative values. Finally, idiograms were constructed based on the absolute values.

Silver staining of nucleolar organizing regions may markedly influence idiogram construction. At high chromosome condensation, secondary constrictions may not be visible, as reported in rye (Merker, 1973). In our study, on D chromosomes of 'Expression' and 'Marco Polo' and on B chromosomes of 'Star Gazer,' secondary constrictions were revealed only when silver staining was used.

In all lily genotypes analyzed, only the longest chromosomes, metacentric or submetacentric A and B, were easily recognizable without measurements, and in cultivars also chromosomes J and F, the shortest ones. In some genotypes, C and D chromosomes were characteristic for their arm indices. Chromosomes possessing satellites are also easily recognizable, especially after silver staining.

In this study, classification of chromosomes in cultivars was more complicated since they are characterized by higher polymorphism in homologous chromosomes. Lily cultivars are highly heterozygous, being close to the primary F1 hybrids obtained through distant crosses. In the species analyzed here, the differences in length of homologous chromosomes were not significant and may have been an artifact of preparation. In the relatively recent hybrid *L. × formolongi* (*L. longiflorum* × *L. formosanum*), polymorphism of homologous chromosomes was not found. This is because the parental species have very similar chromosomes, as noted by Stewart (1947) and confirmed by Withers (1967). Small differences in the lengths of homologous chromosomes were observed in genomes of *Nicotiana glutinosa* and *N. tabacum* (Giddings and Rees, 1992). On the other hand, in *Brassica napus* (Skarzhinshaya et al., 1998) and *Rhoeo spathacea* (Golczyk and Joachimiak, 1999), pairing of chromosomes was so uncertain that each single chromosome was numbered individually.

Distinguishing chromosomes based on length may be charged with significant error due to variation in chromosome contraction within the chromosome complement in one cell (Bajer, 1959) and in

different cells as well. A serious problem in determining correct chromosome lengths may be deformation during preparation, mainly by squashing, which may increase total chromosome length and change the arm indices (Bosemark and Bormotov, 1962). It may depend on the chromosome's position in relation to the center of the squash. These deformations are not evenly distributed over the chromosome arms; on average, long arms are stretched more than short arms (Sybenga, 1959). Chromosome size may also be affected by growth conditions (Swanson 1957, after Heimburger, 1962) and may also depend on the duration of the analysis (Heimburger, 1962). Matern and Simak (1968) concluded from statistical analyses that chromosomes are distinguishable on the basis of length if the average difference between the larger and the shorter chromosome exceeds 8% of the lengths of these two chromosomes, and the risk of reversed classification was lower when the difference was at least 11% of the average lengths of the two chromosomes. A higher difference, over 15% of the mean length of the arms, is necessary for correct classification of the long and short arms in metacentric and submetacentric chromosomes. Despite all the reservations mentioned above, some investigators have drawn conclusions from small differences in length between chromosomes. For instance, Heneen (1962) indicated differences in chromosomes of inbred lines of rye although they did not exceed 1% of the relative length.

In our study, the differences in length between morphologically similar chromosomes of *L. henryi* G and H and H and I equalled 0.58% and 0.37%, whereas the differences between their short arms equalled 7.77% and 4.20%. Following these we did not propose length differences as chromosomal markers in *Lilium*, with a few exceptions concerning chromosomes that visually differ in size, such as chromosomes A and B and in some cultivars chromosome J. Other authors have also verified lily hybrids, mostly on the basis of secondary constrictions (North and Wills, 1969; Okazaki et al, 1994; Fernandez et al., 1996; Roh et al., 1996; Obata et al., 2000). To avoid mistakes in qualifying constrictions, we recommend using silver staining to supplement Feulgen staining.

Although morphological details revealed by Feulgen and silver staining did not provide many markers and distinguished only between 4 and 10 chromosomes, depending on the genotype, these simple methods could be used to verify hybrids obtained from species × cultivars and cultivars × species crosses, leaving no room for doubt. However, it would be more difficult if not impossible to use these methods to verify hybrids obtained by crossing cultivars.

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