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Cytogenetic Analysis of eight Lily Genotypes

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Abstract — Chromosomal banding markers were established for four lily species - *L. candidum*, *L.* × *formolongi*, *L. henryi*, *L. pumilum* and four belonging to horticultural group Oriental hybrids - cultivars - 'Alma Ata', 'Expression', 'Marco Polo' and 'Muscadet'. Banding markers were provided by Ag-NOR, CMA_3/DA/DAPI and C-Giemsa stainings. These techniques enabled to establish markers for identification from 4 to 17 out of 24 chromosomes depending on genotype and technique. Negative DAPI bands were not always observed whereas Ag-NOR and CMA_3 bands were distinctly visible on chromosomes and co-localized the positions of secondary constrictions which enabled to distinguish primary from secondary constrictions, even if these structures were localized close to each other. Additional CMA_3 bands were present intercalary on chromosomes. The C banding method revealed the most markers for chromosomes identification but the technique was difficult to standardisation and results were not fully reproducible. For each genotype tested relative DNA content was evaluated.

Key words: C-banding; CMA_3/DA/DAPI banding; karyotyping; *Lilium* spp.; silver staining.

INTRODUCTION

A large size of lily chromosomes makes them convenient for cytological analyses. However, their morphology - length and centromere position is highly conserved within and between species, therefore only a few chromosomes are recognizable on the basis of above traits (Lim *et al.* 2001; Marasek and Orlikowska 2003). Also statistical analyses of measure data do not help essentially in identification of particular chromosomes (Marasek and Orlikowska 2003). Chromosomal markers in lily refer predominantly to the presence and the position of secondary constrictions (Uhring 1968; North and Wills 1969; Okazaki *et al.* 1994; Fernandez *et al.* 1996; Obata *et al.* 2000; Marasek and Orlikowska 2001; 2003). The number of chromosomes with secondary constrictions varies in lily species from 4 to 16 (Stewart 1947). Staining using acetocarmine or Schiff's reagent does not always reveal all secondary constrictions for their invisibility at high chromosomes condensation, which was a problem in our study (Marasek and Orlikowska 2003) but also was reported for other plants (Hennen 1962; Merker 1973). Due to little morphological polymorphism between majority of lily chromosomes, techniques of longitudinal differentiation (banding) were applied to help in genotypes identification (Smyth *et al.* 1989; Lim *et al.* 2001). These bands are visualised by specific staining of chromosomal proteins accompanying active nucleolar organising regions (Ag-NORs) (Van Kalm and Smyth 1980; Smyth *et al.* 1991), heterochromatin sections (C-bands) (Smyth *et al.* 1989; Smyth 1999), regions rich in A-T or G-C pairs (fluorescent bands) (Kongsuwan and Smyth 1977; Lim *et al.* 2001). Fluorescent in situ hybridisation (FISH) is another technique producing chromosomal markers that show positions of specific genes (Lim *et al.* 2001; Marasek *et al.* 2004a, b).

In the present study idiograms of eight lily genotypes are saturated with markers produced after silver, fluorescent (CMA_3/DA/DAPI) and Giemsa staining. The efficacy of each method for error-free chromosome classification is discussed. For each genotype, the relative DNA content was estimated using flow cytometry.

MATERIALS AND METHODS

Plant Material - The following genotypes were investigated: *Lilium candidum* L., Section Lilium

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(European species), *L.* x *formolongi*, Section Leucolirion (*L. formosanum* Wallace 'Wilson's Giant White' (*L. longiflorum* Thunberg – the cross was made by Wada in 1939, Japan), *L. henryi* Baker, Section Leucolirion (Chinese species), *L. pumilum* De Candole, Section Sinomartagon (Siberian species), cultivars – 'Alma Ata', 'Expression', 'Marco Polo', 'Muscadet' (Section Archelirion). The bulbs of *L. candidum, L. henryi* and *L. pumilum* were purchased from the germplasm collection administrated by the Research Institute of Pomology and Floriculture. Seeds of *L.* x *formolongi* were obtained from Dai – Ichi Seed Co., LTD, Holland. They were germinated and grown until mature bulbs were formed. Bulbs of cultivars were obtained commercially. Cultivars used in this study belong to the horticultural group, which contains genomes of species - *L. auratum*, *L. speciosum*, *L. japonicum*, *L. rubellum*, *L. nobilissimum*, *L. alexandrae* and *L. henryi* (WITHERS 1967).

Flow Cytometry analysis - For flow cytometric analysis, 10 mg of youngest leaves were taken. Onion cultivar 'Wolska' having 38.25 pg DNA per nucleus was used as a standard. Samples were prepared according to MAJEWSKA *et al.* (2000). Fluorescence of propidium iodide was measured in a Partec CA II flow cytometer (Partec GmbH, Germany) equipped with an HBO 100W/2 lamp, KG1, B38, UG1 filters, a CG435 barrier filter, a TK420 dichroic mirror and a 40 x 0.8 quartz objective. In each sample 5,000 to 10,000 nuclei were measured and analysed using the Partec DPAC 2.2 computer programme.

Slide preparation - Root tips were obtained from roots 0.5-1.0 cm long, grown on bulbs scales for metaphase chromosomes examination. Five bulbs for each genotype were scaled and incubated in a mixture of perlite and peat (1:1) in the dark at 13°C. Root tips were treated with 0.1% colchicine for 4 hours, and then fixed in 3:1 ethanol-glacial acetic acid for 4 hours. For fluorescent and silver staining root tips were washed in 0.01 M enzyme buffer (citric acid - sodium citrate, pH 4.8) for 20 minutes and digested in a mixture of enzymes - 20% pectinase (Sigma) and 4% cellulase (Sigma) for 5 hours at 37°C. For C banding root tips were hydrolysed in 1N HCl for 13 minutes. Root tips were squashed in a drop of 45% acetic acid. After freezing and removing of the coverslips, the slides were dehydrated in pre-chilled absolute ethanol, air-dried and kept at 4°C until use.

Silver staining - It was performed according to the modified method of HIZUME *et al.* (1980). Slides were stained in a humid chamber in 50% silver nitrate at 60°C for about 1 hour. When nucleoli organisers were stained black, slides were washed in bi-distilled water, air-dried and embedded in p-Xylene-bis(N-pyridinium bromide) - DPX.

Fluorescence staining - CMA_3/DA/DAPI staining (CMA_3 – chromomycin A3, DA – dystamycin A, DAPI – 4'-6-diamidino-2-phenylindole), was performed according to method of MALUSZYNSKA and OLSZEWSKA (1999). Slides were mounted in 1:1 glicerol-McIlvaine buffer (0.1 M citric acid and 0.2 M Na_2HPO_4 2xH_2O, pH 7.0).

C-banding - C bands were stained according to the method of SMYTH and KONGSUWAN (1980), modified by using saturated barium hydroxide instead of 0.064 M solution. Slides were incubated in $Ba(OH)_2$ for 5 minutes and then in 2 x SSC (30 mM sodium citrate, pH 7.0 and 300 mM NaCl) for 18 minutes, at 60°C. Slides were stained in a 4 % Giemsa solution (MERCK) in phosphate buffer (pH 6.8), air-dried and embedded in DPX.

Slide analysis - After silver and C bands staining, metaphase chromosomes were analyzed and photographed with a camera attached to a Nikon Microphot-FXA microscope and after fluorescence staining with a camera attached to a Nikon Optiphot-2 epifluorescence microscope equipped with the appropriate filter sets (UV-2A for DAPI and B-2A for CMA_3), on Fujicolour 100 ISO or 400 ISO colour negative film respectively.

The length of chromosomes and their arms were measured on 10 well-spread metaphase plates with a ruler. Chromosomes were identified and idiograms were constructed according to STEWART (1947), on the basis of the arm lengths, position of the primary constrictions and the presence and position of the secondary constrictions with all reservations reported in our earlier paper (MARASEK and ORLIKOWSKA 2003). Homologous chromosomes which differed in bands pattern were separately characterised on idiograms. Characteristic bands which were present in each of ten studied metaphase plates were positioned on idiograms.

RESULTS

DNA content and chromosome morphology - Using flow cytometric analysis for genotype identifica-

tion the following value of relative DNA content per nucleus were obtained: *Lilium candidum* – 97.84 ± 0.25 pg, L. x formolongi – 95.60 ± 0.44 pg, *L. henryi* Baker – 84.84 ± 0.53 pg, *L. pumilum* – 95.22 ± 1.85 pg and for cultivars – 'Alma Ata' – 89.69 ± 1.05 pg, 'Expression' – 85.80 ± 0.34 pg, 'Marco Polo' – 88.62 ± 1.37 pg, 'Muscadet'– 88.38 ± 0.39 pg.

All genotypes tested were diploid having 2n=2x=24 chromosomes. The lily chromosomes are large, for example the length of *L. candidum* chromosomes ranged from 17.08 ± 1.56 to 9.39 ± 0.97 μm. However only a few of them could be unquestionably distinguished in metaphase plates based on the size. In all genotypes tested it referred to metacentric or submetacentric chromosomes A and B, and in cultivars also to chromosomes J and F, the shortest ones. The second group of easily recognisable chromosomes in plates were those having satellites. On the other hand, in all genotypes there were chromosomes that were practically indistinquishable on the basis of morphometric analysis - telocentric chromosomes G, H, I.

Banding markers for identification of lily chromosomes - Banding patterns obtained for parental genotypes after staining with silver (Ag-NOR), fluorescent dyes CMA_3/DA/DAPI and Giemsa are shown on Figures 1 and 2.

Silver staining of nucleolar organizing regions (NORs) enabled to recognise from 4 to 10 chromosomes, depending on genotype. The following numbers of NORs were found (Fig. 1 and 2): 2 pairs (*L. henryi*), 3 pairs (*L.* x *formolongi*), 5 pairs (*L. pumilum*), 5 NORs (2II + 1I) (*L. candidum* - Fig. 3a, 'Expression' and 'Muscadet') and (1II + 3I) ('Alma Ata' and 'Marco Polo'). The heteromorphism of the homologous chromosomes was observed for all cultivars: A, C and F chromosomes in 'Alma Ata'; A, B and D chromosomes in 'Marco Polo'; A chromosomes in 'Expression' and 'Muscadet' and I chromosomes in *L. candidum*. After CMA_3/DA/DAPI staining, the fluorescence banding patterns of chromosomes stained by CMA_3 were generally the reverse of the DAPI patterns. CMA_3 bands were visible as brighter regions of chromosome, whereas the same regions after DAPI were visible as unstained gaps (Fig 3d-e). CMA_3/DAPI bands were present in all positions of NORs but also pericentrically and in one case intercalary on long arm of E chromosome of *L. candidum* (Fig. 1a). In genotypes analysed here from 4 to 10 chromosomes were able to recognize on the base of CMA_3/DAPI banding. The following numbers of bands were found (Fig. 1 and 2): 2 pairs (*L. henryi*), 3 pairs (*L.* x *formolongi*), 5 pairs (*L. pumilum* and 'Muscadet'), 8 CMA_3 bands (3II + 2I) (*L. candidum*), 10 bands in 'Alma Ata' (4II + 2I), 9 bands in 'Marco Polo' (3II + 3I) and 7 bands in 'Expression' (3II + 1I). The polymorphism in cultivars referred to B and F chromosomes in 'Alma Ata' (Fig. 2a), A chromosomes in 'Expression' (Fig. 2b), A, B and C chromosomes in 'Marco Polo' (Fig. 2c) and concerned the short arms except for C chromosomes in 'Marco Polo'. For species analysed, polymorphism was only found for C and E chromosomes of *L. candidum* (Fig. 1a and 3c).

Predominately, C bands were located near primary and secondary constrictions but also in intercalary positions both, on the short and the long arms. Some C-bands were unrepeatable between cells, therefore only those seen in all metaphase plates were considered. This method provided markers for identification from 8 to 17 chromosomes. In genotypes analysed here the following numbers of C bands were observed (Fig. 1, 2 and 3b): 12 in *L.* x *formolongi* (6II) and 'Alma Ata' (5II + 2I), 13 in 'Marco Polo' (4II + 5I), 14 in *L. pumilum* (7II), 18 in *L. henryi* (9II), 22 in 'Muscadet' (10II + 2I), 23 in *L. candidum* (10II + 3I) and 'Expression' (9II + 5I). The polymorphism between homologous chromosomes was observed in the number and the size of bands in C, E and L chromosomes in *L. candidum* (Fig. 1a) whereas in cultivars it referred to all genotypes tested: 'Alma Ata' (A and C chromosomes - Fig. 2a), 'Expression' (A, B, C and G chromosomes - Fig. 2b), 'Marco Polo' (A, B, D and E chromosomes - Fig. 2c) and 'Muscadet' (E and J chromosomes - Fig. 2d).

DISCUSSION

According to Birader and Rayburn (1994, cit. after Bennett and Leitch 1995), flow cytometry enabled to reveal as small differences in DNA content between analysed forms as 2%. Van Tuyl and Boon (1997) recorded differences of diagnostic values between analysed *Lilium* species - between and within the sections with the biggest for *L. canadense* and *L. henryi* (34%). In the present work, although the four analysed species belong to different sections, they do not differ markedly in their relative DNA contents, except for *L. henryi*. The highest difference – 12.22 pg/2C was noticed between *L. candidum* and *L henryi*. Among cultivars only 'Expression' could be

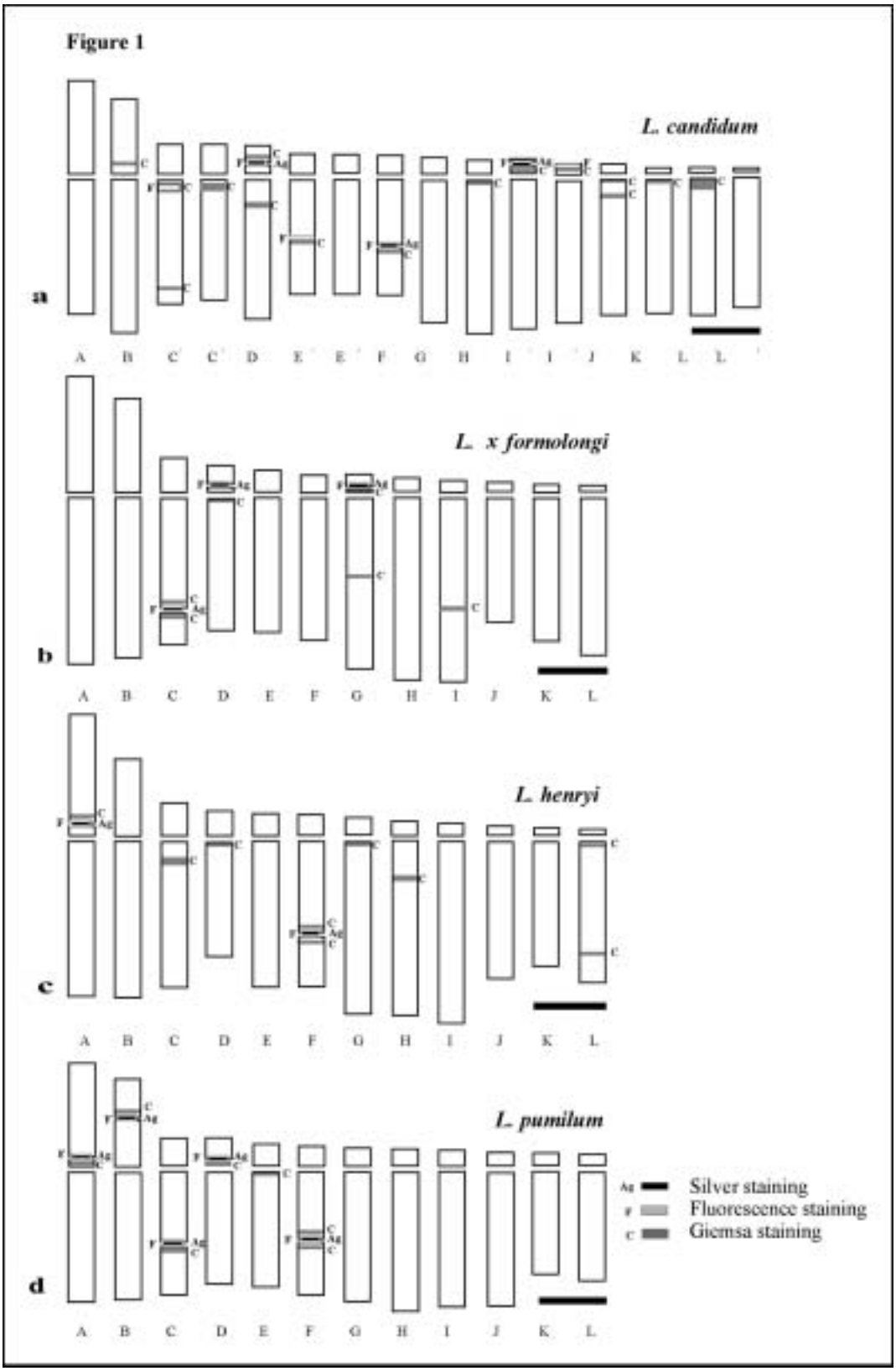

Fig. 1 — Idiograms of metaphase chromosomes of four *Lilium* species depicting the position of silver bands (Ag-black), C bands (C- dark grey) and fluorescence bands (F- pale grey). In each case, chromosomes are arranged in order of descending length of the short arm. Bars represent 5 μm.

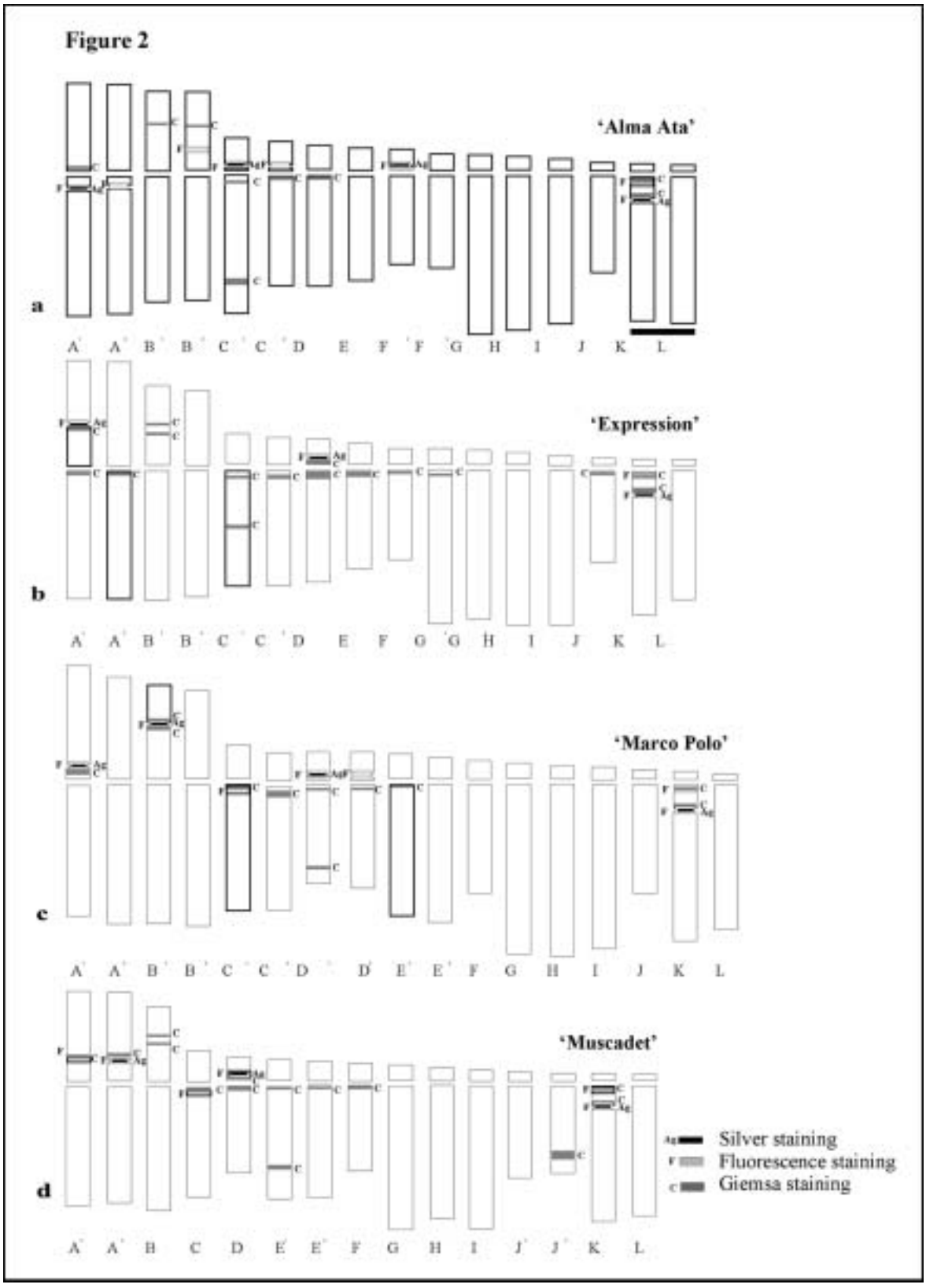

Fig. 2 — Idiograms of metaphase chromosomes of four lily cultivars belonging to Oriental hybrids depicting the position of silver bands (Ag- black), C bands (C- dark grey) and fluorescence bands (F- pale grey). In each case, chromosomes are arranged in order of descending length of the short arm. Bars represent 5 μm.

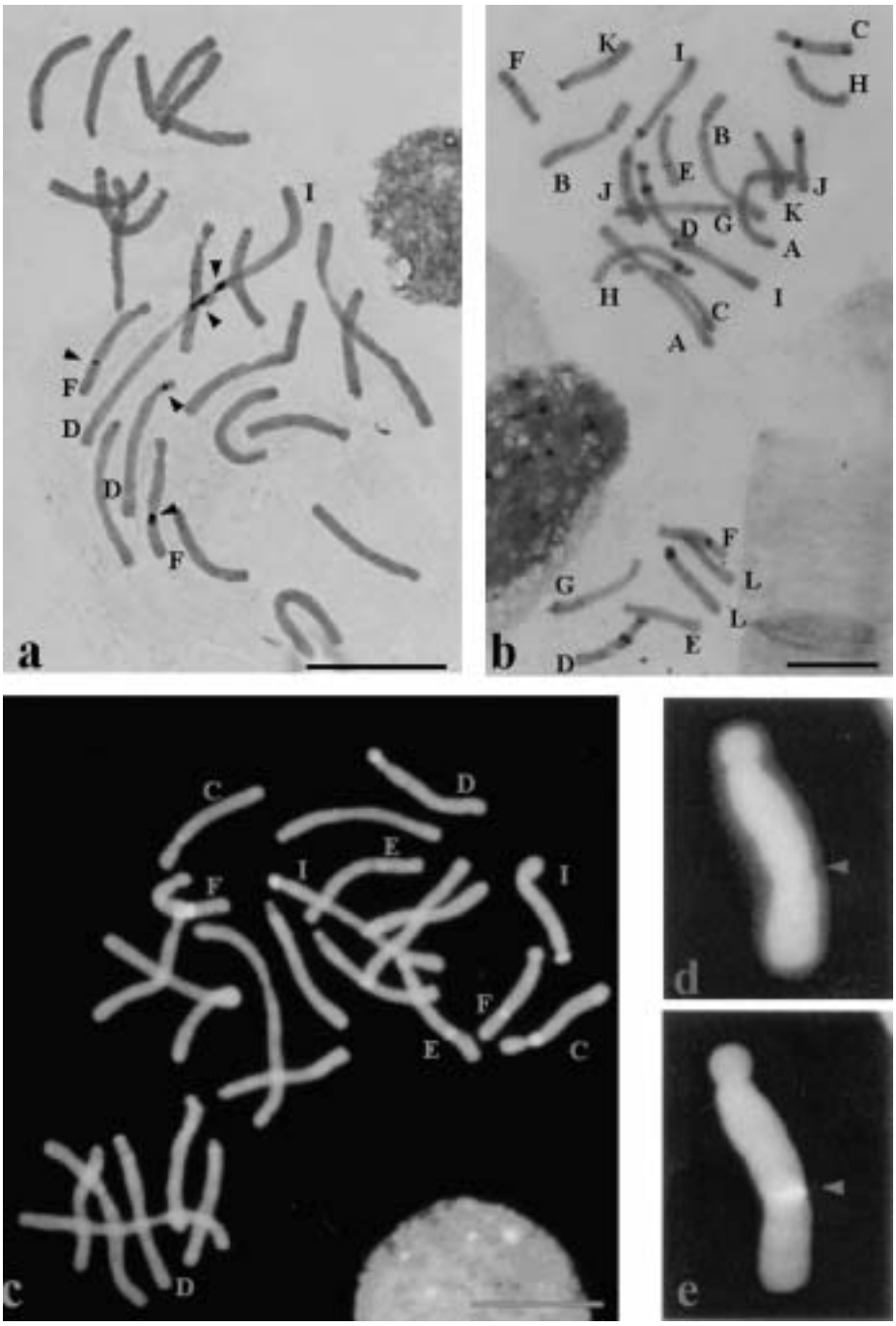

Fig. 3 — Mitotic chromosomes of *Lilium candidum* (a) silver staining – Ag bands at secondary constrictions are indicated by arrowheads, (b) Giemsa staining, (c) CMA_3 staining, (d) enlarged F chromosomes after DAPI staining – negative band marked by arrowhead, (e) enlarged F chromosomes after CMA_3 staining – positive band marked by arrowhead. Bars represent 10 μm.

recognized based on 2C DNA value. The pg value for *L. henryi* obtained in our study was much higher in comparison to that of VAN TUYL and BONE (1997), which could be the result of different dyes used – propidium iodide versus DAPI. The last one binds to specific DNA regions rich in A-T pairs, whereas propidium iodide stains the whole DNA. DOLEZEL *et al.*(1992) (cit. after BENNETT and LEITCH (1995) pointed at statistically significant differences in DNA contents between materials stained with propidium iodide and DAPI.

In *Lilium* visual identification of chromosomes on the base of theirs length, centromere position and the presence and position of secondary constrictions is not effective for majority of chromosomes (MARASEK and ORLIKOWSKA 2003). For example, chromosomes G, H, I are almost completely similar within and between genotypes. In the case of a high similarity in morphology of chromosomes, staining techniques creating bands on chromosomes are recommended (FRIEBE *et al*. 1996) to increase error-free chromosome classification.

Silver staining of nucleolar organizing regions turns out to be helpful for idiograms construction in genus *Lilium* as it reveals easily secondary constrictions, which is especially important when they are close to primary constrictions as in *L. henryi*, *L. pumilum*, 'Alma Ata' and 'Marco Polo' (MARASEK and ORLIKOWSKA 2003). The secondary constrictions on chromosomes D of 'Expression' and 'Marco Polo', revealed after silver staining, were not visible on chromosomes after Feulgen staining probably due to high chromosome condensation.

The number of chromosomes recognizable after a double fluorescence banding with CMA_3/DA/DAPI and silver staining were comparable in species. However, in cultivars and *L. candidum*, fluorescence staining provideded more markers. CMA_3 bands were present mostly in the positions of NORs or close to the centromere. The presence of CMA_3^+ bands in the position of NOR regions is due to the fact that ribosomal DNA is G-C rich (SCHWEIZER 1976; SCHWEIZER 1980) and this is why this staining can be used for quick localization of rDNA. The presence of CMA_3 bands in positions of NOR was recorded in diploid *Brassica* species (HASTEROK and MALUSZYNSKA 2000a; b) and somatic hybrids of *Solanum* (SREBNIAK *et al*. 2002). The number and the distribution of CMA_3^+/$DAPI^-$ and silver bands is generally the same as the localization of 25S rDNA signal revealed using FISH (MARASEK *et al*. 2004a; b). The differences in number of CMA_3 and $AgNO_3$ markers found in 'Alma Ata', 'Marco Polo', 'Muscadet' and *L. candidum* can suggest blocking of NORs activity that might have happened as the consequence of former hybridisation events (KEEP 1962). It was recorded in hybrids e.g. *Hordeum vulgare* x *H. bulbosum* (LANGE 1972) and in 6x and 8x *Triticale* (TARKOWSKI and STEFANOWSKA 1972).

In genus *Lilium*, Giemsa staining for C banding was used both for the study of species taxonomy (SMYTH 1991; SMYTH 1999) and hybrids verification (SMYTH and KONGSUWAN 1980). In our investigation, C banding has provided the most markers out of the three used methods for identification of individual chromosomes. Nevertheless, our attempts for standardisation of this method were not fully satisfying and results obtained not always repeatable. In genotypes analysed here C bands were found in different locations on chromosomes: close to primary and secondary constrictions or in pericentromerical positions. In some cases, the co-localizations or close localizations of C and CMA_3 bands were observed, e.g. in all cultivars on K chromosomes. This may be explained by the fact that both stainings enable to reveal the regions of chromosomes rich in heterochromatin and having highly repetitive DNA (FRIEBE *et al*. 1996). Our results are generally consistent with these of KONGSUWAN and SMYTH (1978) and SMYTH *et al*. (1989) regarding *L. henryi* and *L. candidum*. The minor differences in number of bands may be caused by the slight modification of the procedure - $Ba(OH)_2$ saturated versus 0.065M.

To sum up, each of the three investigated here chromosome banding methods is helpful in saturation of lily karyotypes with cytological markers enabling genotypes identification. The highest heteromorphism was revealed for C banding, but this method was the most troublesome for standardisation and reproducibility. The silver staining and the fluorescence staining CMA_3/DA/DAPI were equally easy, informative and reproducible, where the later one was used here for the first time for characterisation of lily karyotypes. However it should be underlined that none of the method used in this paper provides markers for identification of all chromosomes. Flow cytometric analysis of relative DNA contents did not enable to find statistically significant differences between analysed genotypes.

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