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Cytogenetic characterization of three *Hypericum* species by in situ hybridization

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Abstract The chromosomal positions of the 5S/25S rRNA genes of *Hypericum perforatum* (2n=32), *H. maculatum* (2n=16) and *H. attenuatum* (2n=32) were comparatively determined by FISH, and six, three and seven chromosome pairs of the respective karyotypes were subsequently distinguished. The rDNA loci between *H. perforatum* and *H. maculatum* seem to be identical (with respect to the ploidy difference), indicating that *H. perforatum* probably arose by autotetraploidization from an ancestor closely related to *H. maculatum*. The positional differences between the 5S rRNA gene loci of *H. perforatum* and *H. maculatum* on the one hand and *H. attenuatum* on the other argue against a previous hypothesis according to which *H. perforatum* originated from a remote interspecific hybridization between *H. maculatum* and *H. attenuatum*.

Key words *Hypericum* · FISH · 5S/25S rRNA genes · Karyotype evolution · Polyploidy

Introduction

Saint John's wort (*Hypericum perforatum* L.) is one of the medicinal crops being intensively studied during the last few years. The species produces pharmaceutically important metabolites such as anthraquinones (hypericin and its derivatives) and is used for its antiviral, antidepressive and anticancer activities (Takahashi et al. 1989; Hudson and Towers 1991; Lavie et al. 1995).

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H. perforatum belongs to the herbaceous section of the genus *Hypericum* with the basic chromosome number x=8 (Robson and Adams 1968). It is usually tetraploid (2n=4x=32) with small (0.78–1.52 µm) and morphologically similar chromosomes of which only two chromosome pairs (the largest ones) are distinguishable in uniformly stained preparations. However, both diploid and hexaploid chromosome numbers have also been occasionally reported (Robson and Adams 1968; Robson 1981). This might possibly be associated with facultative apomixis (apospory and parthenogenesis) as a mode of reproduction in *H. perforatum* (Noack 1939; Brutovská et al. 1998). A great variability has also been reported in a number of forms, varieties and subspecies of *H. perforatum* (Schwarz 1965). According to a hypothesis of Campbell and Delfosse (1984) *H. perforatum* is a product of an ancient hybridization between two diploids, *H. maculatum* subsp. *maculatum* Crantz and *H. attenuatum* Choisy (both 2n=2x=16), with subsequent chromosome doubling. The distribution of *H. attenuatum* in northeastern Asia (Gorshkova 1949; Yang 1981) overlaps that of *H. maculatum* in the Altai region of Central Asia. Thus, the putative hybridization might have occurred in Asia from where the hybrid might have expanded into the regions of its current native distribution.

Here we present comparative karyotype analyses for *H. perforatum*, *H. maculatum* and *H. attenuatum* by means of fluorescence *in situ* hybridization (FISH) experiments with 5S/25S rDNA probes. This allowed us to determine the number and chromosomal positions of the corresponding gene loci and to distinguish and compare the chromosomes carrying these loci between the related species. The similarity of nucleolus organizer region (NOR)- and 5S rRNA-bearing chromosomes indicated *H. perforatum* to originate from a common ancestor with *H. maculatum* via autopolyploidization due to facultative apomixis rather than from an interspecific hybridization event.

Materials and methods

Chromosome preparation

Root tips of *Hypericum perforatum* L. (Botanical garden of the Johannes Gutenberg University Mainz, Germany), *H. maculatum* Crantz. (Botanical garden of the University Salzburg, Austria) and *H. attenuatum* Choisy (Botanical garden of the University Sapporo, Japan) were treated for 3 h with 4 µ*M* amiprophosmethyl (APM) at room temperature, fixed for 16 h in ethanol:glacial acetic acid (3:1) and digested in 2.5% pectolyase (Sigma) and 2.5% cellulase (Onozuka R-10, Serva) for 20 min at 37°C. Single root tips were transferred into a drop of 45% acetic acid on a slide and gently squashed. The cover slips were removed after the slides were immersed in liquid nitrogen, and the preparations were dehydrated for 10 min in 96% ethanol. The air-dried slides were used immediately for FISH and genomic *in situ* hybridization (GISH), or were stored at 4°C in glycerol.

Isolation of genomic DNA and probe preparation

Total genomic DNA of *H. perforatum* (2n=32), *H. maculatum* (2n=16) and H. attenuatum (2n=32) was isolated from leaves of young plants grown on RM medium in vitro (Čellárová et al. 1992) according to method of Haberer et al. (1996) with modifications according to Halušková and Čellárová (1997).

Probes corresponding to genes encoding 5S and 25S rRNA were obtained by the polymerase chain reaction (PCR) from total genomic DNA. The amplification mixture contained 20 ng of genomic template DNA, 0.2 m*M* each of dATP, dCTP and dGTP, 0.15 m*M* dTTP, 0.05 m*M* digoxigenin-11-dUTP or 0.05 m*M* biotin-16-dUTP (Boehringer Mannheim), 0.2 µ*M* of each primer, 1× PCR buffer, 2 m*M* $MgCl_2$ and 1 U/50 µl *Taq* polymerase (Boehringer Mannheim). Primers were designed to yield products of 117 bp (5S rDNA) and 220 bp (25S rDNA). The primer sequences were as follows:

for 25S: JF09: 5′-GCG AGC GAA CCG GGA TAA GCC C-3′
JF10: 5′-CGG AAT TTA CCG CCC GAT TGG GG-3′
for 5S: UP46: 5′-GTG CGA TCA TAC CAG C(A/G)(G/T) TAA TGC ACC GG-3′ UP47: 5′-GAG GTG CAA CAC GAG GAC TTC CCA GGA GG-3′

In situ hybridization

FISH on metaphase chromosomes was performed according to Fuchs and Schubert (1995). Slides were incubated in RNase (50 µg/ml in 2× SSC) for 40 min at 37°C. After three 5-min washes in 2× SSC, proteinase K treatment (1 µg/ml in 20 m*M* TRIS-HCl, pH 7.5, 2 m*M* $CaCl_2$) for 30 min at 37°C was performed. For post-fixation the slides were incubated in 4% paraformaldehyde, 2×SSC for 10 min, then rinsed three times, each for 5 min, in 2× SSC, dehydrated in 70% and 96% ethanol and air-dried. The hybridization mixture contained probe DNA (20 ng/slide), 10% (w/v) sodium dextran sulphate, 50% (v/v) deionized formamide and 2× SSC. The chromosomes and the DNA probe were denatured at 80°C for 10 min and incubated overnight at 37°C. Three post-hybridization washes were carried out in 50% formamide, 2× SSC at 42°C, in 1× SSC, 0.2× SSC and 0.1× SSC at 60°C for 5 min each. Signals were detected by rhodamine-conjugated anti-digoxigenin (5S rDNA) and fluorescein isothiocyanate (FITC)-conjugated streptavidin (25S rDNA), both from Boehringer Mannheim. Amplification of signals was done by sequential applications of anti-tetramethyl-rhodamine and anti-rabbit-rhodamine or by biotinylated anti-streptavidine and FITC-streptavidine.

The chromosomes were counterstained with 4′,6′-diamino-2-phenylindole (DAPI, Molecular Probes). Images were captured for each fluorescent dye separately with a cooled CCD camera system (Photometrics) on a Zeiss Axioplan 2 fluorescence microscope, merged with Adobe Photoshop and printed on a thermo-sublimation printer (Sony).

Silver staining

Squash preparations from root tips fixed in ethanol:glacial acetic acid (3:1) were stained with silver according to the method of Lacadena et al. (1984). $AgNO_3$ (1 g) was dissolved in 1 ml sodium citrate buffer, pH 3. A 100-µl aliquot of the solution was poured onto squash preparation and left for 30 min at 55°C in the dark.

Results

Metaphase chromosomes of *H. perforatum* revealed four hybridization signals (two strong and two weaker ones) for 25S rDNA at terminal positions on two small chromosome pairs (Fig. 1b). The observation of up to four nucleoli in interphase nuclei after silver staining (Fig. 2) confirmed the presence of two active pairs of NORs in *H. perforatum*. FISH using a 5S rRNA gene-specific probe yielded 12 signals on eight chromosomes which did not carry 25S rDNA (Fig. 1a,b). The two largest metacentric chromosome pairs showed signals of different intensity on each chromosome arm in the subterminal position. Two small chromosome pairs revealed a signal again in subterminal position. Thus, six chromosome pairs of the tetraploid *H. perforatum* could be discriminated from the remaining ones, and each type of signal was found on two similar chromosome pairs.

For the closely related diploid species *H. maculatum*, one pair of small chromosomes with a signal for 25S rDNA at the terminal position was observed. Loci of 5S rRNA genes were found on each arm of the largest chromosome pair and additionally on a small chromosome pair, all in a subterminal position (Fig. 3).

The plantlets grown from *H. attenuatum* seeds proved to be tetraploid (2n=32). Up to three strong signals were found after FISH with the 25S rDNA probe at a terminal position of the small chromosomes (Fig. 4b). This indicates that probably two pairs of NOR-bearing chromosomes are present that are similar in shape to those of *H. perforatum* and *H. maculatum*. FISH with the 5S rDNA probe revealed up to 10 sites (Fig. 4a). One pair of the large metacentric chromosomes showed two loci (one on each arm), similar to *H. maculatum*. However, on the second large chromosome pair no 5S RNA genes were detectable. Instead, in addition to the subterminal locus on one small chromosome pair, two small chromosome pairs harbour 5S RNA genes in a nearly median position. Such a locus was not observed on *H. perforatum* and *H. maculatum* chromosomes. These data indicate that some rDNA positions are similar for all three species but that one 5S RNA gene locus of *H. attenuatum* is different from those of *H. perforatum* and *H. maculatum*. Because two of the 5S RNA gene-bearing chromosomes occur as only one pair, the chromosome constitution of the *H. attenuatum* accession tested seems to be allo- rather than autotetraploid.

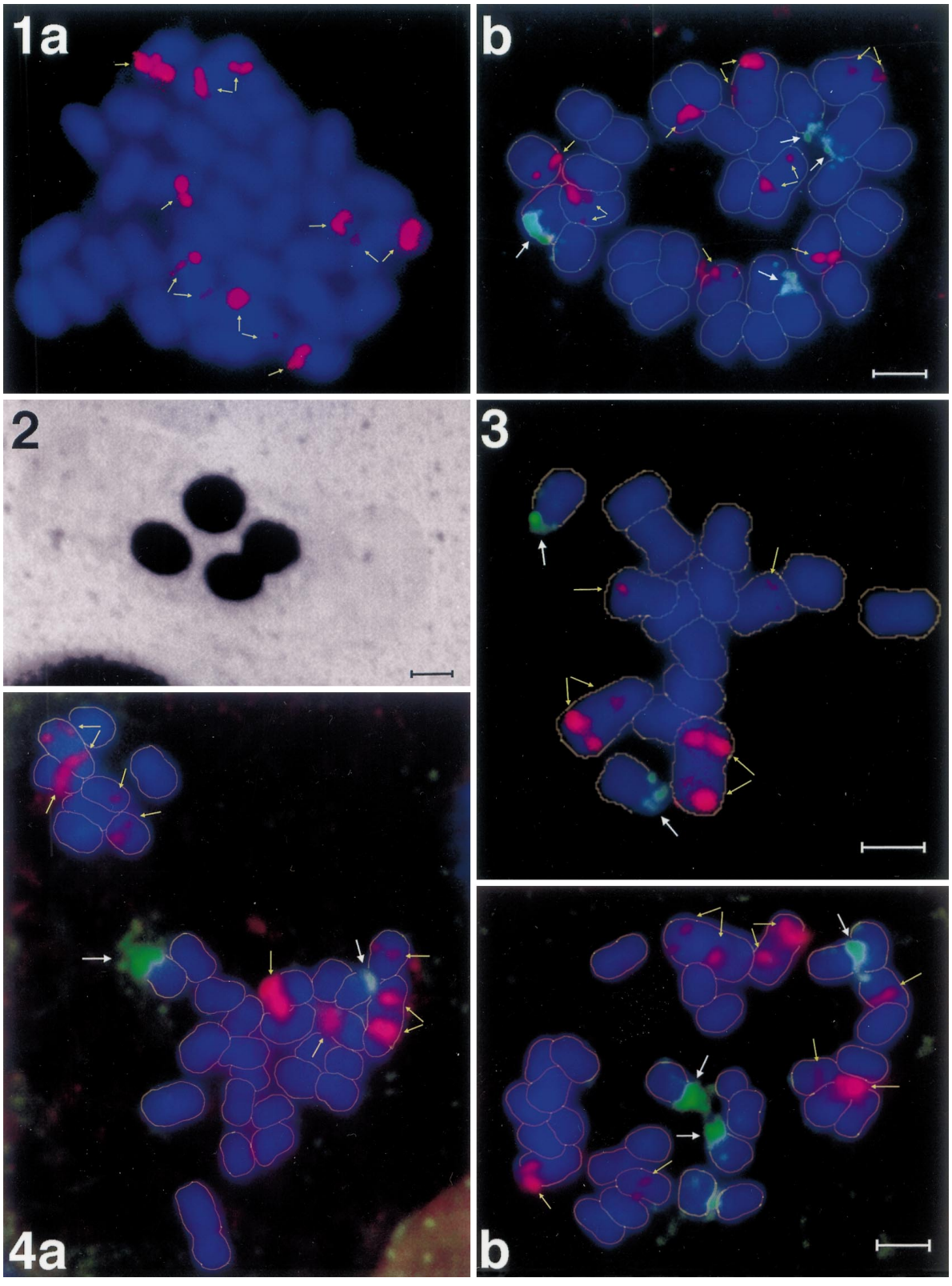

Fig. 1 Chromosomes of *H. perforatum* after FISH with 5S rDNA (**a**, **b** *small arrows*) and with 25S rDNA (*green*) (**b**). *Bar:*=1 µm

Fig. 2 Four nucleoli in an interphase nucleus of *H. perforatum* after silver staining. *Bar:*=1 µm

Fig. 3 Chromosomes of *H. maculatum* after FISH with 25S rDNA (*green*) and 5S rDNA (*red small arrows*). *Bar:*=1 µm

Fig. 4 Two metaphase cells of *H. attenuatum* (2n=32) after FISH with 25 S rDNA (*green*) and 5S rDNA (*red small arrows*). *Bar:*=1 µm

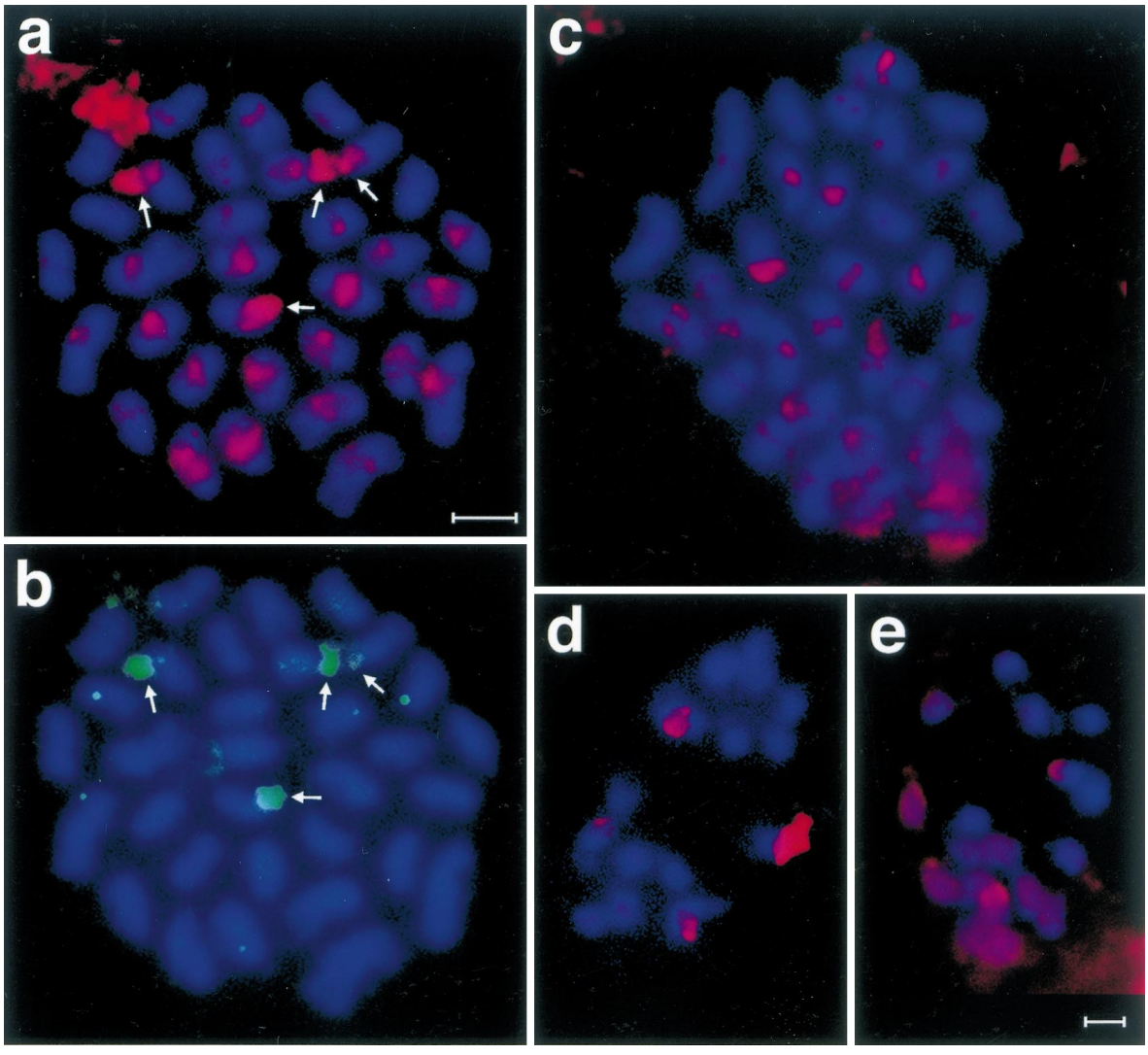

Fig. 5 GISH with rhodamine-labelled probes of *H. perforatum* and *H. maculatum*, respectively, in homologous and in cross-reaction. *Bar:*=1 µm. **a** Genomic DNA of *H. perforatum* on chromosomes of *H. perforatum*, **b** the same metaphase with four signals after reprobing with 25S rDNA (*green*), **c** genomic DNA of *H. maculatum* on chromosomes of *H. perforatum*, **d** genomic DNA of *H. maculatum* on chromosomes of *H. maculatum*, **e** genomic DNA of *H. perforatum* on chromosomes of *H. maculatum*

Discussion

FISH with 5S/25S rRNA gene-specific probes provided the necessary landmarks to discriminate six, three and seven chromosomes of *H. perforatum*, *H. maculatum* and *H. attenuatum*, respectively, which is impossible by conventional karyotype analysis (Brutovská et al. 1999). The observation of a doubled number of all loci in *H. perforatum* as compared to *H. maculatum* corresponds with the tetraploid nature of the former species.

The hypothesis of an allopolyploid origin of *H. perforatum* by a remote interspecific hybridization event between the supposed ancestral species *H. maculatum* and *H. attenuatum* could not be confirmed since the positions of 5S rRNA gene loci observed for *H. attenuatum* are not in favour of such an event involving *H. attenuatum*.

Reciprocal GISH with genomic DNAs from *H. perforatum* and *H. maculatum*, directly labelled by tetramethyl-rhodamine-6-dUTP, was not successful because both probes in homologous (Fig. 5a,d) as well as in cross-hybridization experiments (Fig. 5c,e) labelled only the pericentromeric heterochromatin and the NORs (Fig. 5b) independent of whether unlabelled DNA of the other species was added in excess or not (data not shown). Nevertheless, this result shows that even the repetitive sequences of both species are very similar and that those derived from *H. maculatum* do not distinguish between the chromosomes of *H. perforatum* (Fig. 5c).

This and the similarity in position and hybridization intensity of rRNA gene loci, which supports the idea of a close relationship between *H. perforatum* and *H. maculatum*, suggest that *H. perforatum* could have evolved from *H. maculatum* (or a common ancestor of both species) by autopolyploidization during the course of apomictic propagation. This interpretation seems most rea-

sonable since autopolyploidy was recently confirmed by meiotic chromosome pairing and restriction fragment length polymorphism analysis, respectively, for two other tetraploid and facultatively apomictic species, namely, the monocots *Paspalum rufum* (Quarín et al. 1998) and *P. simplex* (Pupilli et al. 1997).

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