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ON HETEROCHROMATIN IN KARYOSYSTEMATIC STUDIES

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The application of chromosome banding methods for plant karyosystematic studies is analyzed. The authors discuss ways of constructing C-band idiograms and interpretating the results of C-banding studies with respect to the polymorphism of heterochromatin and its histochemical differentiation. The role of quantitative changes of heterochromatin in evolution, its functional effect, and the division into dispensable and indispensable heterochromatin are presented. An overview of the recent literature on this subject is also given.

Key words: C-banding, genome structure, heterochromatin, karyotype analysis, plants.

INTRODUCTION

Banding chromosome studies have been carried out for almost thirty years (for plant chromosomes: CASPERSSON et al., 1969; VOSA and MARCHI, 1972; SCHWEIZER, 1973; MARKS, 1974), and their potential importance for karyotype analysis was noticed at the very beginning. In animal and human karyotype analysis this has resulted in the development of routine methods which are characterized by high reproducibility and resolution, mainly modification of G-banding and R-banding methods (COMINGS, 1978; SUMNER, 1982; JOACHIMIAK, 1983a). These relatively stable, species-specific band patterns can serve as the basis for elaboration of standard karyotypes; they are also useful material for comparative studies. A good example is found in studies on human and great apes karyotypes, which are the basis for interesting elaborations of karyotype evolution within the group Hominidae and of the relationships between species (SEUANEZ, 1979). G- and R- band patterns of some mammalian chromosome segments have been constant for several, even tens of millions of years; for example, the long arm of Chinese hamster chromosome 6 and mouse chromosome 2, containing the group of oncogenes *(SRC, ABL, AK1, ADA* and *TTPA),* possess identical G- band patterns (STALLINGS et al., 1985).

In plant karyotype analysis, the potential advantages of chromosome banding have not been fully utilized so far. Among the many reasons for this, the main ones are these: (1) the lack of routine, highly reproducible methods for proper preparation of the cell material before chromosome banding (e.g., quick methods of in vitro cell culture, cell cycle synchronization, and spreading cell suspension on microscope slides); (2) the small choice of highly reproducible banding methods suitable for routine use; and (3) difficulties in elaboration and interpretation of the results obtained in chromosome banding studies.

The first two reasons are associated with the specific character of plant material: - Because of the presence and cohesion of cell walls, proper preparation of the material for banding studies is difficult; it usually requires mechanical crumbling and squashing of relatively large pieces of meristematic tissue. Such

a procedure guarantees neither accumulation of division stages in the cell material nor sufficient exposure and spreading of chromosomes. A solution to this problem could involve digestion of cell walls and in vitro culture of cell protoplasts, controlled stimulation and synchronization of cell divisions in culture and dropping the cell suspension directly onto microscope slides. Unfortunately, plant protoplast culture is a rather complicated affair (BLACKHALL et al., 1994; DODDS and ROBERTS, 1996). The use of plant protoplast cultures to obtain material for routine chromosome banding studies is nowadays neither possible nor workable.

The degree of chromatin condensation in plant metaphase chromosomes is higher than in animals. This is why some of the most useful methods of karyotype analysis (G-banding and R-banding) cannot be used in plant studies (GREILHUBER, 1977). Among other methods only the following can be used on a larger scale: some fluorescent methods of staining A-T or G-C rich heterochromatin, and non-fluorescent Giemsa C-banding or NOR-staining (mainly silver staining) (JOACHIMIAK, 1983a). C-banding is relatively difficult to control and is not always reproducible: different C-banding procedures can lead to slightly different results (e.g., they differ in accuracy). Its other limitations with respect to plant karyotype analysis will be discussed later. Silver staining (Ag-NOR method) is also relatively difficult for routine application and it presents a number of significant drawbacks such as limited reproducibility, low signal-to-noise, etc. (MEYWALD et al., 1995). Moreover, it can identify only a small number of chromosomes which have active nucleolus organizers. This is why it will not be further discussed.

We can do little about the above difficulties related to the specific character of plant material. Although there have been some attempts to avoid the problems, such as dropping cell suspension on microscope slides instead of squashing the cells (GEBER and SCHWEIZER, 1988), so far they employ mainly previously fixed (thus already dead) plant tissues. Only exceptionally, hypotonic shock preceded by protoplast culture (MOURAS et al., 1978, in *Nicotiana)* or synchronization of cell divisions have been used to obtain chromosomes (MALMBERG and GRIESBACH, 1980, in *Nicotiana* and *Lycopersicon* protoplast cultures; WANG and PHILLIPS, 1984, in *Daucus* cell suspension culture).

CHROMOSOME IDENTIFICATION AND IDIOGRAM CONSTRUCTION

A much more serious but solvable problem is plant chromosome banding itself and proper construction of band idiograms for standard and comparative karyotype analysis. This problem requires a more thorough explanation. It is connected with the specific character of the stained sequences. As already mentioned, only routine methods which can be relatively widely applied for plant karyotype analysis have detected heterochromatin (fluorescent methods: staining with Hoechst, DAPI, Quinacrine, Chromomycine A_3; and mainly non-fluorescent methods: different C-banding procedures). However, this fraction of chromatin is often not very conservative, and it shows considerable intraspecific variability (JOACHIMIAK, 1983b); therefore the C-banding patterns obtained for different specimens of a given species can vary. Moreover, comparative karyotype studies of some plants have shown that heterochromatin can be a mobile element able to jump to different positions in the genome (LOIDL, 1983; GREILHUBER and LOIDL, 1983; JOACHIMIAK et al., 1987; SCHWEIZER and LOIDL, 1987; JOACHIMIAK, 1987). Because of that, it is more difficult to establish a standard karyotype of a species or line, to compare the data obtained by different researchers, and to draw conclusions about microevolution and speciation (so important in the majority of karyosystematic studies).

Another issue is identification of particular chromosome types in the material and construction of proper idiograms which would consider the general morphology of the chromosomes (chromosome length and centromere position) as well as localization of heterochromatin. C-banding leads to loss of a great part of the chromosome material and to significant changes in chromosome morphology. C-banded plant chromosomes differ from classically stained chromosomes in both length and thickness (JOACHIMIAK et al., 1994). When they do not possess clear heterochromatin segments in the centromere, locating this structure very often becomes difficult or even impossible. Many researchers cope with this problem by constructing the primary karyotype of a studied species (specimen) on the basis of classically stained preparations. Afterwards they transfer the heterochromatic bands obtained from C-banding

studies to classically obtained idiograms. Of course, such a procedure requires faultless identification of the classically determined chromosome types in the C-banded material. However, in many cases this is very difficult, so heterochromatin bands may be attributed to certain classical chromosome types in quite an arbitrary way. This calls into question the point of the whole operation. As detailed studies of sequentially stained plant chromosomes (first classically stained, then C-banded) show, alterations in chromosome arm length in C-banded material can cause totally mistaken identification of previously (classically) distinguished chromosome types (JOACHIMIAK et al., 1994). Moreover, in the case of only slightly differentiated chromosomes in a karyotype, it is very difficult to correctly distinguish the respective chromosome types. Some of these difficulties are related to insignificant differences in chromosome length, similarities in centromere position, and dynamic changes in chromosome morphology during the mitotic metaphase stage, attributed to differential condensation of the chromosome arms (KAKEDA and FUKUI, 1994). The best but also the most difficult way to avoid overlapping of the above mentioned mistakes is to establish karyotypes of metaphase plates sequentially stained, first classically and then C-banded (JOACHIMIAK et al., 1987, 1994; KRAWCZYK et al., 1988). If such a procedure is not possible, C-band karyotypes can be constructed directly from analysis of a sufficient number of C-banded metaphase plates obtained from different plant specimens. This can eliminate accidental changes in the length of the chromosome arms. Other difficulties in the construction of idiograms can be avoided if we adopt certain procedures based on more general assumptions derived from numerous studies on animal and plant heterochromatin. These studies have revealed the wide range of heterochromatin polymorphism

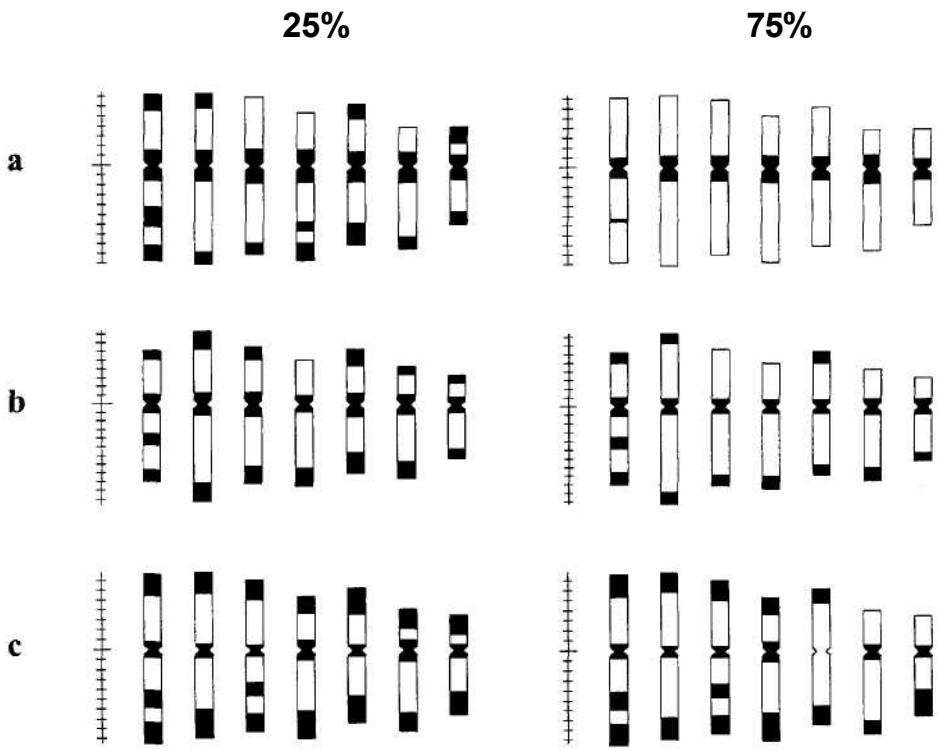

Fig. 1. C-band idiograms of three diploid (2n = 14) taxa of *Phleum:* (a) *P. "commutatum"* (informally named), (b) *P. alpinum* subsp. *rhaeticum* and (c) P. *nodosum.* Left column — majority of banding areas (occuring with at least 20% frequency in chromosome collection); right column — only banding areas occurring with higher frequency (at least 75% in chromosome collection). For chromosome measurements, the MultiScan ver. 4.01 (Computer Scanning Systems Ltd., Poland) and Mr Karyo ver. 3.10 (by Tokarski & Joachimiak) programs were used. Microscope images were transferred via videocamera to the Multiscan system. For calculations, statistical analysis and drawings, the Mr Karyo program was used.

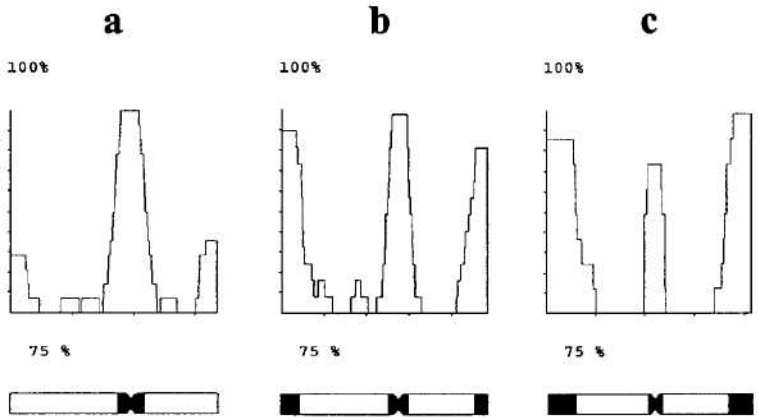

Fig. 2. Profiles of heterochromatin distribution in chromosome 2 of (a) *P. "commutatum",* (b) *P. alpinum* subsp. *rhaeticum* and (c) P. *nodosum* with schematic representation of chromosomes with frequently observed (75% of chromosome collection) heterochromatin distributions. Number of analyzed C-banded chromosomes: a — 70, b — 30, c — 30. Microscope images were transferred via videocamera to the MultiScan ver. 4.01 (Computer Scanning Systems Ltd., Poland). For chromosome measurements, calculations, statistical analysis and drawings, the Mr Karyo program was used (Mr Karyo ver. 3.10 by Tokarski & Joachimiak).

but have demonstrated that this fraction can be broken into two separate subtractions: fixed and polymorphic blocks of heterochromatin (JOHN and KING, 1985; JOHN et al., 1986; KRAWCZYK et al., 1988). Fixed blocks occur in the karyotype of a given species with relative constancy (the majority of chromosomes of a given type possess them); polymorphic blocks occur only as an accessory element located at different sites on chromosomes. Polymorphic blocks either appear or do not appear in the karyotype of a particular specimen, and their frequencies and amounts may vary both within and between populations.

Thus it appears that only the relatively constant fixed blocks of heterochromatin should be considered in constructing the standard karyotype of a given species. Failure to distinguish these two heterochromatin types can lead to misinterpretation and make it difficult or impossible to draw conclusions from comparison of C-banded karyotypes of different forms and species. To illustrate the problem, let us use the example of a group of closely related *Phleum* species belonging to the section *Phleum* Griseb. and examined with the C-banding method (JOACHIMIAK and KULA, 1993,1996). Classically stained genomes of these species merely show only accidental differences. However, advanced banding analysis enables them to be differentiated.

All the diploid *Phleum* species we analyzed show relatively high intraspecific polymorphism of heterochromatin. C-banded karyotypes of plants belonging to these separate taxa could show significant similarities if the fixed and polymorphic blocks of heterochromatin are not distinguished. However, if only the relatively stable heterochromatin segments are considered, differences in the structure of the karyotypes become clear (Fig. 1).

The range of intraspecific heterochromatin polymorphism can be determined only in the course of statistical analysis of numerous karyograms. Each of the analyzed chromosome types within a genome has its specific banding profile characterized by an unequivocal arrangement of banded areas. Some examples of such profiles are presented in Figure 2. Because polymorphic bands, unlike fixed bands, constitute an additional element (occurring/non-occurring in the karyotype of particular specimens within a population/species), they can be omitted in the construction of basic idiograms. This allows interspecies differences in the structure of C-banded karyotypes to be emphasized, if they are present.

INTERPRETATION OF THE RESULTS OF CHROMOSOME BANDING

The above presented procedure allows for better discernment of the structure of karyotypes

of particular forms; it should not, however, influence the interpretation of quantitative data concerning the evolutionary role of heterochromatin. In particular, it should not be concluded that polymorphic blocks of heterochromatin constitute an unnecessary surplus of no functional importance, nor that their persistence in the genomes of some plants is completely accidental. In terms of quantity, the heterochromatin which makes up fixed blocks should not be identified with the **minimum amount** required to ensure survival. In the majority of specimens it is accompanied by some amount of heterochromatin sequences differently localized in the genome (occurring as an addition to fixed blocks or forming separate polymorphic blocks of heterochromatin). This amount can vary and its actual value depends to some degree upon environmental conditions.

For a particular population/species there is probably a certain **optimum amount** of heterochromatin (Fig. 3) providing capacities for survival not only in given conditions but also in case of environmental changes (i.e., making adaptation to new conditions possible). The majority of the breakpoints involved in chromosome evolution, for example in translocations and deletions, occur in the heterochromatin, so the amount and location of heterochromatin can determine the chance for fast rearrangement of the karyotype structure. The significant contribution of heterochromatin in chromosome rearrangements under stress conditions (e.g., in cell and tissue cultures in vitro) has been experimentally confirmed many times (SACRISTAN, 1971; MCCOY et al.,

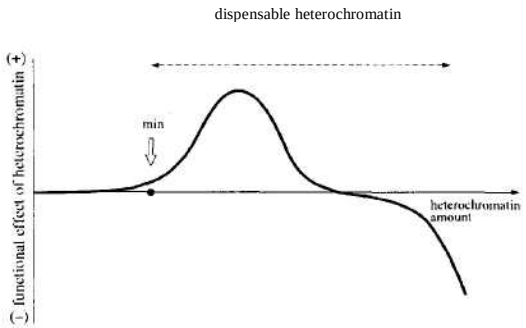

Fig. 3. Functional effects obtained by adding constitutive heterochromatin to a heterochromatin-deprived cell (not viable with zero heterochromatin). min — minimum amount of heterochromatin required for function, (+)/(-) — positive/negative effect on function (according to Fig. 1 in ZUCKERKANDL AND HENNIG, 1995, slightly modified).

1982; LAPITAN et al., 1984; JOHNSON et al., 1987a,b; LEE and PHILIPS, 1988; JOACHIMIAK et al., 1995).

Heterochromatin does not usually contain coding genes, although it may possess mobile 18/25S rDNA sequences (SCHUBERT, 1984; SCHUBERT and WOBUS, 1985; RICROCH et al., 1992; PICH et al., 1996), and its role in the genome has not been precisely identified yet. There is, however, satisfactory evidence proving its direct or indirect influence on a number of important functions of an organism, such as reproduction, regulation of gene expression (position-effect variegation), regulation of crossovers, etc. (JOACHIMIAK, 1983b, and more recent data and hypotheses: PARDUE and HENNIG, 1990; PALUMBO et al., 1994; IRICK, 1994; LOHE and HILLIKER, 1995; ZUCKERKANDL and HENNIG, 1995). It seems that a certain determined amount of heterochromatic sequences has to accompany the centromeres so that chromosomes can undergo proper segregation during mitosis. Too little of this type of heterochromatin can cause premature separation of chromatids, and too much can cause disturbances in their regular movement towards the poles. Beyond these limits malsegregation will occur, leading to aneuploidy (SUMNER, 1991). As a result, chromosomes with too little or too much centromere-associated heterochromatin are eliminated from the chromosome pool of a given species. In some plant species the minimal amount of telomeric heterochromatin may be regulated in a similar way. In Alliaceae the original plants' telomeric repeats (TTTAGGG) became lost during evolution and were replaced by highly repetitive DNA sequences located at the ends of the chromosome arms (FUCHS et al., 1995; PICH et al., 1996). Highly repetitive telomeric heterochromatin segments in *Allium* and related species fulfill telomeric functions in protecting the chromosome termini from degradation and progressive shortening (elimination of chromosomal material from non-protected chromosome ends is predicted from the properties of DNA replication, for review: HOLMQUIST and DANCIS, 1980; BLACKBURN, 1990, 1991). Thus, at least in Alliaceae a minimal amount of telomeric heterochromatin is necessary for stabilization of each chromosome end. These facts prove that the amount of heterochromatin in the karyotype can be,

from the evolutional point of view, an important feature of the nuclear phenotype.

The range of fluctuation in the amount of heterochromatin can differ between species or populations as a result of the partial elimination or accumulation of heterochromatin devoted to fixed positions (PILCH, 1981; GUSTAFSON et al., 1983) and divergent contributions of polymorphic bands in the karyotypes (GREILHUBER and SPETA, 1976; LOIDL, 1979; GREILHUBER, 1982; JOACHIMIAK et al., 1987; KRAWCZYK et al., 1988). This part of the heterochromatin, which is not always (not in all conditions) necessary for survival yet is important for microevolution and speciation, has been described as **dispensable heterochromatin** (ZUCKERKANDL and HENNIG, 1995). In karyologically studied taxa of the *Phleum* section (JOACHIMIAK and KULA, 1993,1996), species-specific ranges of amounts of dispensable heterochromatin in the karyotype can overlap. Thus, for example, heterochromatin-rich karyotypes of some *P. commutatum* specimens are in this respect close to the heterochromatin-poor karyotypes of *P. alpinum* ssp. *rhaeticum.*

Distinguishing between fixed and polymorphic bands in the karyotype of a given species requires examination of a number of specimens. Although this is very difficult in C-banding studies, it seems necessary in karyotype analysis. Examination and comparison of single-specimen karyotypes can yield different results among different researchers. Such an analysis, which does not take into account the variability of heterochromatin, can lead to mistaken conclusions about the origin and affinity of the studied species.

CYTOCHEMICAL DIFFERENTIATION OF HETEROCHROMATIN

Molecular studies on heterochromatin, carried out for many years, have shown that it is not homogeneous in structure. One of the more important aspects studied in karyotype analysis is the differentiation of sequences of certain base pairs in the DNA. Therefore, a detailed study of the molecular composition of positively C-banded chromosome segments has become an important part of cytogenetic and karyological research. Differentiation of the two basic fractions of heterochromatin, AT-rich and GC-rich, is possible thanks to fluorescent chromosome staining methods, which are more and more frequently used. The specific character of fluorescent staining is based on the affinity of fluorochromes with certain base pairs (COMINGS, 1978; SUMNER, 1982). Fluorescent stains such as Hoechst 33258, quinacrine derivatives, DAPI and its derivatives (AT-specific), chromomycin A_3, mithramycin and olivomycin (GC-specific) bind to the DNA by intercalating between base pairs or through ionic interactions with phosphate groups, or by means of external interactions with the DNA double helix (MULLER and GAUTIER, 1975; COMINGS et al., 1978; SCHWEIZER, 1979; LATT et al., 1980; AHRBERG and SCHWEIZER, 1982; SCHWEIZER et al., 1987). Generally, however, fluorochrome binding with chromatin is reversible, which enables several fluorescent stains to be used in succession with the same material, or fluorescent and nonfluorescent (e.g., C-banding) staining can be combined (sequential staining). The intensity of fluorescence of certain chromosome regions depends not only on their nucleotide sequence but also on the degree of DNA repetitiveness and other unidentified factors. The composition and repetitiveness of base pairs determine various properties of chromatin, such as denaturation and reassociation in different conditions. This feature can be used in studies in which acridine orange (AO), a fluorochrome which stains single- and double-strand nucleic acids differently, is used. After staining with AO, double-stranded DNA becomes green, and single-stranded (denaturated) DNA becomes red. After prior denaturation, highly repetitive heterochromatin regions will reassociate faster (green fluorescence with AO) than the rest of the chromatin (red fluorescence). This enables quick detection of heterochromatin regions in chromosomes and interphase nuclei (STOCKERT and LISANTI, 1972; SATO, 1988; GRABOWSKA and JOACHIMIAK, 1993). To increase the specificity of staining and to intensify the band patterns' fluorescence, so-called fluorescent counterstaining is advisable. It consists in using fluorochromes and nonfluorescent compounds, such as AT-specific distamycin A and GC-specific actinomycin D in double or triple staining, making it easier to contrast the chromosome segments with bright and quenched fluorescence (SCHWEIZER, 1976a, 1976b, 1979, 1981; SCHWARZACHER and SCHWEIZER, 1982).

Application of fluorochromes makes it possible to establish the nucleotide composition of genome elements characterized by large variability — NORs, NOR-associated heterochromatin, satellites, as well as (quite rare in plants) cold-sensitive regions (CSRs) (MOSCONE et al., 1996; BERG and GREILHUBER, 1993). Additionally, fluorescent methods are highly sensitive and do not require as much preparation as C-banding.

Fluorescent methods for differential staining of chromosomes allow us to indicate differences in the arrangement of certain kinds of heterochromatin in chromosomes of particular species within a genus, and often also intraspecifically. Therefore they facilitate tracing of trends in karyotype differentiation within and between taxa and elucidation of the evolutionary relationships between species. Their use is especially helpful in identifying particular chromosome types and thereby distinguishing between groups of homologues. They are essential in the case of slightly differentiated karyotypes and are an aid in comparative karyosystematic studies. The possibility of precisely locating heterochromatin regions of a certain type plays an important role in karyotype analysis and idiogram construction.

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