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The evolution of the ribosomal loci in the subgenus *Leopoldia* of the genus *Muscari* (Hyacinthaceae)

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Abstract. In the subgenus *Leopoldia* of the genus *Muscari*, *M. comosum* is an exceptional species because it presents the most asymmetrical karyotype of the group and because its only active NOR is located in the fifth chromosome pair, while in the other species it is located in the first or second chromosome pairs (all the species have 2n = 18 chromosomes). Since *M. comosum* has a derived karyotype different from those of the other species of the group, the resulting question is whether, in the first and second chromosome pair of this species, ribosomal cistrons persist. Observations after fluorescence in situ hybridization (FISH) using rDNA probes indicate that there are indeed ribosomal loci in the first and second chromosome pairs of this species, although these loci are inactive with respect to nucleolus organization. The location of rDNA regions in another three species of the same genus (*M. atlanticum*, *M. dionysicum* and *M. matritensis*) provides a basis for examining the significance of these findings in relation to the evolution of the ribosomal loci in this genus. Our observations indicate that in the genus *Muscari*, the largest sites for rRNA genes are not necessarily active, and, therefore, the activation of these regions is not related to the number of copies but to a specific regulation mechanism.

Key words: In situ hybridization, evolution, NOR, rDNA, *Muscari*.

Introduction

Among the widely analyzed regions of the eukaryotic genome are the nucleolar organizing regions (NORs). For this there may be mainly two reasons. First, these regions are the sites of the key genes for cell metabolism because they contain the 18S, 5.8S and 2.5S ribosomal cistrons (McClintock 1934, Ritossa and Spiegelman 1965). Second, the NORs can be stained by means of specific methods such as N-banding and, especially, Ag-staining (Matsui and Sasaki 1973, Goodpasture and Bloom 1975). However, the broad interest in this region may also be due to a third reason: the NORs are highly variable regions of the genome. In fact, this variability, which affects the number and size of the NORs, occurs form the cellular level in one individual (Díez and Puertas 1986) to different species in a taxonomic group (Stebbins 1971).

However, most studies attempting to determine the variability of the NORs present the problem that the above-mentioned methods make it possible to determine only the active ribosomal loci (Howell 1982). Therefore, when the NORs are analyzed with an evolutionary approach (that is, comparing the

NORs of a group of species) the general conclusion after Ag-staining is that there does not seem to be any particular consistency among species in the numbers or even the positions of the NORs (Stebbins 1971, White 1973). Nevertheless, it is possible that some inactive NORs, remnant of chromosomal changes involved in the evolution of the species, or even minor loci, are still present in the karyotypes. Thus, such techniques (Ag-staining, N-banding) are inadequate to resolve the problem of the number and position of the ribosomal loci present in a species, irrespective of their activated or inactivated status. Therefore, it is necessary to use molecular techniques such as fluorescence in situ hybridization (FISH) with ribosomal probes (Fukui et al. 1994).

Here, we analyze the ribosomal loci present in the monocotyledoneous plant *Muscari comosum* (L.) Mill. = *Leopoldia comosa* (L.) Parl. (Hyacinthaceae, order Asparagales), and compare them with the loci present in two species of the subgenus *Leopoldia* (Parl.) Zahar. of the genus *Muscari* Mill. (*M. matritensis* C. Ruiz Rejón et al. and *M. dionysicum* Rech. fil.) as well as with an outgroup species (*M. atlanticum* Boiss. et Reuter of the subgenus *Muscari*). The subgenus *Leopoldia* contains 6–7 species in Europe (Bentzer 1973, Tutin et al. 1980), all of which have the same chromosome number (2n = 18) and normally have one active NOR. However, while in all the species of the group the NORs are usually located in the first or second chromosome pairs (Bentzer 1974), in *M. comosum* the NOR is located in the fifth chromosome (in this last case this observation is confirmed by means of Ag-staining; see Ruiz Rejón C. et al. 1990a). Thus, we have investigated the ribosomal loci (regardless of its active/inactive status) present in the four above-mentioned species in order to ascertain whether these genes exist in the first and second chromosome pairs of *M. comosum*, as they do in the other species of this subgenus and even in other subgenera as *Muscari*.

Materials and methods

The material analyzed included four species, five plants each, belonging to the genus *Muscari*: *M. comosum*, *M. matritensis*, *M. dionysicum* and *M. atlanticum*. For mitotic analyses, roots from bulbs were pretreated in 0.15% colchicine aqueous solution at room temperature for 4 h, fixed in 1:3 acetic acid:ethanol and stored at 4 °C until used. Anthers from young flowers were fixed without pretreatment in 1:3 acetic acid:ethanol. The fixed material was squashed in a drop of 45% acetic acid and coverslips removed at −80 °C. Silver staining of the NORs and nucleoli was performed according to Lacadena et al. (1984) with minor modifications (33–50% silver nitrate for 30–60 min at 60 °C). Preparation of synaptonemal complex spreads was prepared following the method described by Cuñado et al. (1996) with minor modifications. Fresh unfixed anthers were squashed onto a cavity slide with a drop of digestion medium (0.05 g snail gut enzyme "Cytohelicase", Sigma, 0.125 g polyvinyl pyrrolidone, and 0.19 g sucrose in 12.5 ml sterile distilled water). For silver impregnation, a few drops of 33% silver-nitrate solution were placed on the slides, which were then covered with a patch of nylon cloth in a moisture chamber at 30–40 °C until they turned a yellow color. Nuclei were examined using a Jeol 1200EX electron microscope and photographed on Agfa-Scientia film.

For fluorescence in situ hybridization (FISH), a rDNA probe, pTa71 from wheat, containing the 18S, 5.8S, and 2.5S genes and the intergenic spacer (Gerlach and Bedbrook 1979), kindly provided by A. Cuadrado, was used. This probe was labeled with digoxygenin-11-UTP (Boehringer Mannheim) by nick translation according to the manufacturer's instructions. FISH methodology was that indicated by Cuñado and Santos (1998) with minor modifications. Chromosome preparations were pretreated with DNase-free RNase (100 μg/ml) and pepsin (500 μg/ml), dehydrated in an ethanol series, and air-dried. The hybridization mixture, consisting of 50 ng of DNA probe and 500 ng of sheared salmon sperm DNA in 50% (v/v) formamide, 10% (w/v) dextran sulphate and 2× SSC (SSC is 50 mM NaCl, 15 mM sodium citrate), was denatured on a heating block at 90 °C for 10 min and placed on ice for 5 min. The probe was applied to the slides (15 μl/slide), covered with a coverslip and sealed. The slides were incubated at 75 °C for 4 min and then at 37 °C overnight in a modified thermocycler.

Labeled probe was detected with 5 μg/ml monoclonal antidigoxygenin (Sigma) and 10 μg/ml antimouse Ig fluorescein (Boehringer Mannheim). Preparations were counterstained with propidium iodide (1 μg/ml) and DAPI, 4′6-diamidino-2-phenylindol, (1 μg/ml) and mounted with Vectashield (Vector Laboratories).

Results

Muscari comosum. The karyotype of *M. comosum* (2n = 18) consists of one large subtelocentric chromosome (1), one submetacentric chromosome of medium size (2) and seven small metacentric chromosomes (3–9). Several $DAPI^+$ C-bands are interstitially located in the long arm of the first pair, appearing as a large band when the chromosome is condensed, and in the centromere of all chromosomes. A large C-band, CMA_3 (Chromomycin A_3) positive, also exists in the fifth pair, which is associated with the NOR as revealed after Ag-staining. Additional thin CMA_3 positive C-bands, lacking active ribosomal genes, exist in the telomeric regions of the long arms of the first and second chromosomes (for details of these aspects see also Ruiz Rejón C. et al. 1990a).

In situ hybridization of a labeled rDNA probe to mitotic and meiotic cells of *M. comosum* revealed not only the rDNA locus in the fifth chromosome, but also new sites which coincide with the CMA_3^+ bands in the long arms of chromosome 1 (two loci, one smaller than the other) and of chromosome 2 (one locus) (Fig. 1a). However, the decon-

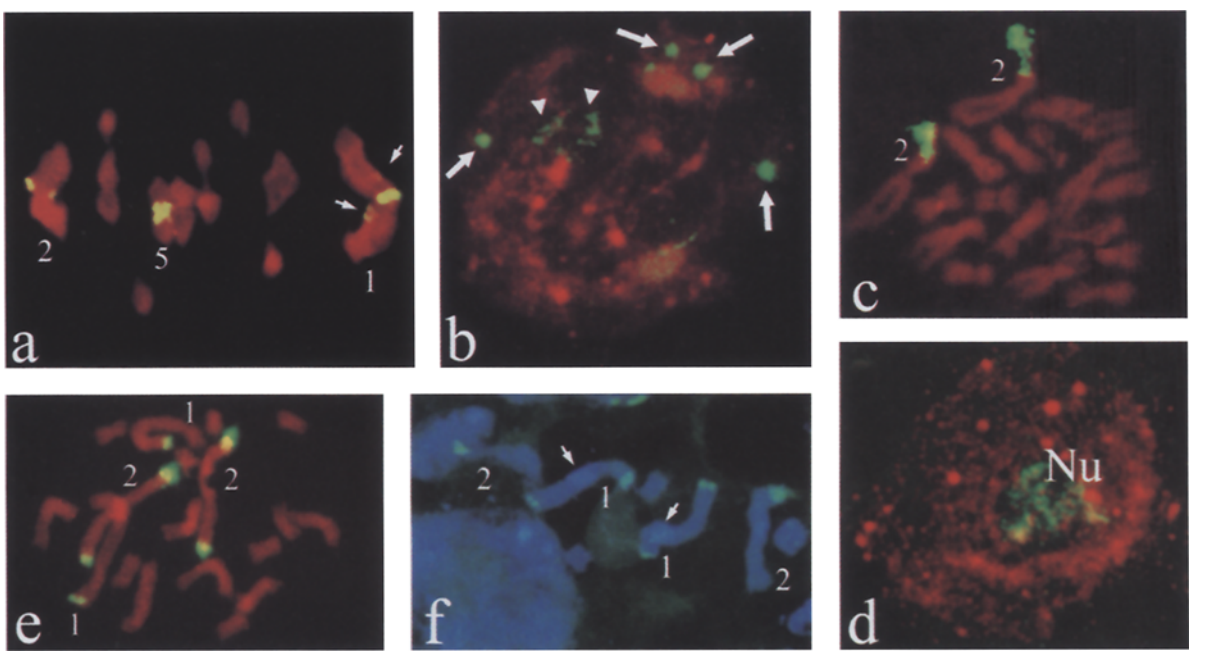


Fig. 1. Different nuclei of the *Muscari* species analyzed after in situ hybridization with a rDNA probe. (**a–b**) *M. comosum.* (**a**) Location of rDNA sequences (yellow-green) in metaphase I chromosomes. Orange-red fluorescence shows DNA counterstained with propidium iodide. Numbers indicate those bivalents carrying these sequences. Arrows indicate a minor rDNA locus. (**b**) Interphase somatic nucleus showing six major sites of rDNA hybridization (green). Four of them are condensed (arrows) and two are decondensed (arrowheads). (**c–d**) *M. atlanticum.* (**c**) Somatic metaphase showing two major sites of rDNA hybridization located in the short arms of chromosome pair 2. (**d**) Interphase somatic nucleus in which the decondensed rDNA (green) is inside the nucleolus. (**e**) Somatic metaphase chromosomes of *M. matritensis* in which the chromosomes carrying rDNA sequences are numbered. (**f**) Partial view of a somatic metaphase of *M. dionysicum.* In this case the chromosomes have been counterstained with DAPI, rDNA loci are in green. Arrows indicate a DAPI positive region located in the long arm of chromosome pair 1

densed appearance shown in interphase nuclei by two of the eight rDNA hybridization sites may be considered as an indication of transcriptional activity in only one locus (Fig. 1b). Indeed, the three new ribosomal loci detected here, in contrast to the rDNA locus in the fifth pair, were not found to be associated with the nucleolus, either in mitotic or in prophase I meiotic nuclei (Fig. 2). Therefore, none of them apparently display nucleolar organizing activity.

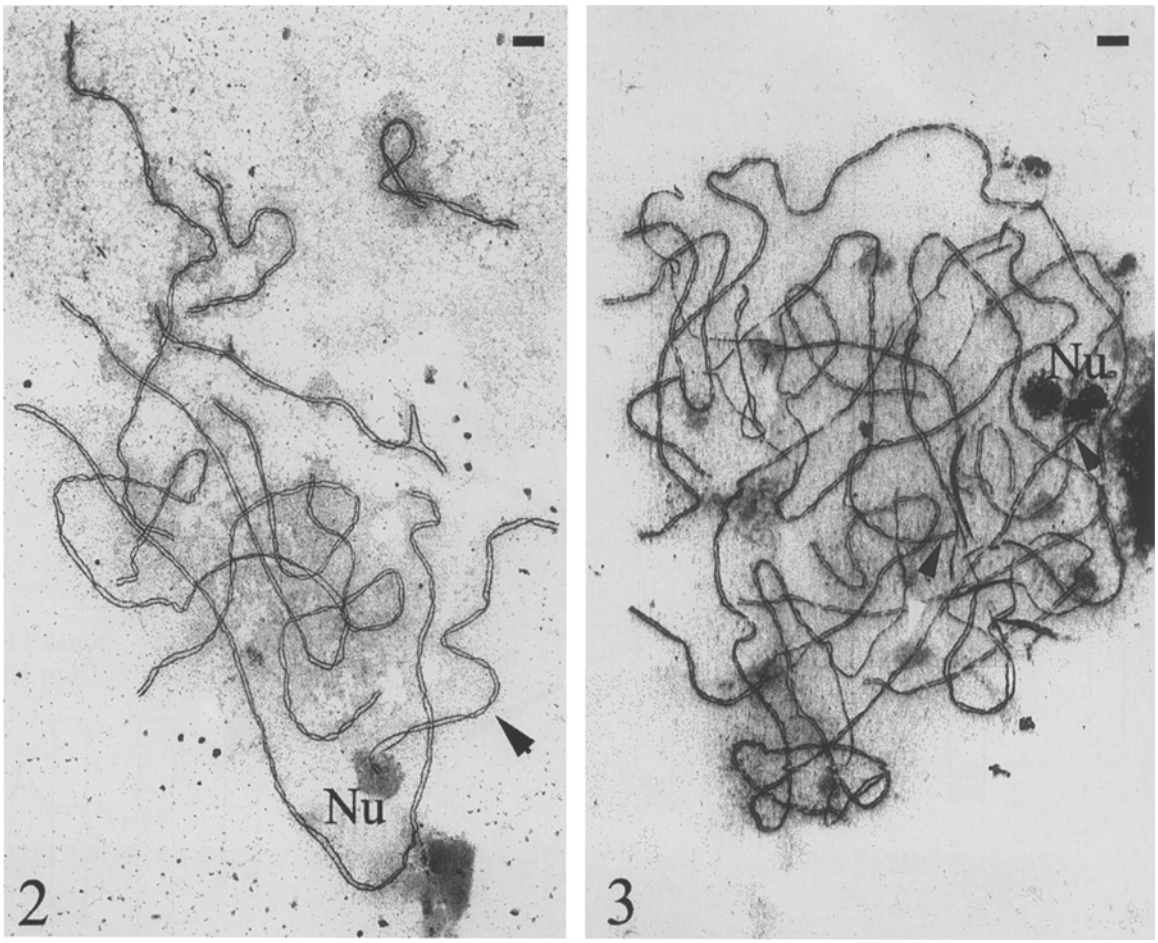
The remaining species. The three species here analyzed (*M. dionysicum, M. matritensis and M. atlanticum*) also have (2n = 18) chromosomes. *M. atlanticum* belongs to the subgenus *Muscari* with a symmetrical karyotype, in which chromosomes do not present detectable heterochromatic bands and the satellite is located in the short arm of the second chromosome pair. *M. matritensis* and *M. dionysicum* are included in the subgenus *Leopoldia* and have more asymmetrical karyotypes with some thin heterochomatic CMA_3^+ bands located in the chromosomes 1 and 2, and a considerable $DAPI^+$ band interstitially located in the long arm of the first chromosome pair in the case of *M. dionysicum*. The satellite appears in the short arm of the second chromosome in *M. matritensis* and in the short arm of the first pair in *M. dionysicum*.

After FISH, in agreement with observations from Ag-staining, a single rDNA locus located in the short arm of the chromosome 2, was detected in *M. atlanticum* (Fig. 1c). This rDNA appeared decondensed and associated with the nucleolus in interphase somatic nuclei (Fig. 1d). In contrast, there were three rDNA loci in *M. matritensis* (Fig. 1e) and *M. dionysicum* (Fig. 1f). Ag-staining of somatic cells revealed that only one of these is apparently active in both species, being located in the short arm of the second pair in *M. matritensis* (Ruiz-Rejón C. et al. 1990a) and in the short arm of the first pair in *M. dionysicum*. Furthermore, the nucleolus was also found to be associated with these same chromosome arms at pachytene (see Fig. 3).

Discussion

Bearing in mind that the ribosomal loci (independently of their active/inactive status) are frequently associated with GC-rich heterochromatic bands (Amemiya and Gold 1988), the presence of CMA_3^+ bands in the long arms of the first and second pairs of *M. comosum* (Ruiz Rejón C. et al. 1990a) could indicate the presence of ribosomal loci in these chromosomes. Here, we confirm this hypothesis by means of FISH. In fact, in the terminal region of the long arm of the first chromosome pair of this species, there are two ribosomal loci, while in the same region of the second chromosome there is only one locus (Fig. 1a). However, these ribosomal loci are apparently inactive, given that the bivalents carrying them were not found to be associated with the nucleolus in meiotic cells (Fig. 2). We also report that other species of the subgenus *Leopoldia* such as *M. matritensis* and *M. dionysicum* have inactive rDNA loci at the same positions as in *M. comosum*.

If we consider the observations on the active ribosomal loci of the species of the subgenus *Leopoldia*, and the presence of only one locus but located in the first, second or fifth chromosome pairs in *M. dionysicum*, *M. matritensis* and *M. comosum*, respectively, we can initially speculate that the position of active NOR changes at random during the evolution of *Muscari* species. However, the comparison of the karyotypes and of the position of all the rDNA loci in the species of the subgenus *Leopoldia* and in an outgroup species such as *M. atlanticum* belonging to the subgenus *Muscari* supports a different hypothesis (Fig. 4). The evolutionary process could begin with species with symmetrical karyotypes, such as *M. atlanticum*, in which there is only one active locus located in the short arm of chromosome 2. The following step could be a species with more asymmetrical karyotypes as *M. matritensis*, in which: (a) a small DAPI+ heterochromatic C-band appears in the second chromosome, and (b) the active NOR persists in the second pair but two



Figs. 2–3. Electron micrographs of silver-stained pachytene nuclei of two species of *Muscari*. *Nu* = nucleolus. Bars represent 2 μm. (**2**) *M. comosum*. Nucleolus is attached at one end of the fifth bivalent (arrow). (**3**) *M. matritensis*. Black arrowheads denote the ends of the second bivalent. White arrowhead indicates the centromere location

additional ribosomal sequences are distributed to the termini of the long arm of chromosomes 1 and 2 and are inactive there. The process could continue in species such as *M. dionysicum*, in which an appearance/amplification of a heterochromatic DAPI positive region is accumulated in the interstitial region of the long arm of chromosome 2, which then becomes longer and is counted as chromosome 1, but without changing the position of the rDNA loci. Finally, the process appears to end with *M. comosum*, in which the heterochromatic DAPI+ region is highly amplified and in which a segment of chromosome 1 with the active NOR is translocated onto a small chromosome 5 (alternative A in Fig. 4). Another possibility could be a translocation of the major part of the long arm of chromosome 1 to a small chromosome which then becomes no. 1, but without affecting the active NOR (alternative B in Fig. 4).

In some cases, such as *Allium* (Schubert 1984, Schubert and Wobus 1985) or Triticeae (Dubcovsky and Dvorak 1995), the active NORs change frequently their position during evolution. The case of *Muscari* seems to be different with respect to the cases of *Allium* and Triticeae, since in *Muscari* the active NORs are apparently fixed in position, although during evolution some structural rearrange-

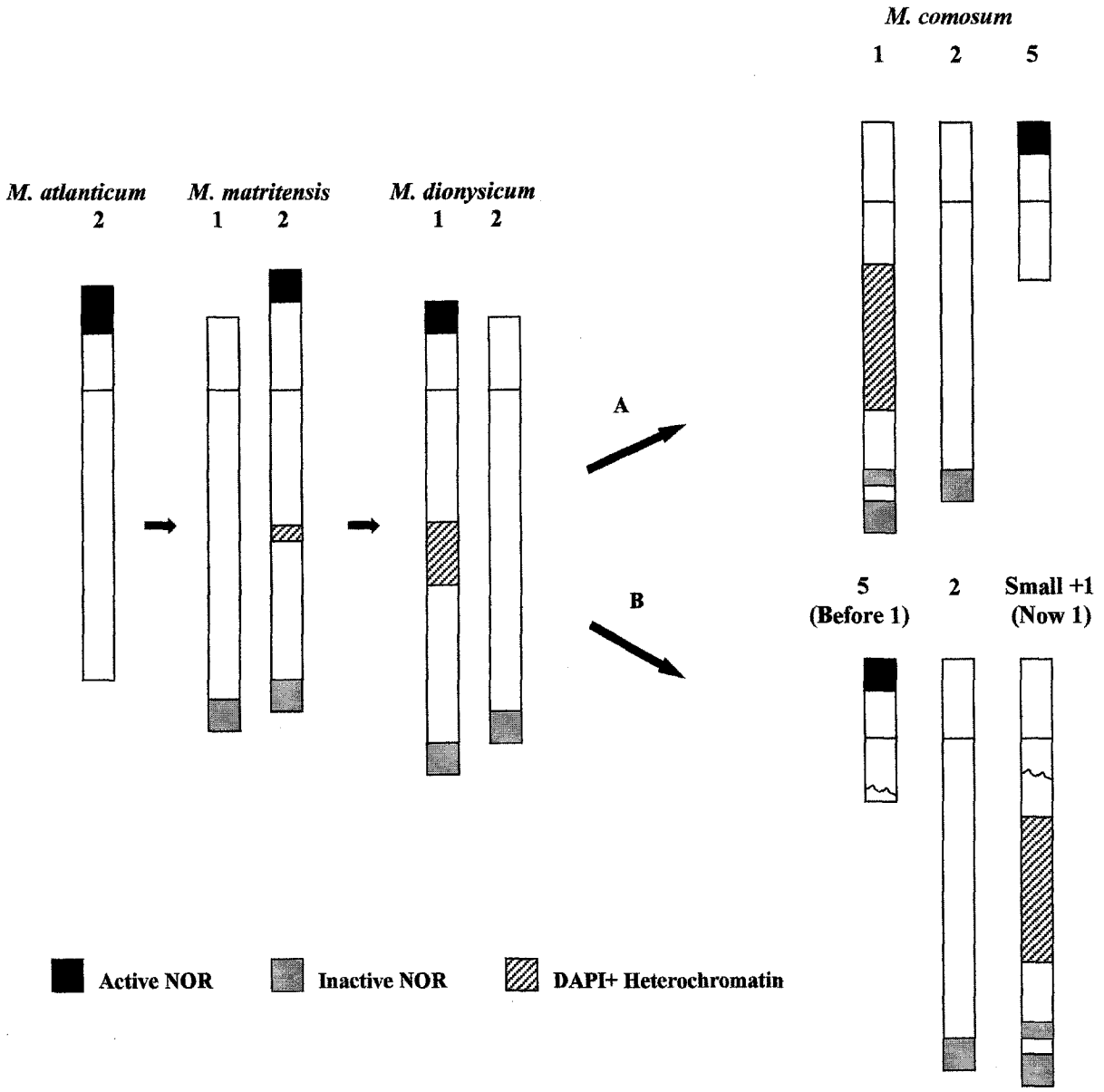


Fig. 4. A hypothetical scheme for the evolution of active and inactive rDNA loci in the *Muscari* species analyzed. See text for explanation

ments occur which affect the karyotypes. In fact, in *M. comosum*, chromosome rearrangements such as pericentric inversions and supernumerary segments are frequently detected as polymorphisms (Ruiz Rejón M. and Oliver 1981, Ruiz Rejón C. et al. 1987, Ruiz Rejón C. et al. 1990b). It is also possible that the asymmetrical karyotype shown by this species could be explained as a consequence of unequal translocations (Ruiz Rejón C. et al. 1990a).

On the other hand, it has been suggested that the major sites with higher repeat copy numbers correspond to active arrays located at NORs, whereas minor sites with few subunit repeat copies may not (see for references McGrath and Helgeson 1998). Our observations indicate that in the genus *Muscari*, major sites for rRNA genes are not necessarily active; therefore, the activation of these regions is not related to the number of copies but to a specific

mechanism that could be based on the differential regulation of methylation patterns of rDNA loci. This situation has been reported in some wheat/rye genotype combinations in which an almost total inactivation rRNA genes of rye origin is associated with a high level of cytidine methylation within the intergenic sequences (Neves et al. 1995, Amado et al. 1997).

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References

Amado L., Abranches R., Neves N., Viegas W. (1997) Development-dependent inheritance of 5-azacytidine-induced epimutations in Triticale: analysis of rDNA expression patterns. Chromosome Research 5: 445–450.

Amemiya C. T., Gold J. R. (1988) Chromomycin A_3 stains nucleolar organizer regions of fish chromosomes. Copeia 1: 226–231.

Bentzer B. (1973) Taxonomy, variation and evolution in representatives of *Leopoldia* Parl. (Liliaceae) in the southern and central Aegean. Botaniska Notiser 126: 69–132.

Bentzer B. (1974) Karyotypes and meiosis in *Leopoldia* Parl. (Liliaceae) from the Southern and Central Aegean (Greece). Botaniska Notiser 127: 69–86.

Cuñado N., Callejas S., García M. J., Fernández A., Santos J. L. (1996) The pattern of zygotene and pachytene pairing in allotetraploid *Aegilops* species sharing the U genome. Theor. Appl. Genet. 93: 1152–1155.

Cuñado N., Santos J. L. (1998) A method for fluorescence in situ hybridization against synaptonemal complex-associated chromatin of plant meiocytes. Exp. Cell Res. 239: 179–182.

Díez M., Puertas M. J. (1986) Quantitative analysis of the formation of nucleoli in *Locusta migratoria*. Can. J. Genet. Cytol. 28: 207–218.

Dubcovsky J., Dvorak J. (1995) Ribosomal-RNA multigene loci-nomads of the Triticeae genomes. Genetics 140: 1367–1377.

Fukui K., Ohmido N., Khush G. S. (1994) Variability in rDNA loci in the genus *Oryza* detected through fluorescence in situ hybridization. Theor. Appl. Genet. 87: 893–899.

Gerlach W. L., Bedbrook J. R. (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acid Res. 7: 1869–1885.

Goodpasture C., Bloom S. E. (1975) Visualization of nucleolar organiser regions in mammalian chromosomes using silver-staining. Chromosoma 53: 37–50.

Howell W. M. (1982) Selective staining of nucleolus organizer regions (NORs). Cell Nucleus 11: 80–142.

Lacadena J. R., Cermeño M. C., Orellana J., Santos J. L. (1984) Evidence for wheat-rye nucleolar competition (amphiplasty) in Triticale by silver-staining procedure. Theor. Appl. Genet. 67: 207–213.

Matsui S. I., Sasaki M. (1973) Differential staining of nucleolus organizers in mammalian chromosomes. Nature 246: 148–150.

McClintock B. (1934) The relation of a particular chromosomal element to the development of the nucleoli in *Zea mays*. Z. Zellforsch. Mikrosk. Anat. 21: 294–328.

McGrath J. M., Helgeson J. P. (1998) Differential behavior of *Solanum brevidens* ribosomal DNA loci in a somatic hybrid and its progeny with potato. Genome 41: 435–439.

Neves N., Heslop-Harrison J. S., Viegas W. (1995) RNA activity and control of expression mediated by methylation and imprinting during embryo development in wheat × rye hybrids. Theor. Appl. Genet. 91: 529–533.

Ritossa F. M., Spiegelman S. (1965) Localisation of DNA complementary to ribosomal RNA in the nucleolus organiser region of *Drosophila melanogaster*. Proc. Nacl. Acad. Sci. USA 53: 737–745.

Ruiz Rejón C., Lozano R., Ruiz Rejón M. (1987) Genetic variability in *Muscari comosum* L. (Liliaceae). II. Characterization and effects on chiasma formation of the polymorphic variants of chromosome 2. Genome 29: 19–26.

Ruiz Rejón C., Lozano R., Ruiz Rejón M. (1990a) A cytogenetic analysis of the chromosomes in two related species of the genus *Muscari* (Liliaceae). Genome 33: 729–732.

Ruiz Rejón C., Lozano R., Ruiz Rejón M. (1990b) Genetic variability in *Muscari comosum* L. (Liliaceae). IV. Geographical distribution and adap-

tative role of the polymorphic variants of chromosome 2. Genetica 81: 133–142.

Ruiz Rejón M., Oliver J. L. (1981) Genetic variability in *Muscari comosum* (Liliaceae). I. A comparative analysis of chromosome polymorphism in Spanish and Aegean populations. Heredity 47: 403–407.

Schubert I. (1984) Mobile nucleolus organizing regions (NORs) in *Allium* (Liliaceae s.lat.)?-Inference from the specificity of silver staining. Plant Syst. Evol. 144: 291–305.

Schubert I., Wobus U. (1985) In situ hybridization confirms jumping nucleolus organizing regions in *Allium*. Chromosoma 92: 143–148.

Stebbins G. L. (1971) Chromosomal evolution in higher plants. Arnold, London.

Tutin T. G. et al. (eds.) (1980) Flora Europaea, vol. 5. Cambridge University Press, London.

White M. J. D. (1973) Animal Cytology and Evolution, 3rd edn. Cambridge University Press, Cambridge.

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