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DNA VARIATION AND RIBOSOMAL-DNA CONSTANCY IN TWO *CREPIS* SPECIES AND THE INTERSPECIFIC HYBRID EXHIBITING NUCLEOLAR-ORGANISER SUPPRESSION*

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Summary

The interspecific hybrid between *Crepis capillaris* and *C. dioscorides* exhibits suppression of the secondary constriction in the chromosome derived from *C. dioscorides*; each species normally has one chromosome pair with secondary constrictions. Such structures are known to be associated with the formation of nucleoli and are the probable sites of ribosomal-DNA (rDNA). The two species and the hybrid were studied with respect to DNA and rDNA variation to determine whether reduction of the highly redundant rDNA might occur as a consequence of nucleolar organiser suppression.

The DNA content of *C. dioscorides* was three times greater than of *C. capillaris*. This is consistent with trends in the genus *Crepis*. Evolutionary advancement is correlated with a reduction of both chromosome number (*C. dioscorides*, $2n = 8$; *C. capillaris*, $2n = 6$) and chromosome size. The chromosomes of the more advanced species, *C. capillaris*, are smaller than those of *C. dioscorides*. No alterations of the parental contributions of rDNA were observed in the hybrid, however; the two species and the hybrid each contained about 5000 rRNA genes. It was concluded that nucleolar organiser suppression must be at the transcription level.

1. Introduction

The DNA (rDNA) complementary to 18 and 28S ribosomal RNA (rRNA) is located in the region of the nucleolar organiser (NOR) in *Drosophila* (Ritossa and Spiegelman, 1965), *Xenopus* (Wallace and Birnstiel, 1966) and maize (Phillips *et al.*, 1971) and to the nucleolus organising body itself and the distal achromatic gap of the maize chromosome (Givens, 1974; Ramirez and Sinclair, 1975; Doerschug, 1976). The redundancy in rDNA cistrons and the broad variation in degree of redundancy have been demonstrated in many higher organisms. Years ago, Navashin (1934) described a striking relationship between the number of chromosomes bearing a secondary constriction formed in hybrids of *Crepis* and the source of parental chromosomes. Normally there are two such chromosomes in diploid plants and animals; these were named SAT chromosomes since the secondary constriction seemingly lacked nucleic acid (*sine acido thymo nucleinico*) (Heitz,

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1931). In certain interspecific hybrids secondary constriction formation was suppressed in the SAT chromosome from one of the parental species. The affected chromosome was always from the same parental species in a given cross. A hierarchy of dominance existed such that within a hybrid secondary constriction formation was suppressed in the chromosome contributed by the species of lower rank. Recently Wallace and Langridge (1971) showed that, in the hybrid *C. neglecta-capillaris*, the nucleolus was always associated with the SAT chromosome of *C. capillaris* and not with that of *C. neglecta*. They attributed the observed behaviour to a repression of RNA transcription in the NOR of the *C. neglecta* genome. We have explored the possibility that the absence of a secondary constriction in the apparently repressed NOR-bearing chromosome with *Crepis* hybrids might be due to an elimination of the redunandant rDNA genes subsequent to fertilisation. This latter interpretation seemed tenable since increases or decreases of rDNA are known to occur (Ritossa, 1968; Tartoff, 1971, 1973). Our studies were based on analysis of DNA and rDNA gene levels in *Crepis capillaris*, *C. dioscorides*, and the interspecific hybrid. Our results clearly indicate that there is no rDNA loss, and unexpectedly that the two species contain the same number of rDNA genes even though one contains almost three times as much DNA.

2. Materials and methods

(i) *Propagation of* Crepis *species and genetic crosses*

Seeds of *Crepis capillaris* were obtained from G. L. Stebbins, University of California, Davis, and the Botanisk Have, Copenhagen. *Crepis dioscorides* was also obtained from the latter source. The plants were grown in growth chambers and the greenhouse and genetic crosses were made according to the methods of J. L. Collins (1922). *C. capillaris* was used as the female parent in crosses with *C. dioscorides* because of its self-incompatibility, whereas *C. dioscorides* is partly self-fertile.

(ii) *Cytogenetic analysis*

The karyotypes of the parent species and the interspecific hybrid were determined as follows: Vigorous young root tips were excised and placed in freshly prepared 0·002M 8-hydroxyquinoline for 3 hours at 16-18°C. (Tjio and Levan, 1950). The tips were rinsed and fixed overnight in 3 parts 95 per cent ethanol: 1 part glacial acetic acid. Cytological preparations were made by the Feulgen Squash Method (Darlington and LaCour, 1962); maceration by hydrolysis in N HCl at 60° for 7 minutes, staining in Schiff's Reagent 1-3 hours followed by tapping the coverslip directly over the root tip (Ghidoni, Achille, personal comm.) that had been placed in a drop of acetic-orcein (1 per cent in 45 per cent acetic acid). The preparation was pressed to flatten the cells and sealed. Many late-prophase and metaphase figures were clearly observed.

(iii) *Culture of* Crepis capillaris

Sterile cultures of *Crepis capillaris* were initiated and grown in the basal medium designed by Eriksson (1965) supplemented with 10 mg/l myo-inositol and 50 ml/l coconut milk. They were grown in the dark at room

temperature (about 21°-23°C) and consisted predominantly of masses of buds on a callus base. The cultures were used for the incorporation of ^{32}P orthophosphate to keep the plant material free of contaminating bacteria and fungi.

(iv) *DNA isolation*

After exposure to " long day " photoperiods (16 hours light, 8 hours dark) after a short day regime (10 hours light, 14 hours dark), *Crepis* plants bolt by producing a profusion of buds. These buds, which are masses of compact cells, were collected, and DNA was isolated by a modification of the method of Hotta *et al.* (1965). Tissues were ground in ethanol cooled to −20°C in a ground-glass homogeniser until the homogenate consisted of broken cells, single and small clumps of cells. After washing two times with 70 per cent ethanol, the cells were disrupted by stirring them into 2 volumes of 1 per cent sodium lauryl sulfate (SLS), 0·01M ethylenediaminetetraacetic acid (EDTA) and 0·05M Tris buffer, *p*H 8·5. The mixture was heated rapidly to 65°C and maintained for 20 minutes, followed by centrifugation and re-extraction of the pellet with an aliquot of the original extraction fluid. Two volumes of 95 per cent ethanol were mixed and the precipitate collected by centrifugation. After washing two times with 70 per cent ethanol, the precipitate was redissolved by slow stirring at room temperature in 0·01M Tris *p*H 7·5, 0·01M EDTA, 1M NaCl, and 1 mg/ml of pronase (the enzyme solution had been pretreated at 65°C for 10 minutes to remove contaminating deoxyribonucleases). After incubation for at least 4 hours, the solution was deproteinised two times with chloroform: isoamyl alcohol (24:1 v/v). The DNA in this and subsequent steps was " spooled out " by slowly adding 0·55 volumes of isopropanol. The remainder of the purification was according to Marmur (1961) and included two ribonuclease treatments (50 μg/ml, 15 minutes, 37°C) followed by an additional pronase treatment (200 μg/ml, 2 hours, 37°C) and extraction with chloroform: isoamyl alcohol until no precipitate formed at the interface. After the final precipitation with isopropanol, the DNA was stored in 95 per cent ethanol at −20°C.

(v) *RNA isolation*

Ribosomal-RNA was isolated by an adaptation of the method of Kirby (1965). The sterile ^{32}P-labelled bud material was washed thoroughly with water, drained, weighed, and frozen in liquid nitrogen. After grinding with a frozen mortar and pestle, the tissue powder was homogenised (3 times, 1 minute each) at three-fourths maximum speed in a Virtis cup containing 0·5 per cent disodium naphthalene disulphonate in 0·05 Tris *p*H 7·6 (5 volumes) and phenol-cresol mixture (5 volumes). The latter contained 550 ml water-saturated phenol (freshly distilled), 70 ml cresol (freshly distilled, colourless), and 0·5 g 8-hydroxyquinoline. After high-speed blending the mixture was shaken vigorously for 20 minutes, chilled, and centrifuged in a Sorvall angle head (15,000 r.p.m., 15 minutes). The aqueous layer was made 3 per cent in sodium chloride, 1 per cent in tri-isopropyl-naphthalene-sulfonate (TIPNS), and shaken twice with phenol-cresol before precipitating with 2·5 volumes of 80 per cent ethanol containing

0·02M potassium acetate. The mixture was stored in the freezer for at least 2 hours to obtain full precipitation of the RNA. The precipitate was collected, washed with 70 per cent ethanol and redissolved in a small volume of 1 per cent TIPNS, 0·01M Tris *p*H 7·6, and an equal volume phenol-cresol mixture. After dissolving, the aqueous portion was made 3 per cent in sodium chloride. The mixture was shaken and centrifuged as before until no material remained at the interface. The RNA was precipitated, stored, collected, washed and dissolved in 0·05M Tris with 3mM $MgCl_2$ and 20 μg/ml DNase (electrophoretically pure) for 15 minutes at 37°C. The RNA solution was diluted with 0·05M phosphate buffer and immediately added to a MAK column (Sueoka and Cheng, 1962), washed and eluted with a 150 ml linear gradient (0·4M to 1·2M sodium chloride in 0·05M phosphate buffer). The ribosomal RNA eluted as a single peak at about 0·8 sodium chloride. The fractions were pooled and the RNA dialysed against two litres 2 × SSC for 24 hours (SSC contains 0·15M sodium chloride and 0·015 sodium citrate). The RNA was used only if it was stable at 65°C for the duration of the hybridisations and if it contained less than 0·05 per cent base stable material (detected by treating a portion of the RNA with 0·2N sodium hydroxide for 20 minutes at 70° and comparing trichloroacetic acid precipitable counts before and after hydrolysis (Gillespie and Spiegelman, 1965).

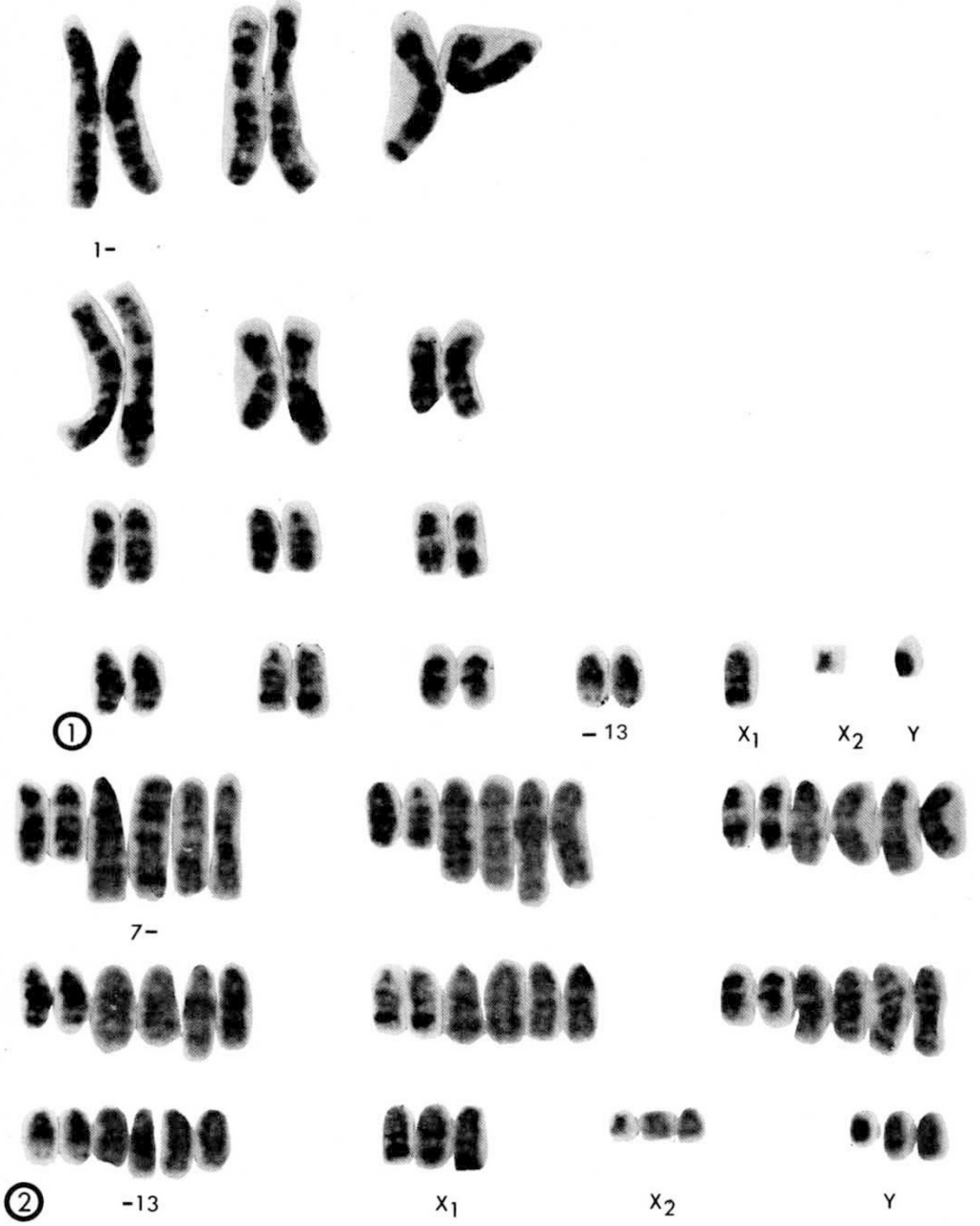
(vi) *Molecular hybridisations*

The DNA's were denatured with alkali and bound to nitrocellulose membrane filters (type B-6, Schleicher and Schuell) with 30 μg DNA per filter (determined by optical density before and after loading) according to the method of Gillespie and Spiegelman (1965). Saturation-type hybridisation reactions were performed according to the method of these authors using the rRNA concentrations specified. The reactions were carried out in 2 × SSC at 65°C for 15 hours. A blank and a DNA filter were incubated in each vial. The filters were then washed, treated with RNase (20 μg/ml, 1 hour) and washed again with 2 × SSC, after which they were dried and counted.

To compensate for differential retention of the DNA by the filters, the amount of DNA on each filter was determined after the hybridisation reactions. The filters were washed several times in chloroform to remove the toluene and fluors and then air dried. Each filter was cut into small pieces, placed in a tube with 1 ml 5 per cent perchloric acid, sealed, and hydrolysed for 20 minutes at 70°C. The amount of DNA in each sample was determined by the diphenylamine reaction (Burton, 1968). A standard curve was prepared with blank filters to correct for colour absorption.

(vii) *DNA determination*

Chicken erythrocytes, which served as an internal standard, were smeared on microscope slides. The slides and excised root tips were fixed in Carnoy's fixative for 3 hours and rinsed several times in 70 per cent ethanol. The root tips and erythrocyte smears were hydrolysed for 20 minutes in 5N HCl at room temperature. The root tips were squashed on a cleared area of the smear slides.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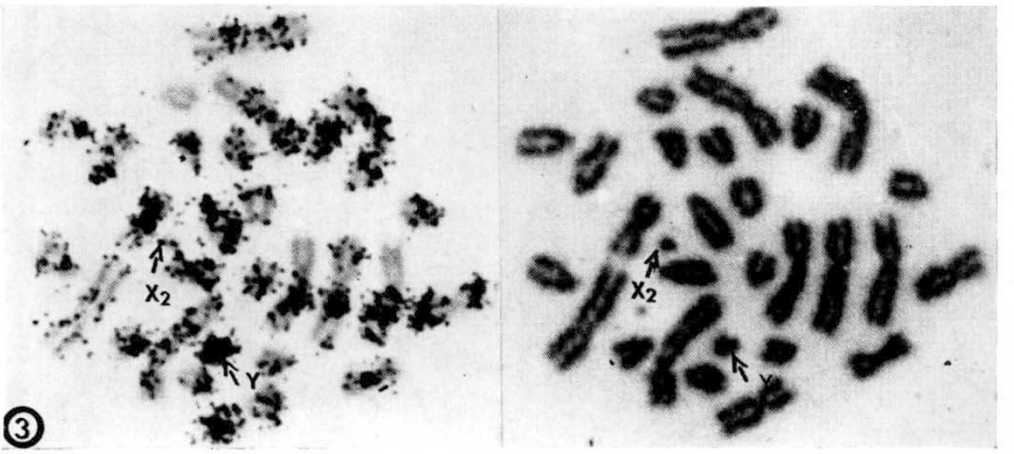
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The erythrocyte nuclear dry mass was determined from 150 polarising interference microscope readings by using the formula given by Berlyn and Miksche (1976). The procedure was repeated after treating the cells with DNase, the difference in dry weights representing the amount of DNA/nucleus.

The cells were stained with Schiff's reagent, and cytophotometric absorbancies were determined as described by Jacqmard *et al.* (1972) and Berlyn and Miksche (1976). The ratio of nuclear dry mass to Feulgen absorption in erythrocyte nuclei was used to calculate the absolute amounts of DNA in *Crepis* nuclei. The DNA content of erythrocyte nuclei as determined by inteferometry ($2{\cdot}62 \times 10^{-12}$ g) is close to published values determined chemically (Sober, 1970).

3. Results

(i) *The cytogenetics of nucleolar organiser suppression in an interspecific hybrid*

Interspecific hybrids were made between *Crepis capillaris* (♀♀) and *Crepis dioscorides*. The results of Navashin (1934) were confirmed by examining the chromosomes of the hybrids and the parental species. The chromosome number of *C. capillaris* is $2n = 6$ and that of *C. dioscorides* is $2n = 8$, causing hybrids to have a total of 7 chromosomes; thus the hybrids were easily distinguished cytogenetically from self-pollinated contaminants of *C. capillaris*. Two homologous satellited chromosomes always were observed in cytological preparations of the parental species (plate I). In all 25 hybrid plants (7 chromosomes), one of the normally satellited chromosomes had no satellite; instead the terminal chromosomal segment was indistinguishable from the main body of the acrocentric chromosome (plate I). The NOR-bearing chromosomes of the two species are easily distinguished in the hybrids because the SAT chromosome derived from *C. capillaris* is shorter than that from *C. dioscorides*. In every hybrid plant the satellite was missing from the putative SAT chromosome derived from the *C. dioscorides* parent.

(ii) *DNA contents and number of rRNA genes*

The nuclear DNA contents of *C. dioscorides*, *C. capillaris* and *C. dioscorides* × *capillaris*, as determined by microspectrophotometry are listed in table 1. The ratio in values obtained for metaphase and telophase cells are approximately 2 for all the plant types, thus pointing to the validity of the method for determining the relative DNA contents of species and hybrid. The data indicate that *C. dioscorides* has about 2·8 times as much DNA as *C. capillaris* even though the two species differ by only one chromosome in their haploid number. The absolute diploid DNA values are about 11 pg for *C. dioscorides*, 4 pg for *C. capillaris* and, as expected, 8 pg for the hybrid. These values are used in subsequent calculations of rDNA content per genome.

Molecular hybridisation experiments (Gillespie and Spiegelman, 1965) were performed with ^{32}P-rRNA extracted from sterile cultures of *Crepis capillaris* which had been grown for two weeks in a medium containing 25 μc/ml inorganic ^{32}P-phosphate. The saturation levels for rRNA/DNA hybridisations in each of the parent species and in the interspecific hybrid

are recorded in fig. 1. Ribosomal-DNA accounts for 0·4 per cent of the total DNA in *C. capillaris*, 0·15 per cent in *C. dioscorides*, and 0·2 per cent

TABLE 1

The relative and absolute DNA contents of Crepis capillaris, C. dioscorides, *and the interspecific hybrid. The 2c (telophase) and 4c (metaphase) amounts of DNA were determined by comparing microspectrophotometer absorbancies of root cell nuclei with chicken erythrocyte nuclei which contain the 2c amount of DNA (2·62 pg) as determined by interference microscopy. Fifty cells of each type were measured*

		Metaphase		Telophase		Erythrocyte
Source	Test	Relative absorbance*	DNA (4c) (pg)	Relative absorbance	DNA (2c) (pg)	Relative absorbance
C. capillaris	1	34·0±0·7	8·5	17·0±0·5	4·3	10·5±0·5
	2	34·0±0·7	7·8	19·1±0·5	4·0	12·3±0·6
C. dioscorides	1	87·2±2·2	22·3	41·8±0·5	10·6	10·3±0·4
	2	84·8±1·6	21·3	46·4±1·5	11·7	10·4±0·4
C. capillaris	1	64·3±1·8	15·1	31·1±1·0	7·3	11·1±0·5
×*C. dioscorides*	2	69·1±2·1	19·8	33·3±1·0	9·6	9·1±0·4

* ±standard error of the mean.

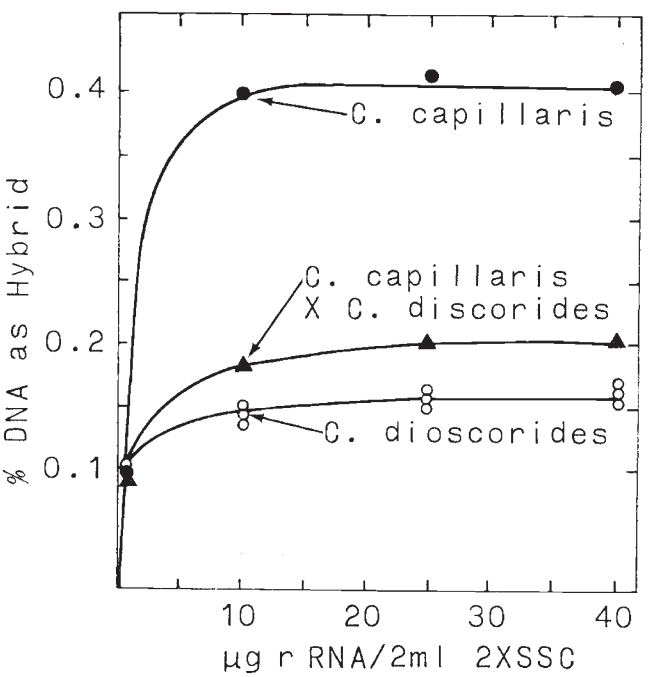

FIG. 1.—Saturation levels in rRNA/DNA hybridisation experiments. 30 µg of the indicated DNA's were attached to membrane filters and incubated 15 hours at 65°C with increasing amounts of ^{32}P-rRNA.

in the hybrid. Three repetitions of the experiment (involving separate DNA extractions) showed the same relationship.

The numbers of rRNA genes per haploid genome in the two parent species and hybrid were calculated from the data provided in table 1 and fig. 1 and are shown in table 2. Assuming a molecular weight of 2×10^6 daltons for the two rRNA types combined, the number of rRNA cistrons per haploid genome is about 2500. That number is as constant for the two parent species as it is for the hybrid. Thus, regardless of genome size, or of chromosomes with an active nucleolar organiser, the total amount of rDNA per diploid nucleus remains unchanged. Failure of nucleolar formations in the hybrid is clearly independent of rDNA presence.

TABLE 2

The number of rRNA genes in a 2c amount of DNA for Crepis capillaris, C. dioscorides, *and the interspecific hybrid. The calculations were based on the following values. DNA contents:* C. capillaris ($4{\cdot}1 \times 10^{-12}$ g), C. dioscorides ($11{\cdot}0 \times 10^{-12}$ g), *hybrid* ($8{\cdot}0 \times 10^{-12}$ g); *molecular weight of rRNA* (2×10^6 *daltons*)

Plant	rRNA genes
C. capillaris	4920
C. dioscorides	4950
C. capillaris × *C. dioscorides*	4800

4. Discussion

(i) *Variation of DNA*

The evolution of the karyotype has been studied in at least 113 *Crepis* species representing 23 of the 27 taxonomic sections of the genus (Babcock, 1947). Progressive morphological and physiological changes within the genus were correlated with a decrease in haploid chromosome number from 6 to 3, such decrease having occurred by stepwise losses of single chromosomes. Paralleling the tendency toward reduction in chromosome number has been a trend toward reduction in chromosome size.

The species analysed in this study reflect both evolutionary trends. *C. capillaris*, the more advanced species, has three chromosomes per haploid set, (plate I*a*), whereas *C. dioscorides* has four which are of larger size. Our finding that *C. dioscorides* has almost three times as much nuclear DNA as *C. capillaris* is in agreement with the cytological findings.

DNA content and evolutionary advancement was studied among nearly 1000 plant species and compared with DNA values for various taxonomic groups of organisms (Sparrow *et al.*, 1972). Although a general trend of increasing DNA content with advancing organismic complexity was seen, within any given taxonomic group advancing complexity or evolutionary specialisation was often associated with decreasing DNA content. The nature of the DNA lost is unknown although it has been suggested that variation in the amount of redundant sequences may account for much of the DNA differences in higher organisms (Britten and Davidson, 1969; Flavell *et al.*, 1974). Comparisons of DNA differences among closely related *Vicia* species as well as cytological comparisons of the chromosomes led to the conclusion that linear amplifications or deletions of sequences occurred at many sites throughout the chromosomes and account for the major

proportion of DNA variation in *Vicia* (Chooi, 1971). Similar alterations are probably the basis for karyotype evolution in *Crepis*.

(ii) *rDNA constancy*

When the differences in DNA content between the two *Crepis* species and hybrid were taken into account, the calculations of number of rRNA genes per diploid genome revealed that all three contained about 5000 sequences complementary to 18 and 25S rRNA. This number is consistent with the high multiplicities of rRNA genes found in higher plants which range from a few thousand to over 20,000 copies (Birnstiel *et al.*, 1971). The striking observation, however, was that even with almost a threefold difference in DNA between the two species, the amount of rDNA per cell remained constant. Either the NOR was not included in the chromosomal alterations leading to the two species or some stringent physiological requirement exists for this number of rRNA genes per cell. Support for the latter interpretation was found in comparisons of diploid and tetraploid *Nicotiana* species where there was a measured twofold difference in DNA content, but both had the same number of rRNA genes (Siegel *et al.*, 1973). In a similar study of *Nicotiana* species Cullis (1975) found a similar relationship between the rRNA gene number of two tetraploids and their diploid progenitors.

(iii) *Explanations for the suppression of the nucleolar organiser*

The determinations of rRNA gene numbers presented here demonstrate clearly that no measurable loss of rDNA occurs concomitant with NOR suppression in the *capillaris-dioscorides* hybrid. The control of NOR activity then must be at the transcription level. Wallace and Langridge (1971), in a study of *Crepis neglecta-C. capillaris* hybrids suggested that differential suppression may be effected by allelic repression among the four iso-alleles found in the *Crepis* hierarchy of species. In their investigation they tried to differentiate between the rRNA's of the two species by base composition but did not present convincing evidence that the interspecific hybrid, exhibiting NOR suppression in the *neglecta* SAT chromosome, synthesised only *capillaris* rRNA. Thus there is yet no direct evidence that rRNA transcription is prevented by NOR suppression.

The hypothesis that NOR suppression would derive from a major reduction of rDNA was attractive inasmuch as it would point to a renewal of all reiterated rDNA at fertilisation. Our findings, however, do not support this idea.

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