# COMPARISON OF THE DNA OF SIX VICIA SPECIES BY THE METHOD OF DNA-DNA HYBRIDIZATION

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Received December 18, 1970

IN this communication, a comparison is made between the nucleotide sequences of six species of the genus Vicia. Cytological studies (CHOOI 1971) have shown that the DNA contents per cell of these six species range from 18.2 to 100 arbitrary units, i.e., they have an approximately six-fold variation in DNA content per cell. In addition, closely related varieties of the same species appear to have significantly different DNA contents per cell. Most of the evidence from these cytological studies has indicated that increase in DNA content per cell in the genus Vicia had taken place through either local multiplicity or segmental duplications. The possibility that some of the increase in DNA content per cell could have taken place via lateral multiplicity was, however, not entirely discounted. The purpose of this investigation was to determine: (1) whether differences in DNA content per cell between the six species are due to repetitious DNA in the sense of BOLTON *et al.* (1967); and (2) whether the differences in DNA content per cell between these six species can best be accounted for by local multiplicity or lateral multiplicity (CHOOI 1971).

The nucleotide sequences of these six species have been compared using the method of DNA-DNA hybridization (reassociation rate and competition experiments). Although segmental duplications and local multiplicity are not identical, they are not distinguishable by the methods used in this work and can be grouped as local multiplicity.

## MATERIALS AND METHODS

*Preparation of $^{32}P$-labeled DNA:* Seeds were surface sterilized with 1% sodium hypochlorite for 20 min and then germinated in vermiculite. Seedlings with lateral roots were grown for 5 days in 500 ml distilled water containing $^{32}P$-orthophosphate (specific activity = 2.3 $\mu$c/ml). Sodium bicarbonate was added to bring the pH of the solution to 7. After the roots had been washed free of the supporting medium, DNA was extracted from them using the method of BENDICH and BOLTON (1967). All the labeled plant DNA used was labeled with $^{32}P$-orthophosphate. Only *E. coli* DNA was labeled with $^{3}H$-thymidine. The specific activity of the labeled plant DNA obtained was 10,000 to 60,000 cpm/$\mu$g.

*Bacterial culture: E. coli* K12 (JC 2918) was grown at 37°C with aeration in 1.5 liters of Tris-glycerol media (NOMURA *et al.* 1962) containing 0.2% glucose plus threonine, leucine, histidine, arginine, proline, and thiamine (all 20 $\mu$g/ml), and streptomycin (100 $\mu$g/ml).

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For the preparation of $^3$H-thymidine labeled *E. coli* DNA, $^3$H-thymidine (0.013 μc/ml) was added to the culture when the cell density reached an optical density of 0.4 at 650 mμ. The cells were allowed to grow for another hour by which time 70% of the $^3$H-thymidine in solution had been incorporated into the cells. When the cell density reached an optical density of 0.8 at 650 mμ, the cells were harvested by centrifugation at 7,000 rpm for 20 min. The cells were washed in a solution of 0.15 M sodium chloride and 0.1 M EDTA (saline EDTA) at pH 8.0 and resuspended in 50 ml of the same solution.

*Isolation of bacterial DNA:* The procedure of MARMUR (1961) was employed in the isolation of the first crude extract of bacterial DNA. Further purification of this extract was carried out using the method of BENDICH and BOLTON (1967). The specific activity of the $^3$H-thymidine labeled *E. coli* DNA obtained was 250–260 cpm/μg.

*Denaturation of DNA:* Native (double stranded) DNA preparations dissolved in 0.1 × SSC were denatured (single stranded) by heating at 100°C for 5 min followed by rapid cooling to 0°C. A hyperchromicity of 29.5% indicated that the denaturation of the DNA was complete (DENHARDT 1966).

DNA for use in the incubation mixture for hydridization was sonicated for 30 sec at maximum output in an MSE 1000 watt Ultrasonic Disintegrator (DENHARDT 1966). The average molecular weight of the sonicated DNA was determined by sucrose gradient analysis (Figure 1).

*Immobilization of denatured DNA on nitrocellulose filters:* The procedure of GILLESPIE and SPIEGELMAN (1965) was used for the immobilization of denatured DNA on nitrocellulose filters (Millipore 025 00 25 mm 0.45 μ) but with one modification—that the filters were dried at 80°C at atmospheric pressure instead of under vacuum at 80°C.

The DNA preparation, dissolved in 0.1 × SSC, was denatured and the concentration was adjusted to approximately 80 μg/ml in 6 × SSC. It was then diluted 10 times with 6 × SSC and passed through a filter (presoaked for 1 min in 6 × SSC and washed with 10 ml of the same solution), and then washed with 100 ml of 6 × SSC. In all the experiments conducted, 20 μg of DNA was immobilized on each filter. The amount of DNA on sample filters was estimated by heating the filter loaded with DNA in 4 ml of 5% perchloric acid at 90°C for 30 min and estimating the $OD_{260}$ of the resulting solution.

*DNA-DNA hybridization—competition experiments:* Hybridization of filter-bound DNA was carried out in vials containing one ml of incubation mixture consisting of 4 μg of sonicated, denatured, labeled DNA and 10 to 220 μg of homologous or heterologous unlabeled, sonicated, denatured DNA in 2 × SSC buffered with 0.01 M Tris-HCl, pH 7.0. After incubation at 60°C for 24 hr, the filters were removed and washed as described by WANAAR and COHEN (1966). After the filters were dried, the amount of radioactivity bound to the filters was determined by liquid scintillation spectrometry.

Other experiments were conducted as described in the figure legends.

*Interpretation of results:* The nucleotide sequences of *V. faba* were used as a standard for comparison of the nucleotide sequences of the DNA of *V. melanops, V. narbonensis, V. benghalensis, V. atropurpurea,* and *V. sativa.*

In a typical DNA-DNA competition experiment (e.g., Figure 5), 20 μg of unlabeled *V. faba* DNA was immobilized on each of the two filters. To each was added: (1) 4 μg of denatured labeled *V. faba DNA* and (2) either denatured unlabeled *V. faba* DNA (10 to 220 μg) or denatured unlabeled heterologous DNA (10 to 220 μg).

The amount of labeled *V. faba* DNA hybridized at the various concentrations of competitor DNA served as a standard equivalent to 100% homology.

The unlabeled heterologous DNA competed with the labeled *V. faba* DNA for sites on the filter-bound DNA. Hence, the amount of labeled *V. faba* DNA hybridized to filter-bound DNA was decreased. The degree to which the hybridization of labeled *V. faba* DNA was decreased at the various concentrations of competitor DNA gave a measure of homology.

Heterologous competition was monitored by the use of *Triticum vulgare* and *E. coli* DNAs. It was assumed that the DNA of *T. vulgare* was not closely related to the DNAs of Vicia species (BENDICH and BOLTON 1967) and that the DNA of *E. coli* was not related to that of plants.

Reciprocal experiments were also carried out to extend the interpretation of nucleotide

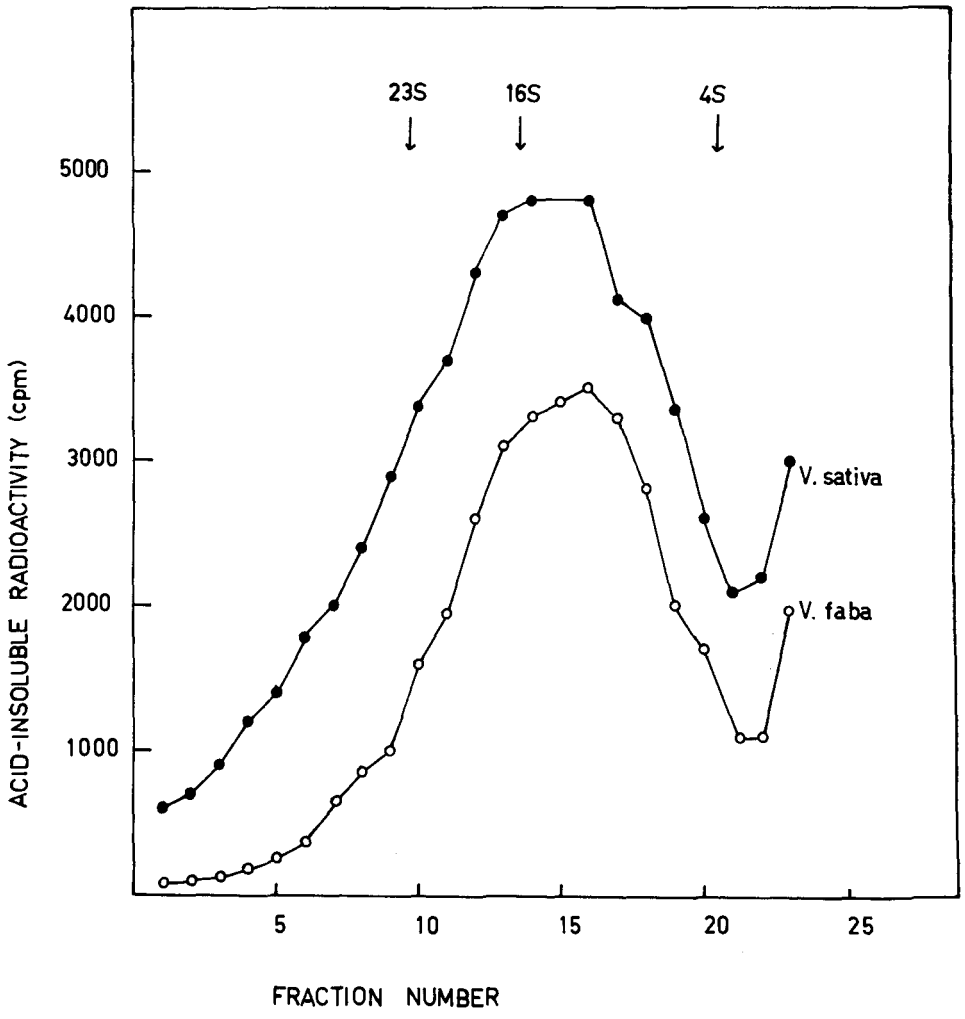


FIGURE 1.—Sedimentation profile in sucrose gradient of sonicated undenatured DNA of *V. faba* (O—O) and *V. sativa* (●—●). 0.2 ml of $^{32}$P-labeled *V. faba* and *V. sativa* DNA fragments were each layered onto 4.4 ml linear 5–20% sucrose gradients. The *E. coli* RNA (23 S, 16 S, and 4 S, prepared by the method of BOLTON 1966), which was used as a marker, was layered onto a separate 4.4 ml 5–20% linear sucrose gradient. The samples were centrifuged in a Beckman SW 39 rotor at 33,000 rpm for 5 hr. 0.2 ml (4-drop) fractions were collected from the bottom of each tube and used for the estimation of acid-insoluble radioactivity. The optical density at 260 mμ of the marker RNA was determined, and the positions of the 23 S, 16 S, and 4 S peaks were noted. The method of STUDIER (1965) was used in the calculation of molecular weight. The acid-insoluble radioactivity (cpm) is plotted against the fraction number. The positions of the 23 S, 16 S, and 4 S peaks of *E. coli* RNA are shown.

sequence homologies between the six Vicia species. When reciprocal experiments were done, the homologous DNA-DNA interaction was also carried out simultaneously. This was necessary since variable results were obtained with different preparations.

The percentage homology between different species was calculated by the method of BEN-

DICH and BOLTON (1967). BENDICH and BOLTON (1967) calculated the total percentage homology shown by the DNAs of any two species at plateau level. In the results presented here, however, the percentage homology shown by the DNAs of any two species was calculated at 20, 40, 80, 120, and 160 $\mu$g of competitor DNA in the incubation mixture. When the percentage homology shared by the DNAs of the two species was calculated at the various concentrations of competitor DNA, the differences in the degree of multiplication of nucleotide sequences in the two DNAs compared were more clearly defined.

Actual percentages of labeled *V. faba* DNA hybridized are shown in Figures 4 and 8. From these, percentage homologies can be calculated and these are shown in Figures 5, 6, 7, 9, and 10.

In one series of experiments (Figure 8), each reading was replicated ten times so that the error could be calculated. For any reading of percentage labeled DNA hybridized, the standard error was 1.4 to 2%.

In the interpretation of the results of competition experiments, a high percentage homology obtained at a low concentration of competitor DNA was assumed to be due mainly to the hybridization of the most highly repeated DNA shared by both species. It was assumed that the nucleotide sequences that were not highly repeated did not take part to a great extent in the hybridization experiments (BOLTON *et al.* 1967).

Theoretically, if the difference in DNA content per cell between two species were due only to even multiplication of DNA messages (lateral multiplicity), the type of nucleotide sequences present and the degree of multiplication of these would be similar for the two species at the same concentration of competitor DNA. Hence the percentages of labeled DNA hybridized at the various concentrations of heterologous competitor DNA should be the same as those in the presence of the same concentrations of homologous competitor DNA (Figure 2). The same results should be obtained in the reciprocal experiment.

If the difference in DNA content per cell between two species were due to uneven multiplication of DNA messages (local multiplicity), more labeled DNA would be hybridized at lower concentrations of heterologous competitor DNA if the heterologous DNA consisted of lower multiplications of some nucleotide sequences (Figure 2). This is because nucleotide sequences which are present in higher multiplications would be able to compete more effectively for sites on the filter-bound DNA. Hence the amount of labeled homologous DNA hybridized to filter-bound DNA would be higher if the heterologous DNA consisted of lower multiplications of some nucleotide sequences. Similarly, less labeled DNA would, however, be hybridized at lower concentrations of heterologous competitor DNA if the heterologous competitor DNA consisted of higher multiplications of some nucleotide sequences. Smaller differences between the two species would be obtained at high concentrations of competitor DNA because at high concentrations of competitor DNA, both highly and less highly repeated DNA have more even chances of hybridizing with filter-bound DNA. In the case of difference due to local multiplicity, different results would be obtained in reciprocal experiments.

## RESULTS

*Molecular weight of sonicated DNA:* The molecular weights of the DNA fragments of *V. faba* and *V. sativa* were found to lie mainly between 600,000 (8 S) and $10 \times 10^6$ (23 S). The average molecular weights of the DNA fragments of *V. faba* and *V. sativa* were assumed to be about $2 \times 10^6$ (13 S) (Figure 1). Since the DNA fragments of *E. coli* and other Vicia species were produced using the same method, their average molecular weights were also assumed to be approximately $2 \times 10^6$.

*Reassociation rates:* Figure 3 shows that except for *E. coli*:

1. The percentage of labeled DNA of each of the six Vicia species hybridized to filter-bound DNA increased rapidly with time until a more or less plateau

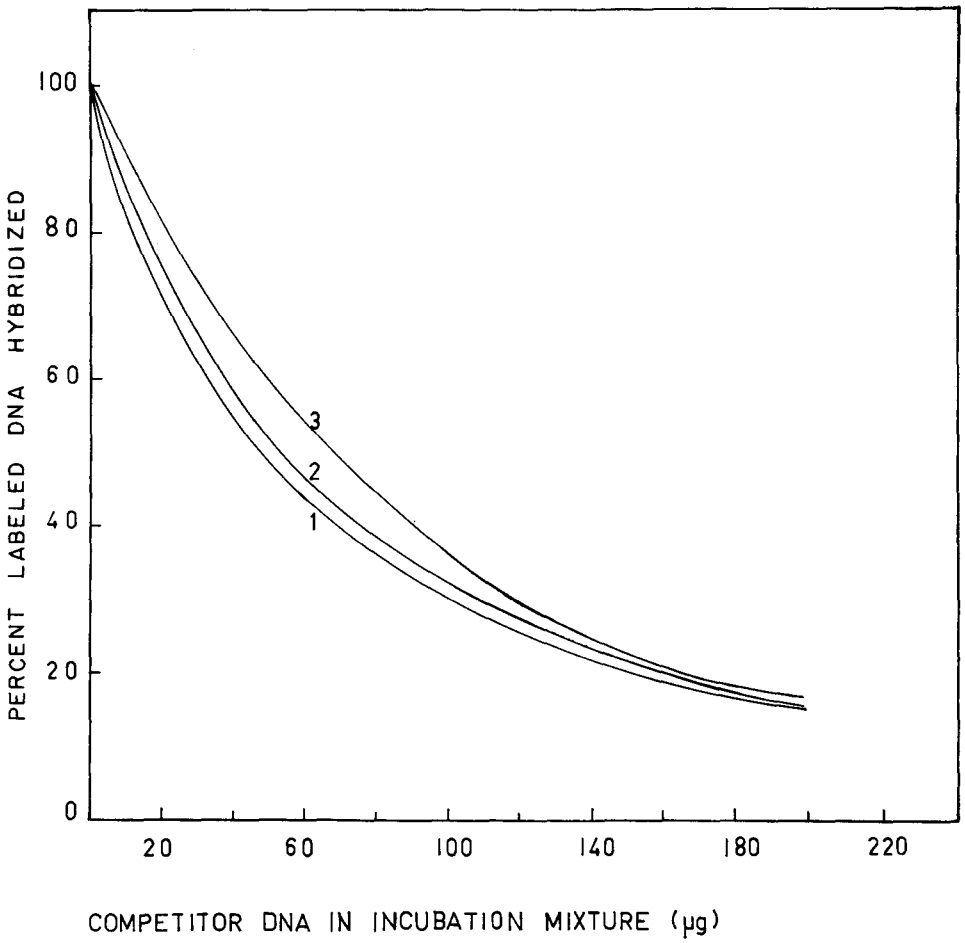


FIGURE 2.—Hypothetical graph showing:

1. Competition by unlabeled DNA fragments of species *X* in the reaction between labeled DNA fragments of species *X* and filter-bound DNA of species *X* (standard).

2. Competition by unlabeled DNA fragments of species *Y* in the reaction between labeled DNA fragments of species *X* and filter-bound DNA of species *X* (difference in DNA content per cell between species *Y* and *X* resides in lateral multiplicity).

3. Competition by unlabeled DNA fragments of species *Z* in the reaction between labeled DNA fragments of species *X* and filter-bound DNA of species *X* (difference in DNA content per cell between species *Z* and *X* resides in local multiplicity).

level was reached. At the plateau level, the amount of DNA hybridized increased at a relatively smaller degree with time.

2. At the plateau level the rates of reassociation of the DNAs of each of the six species were approximately the same. In other words, although the rates of reassociation of the DNA of each of the six species were significantly different initially (i.e., before the plateau level), they were approximately the same at the plateau level.

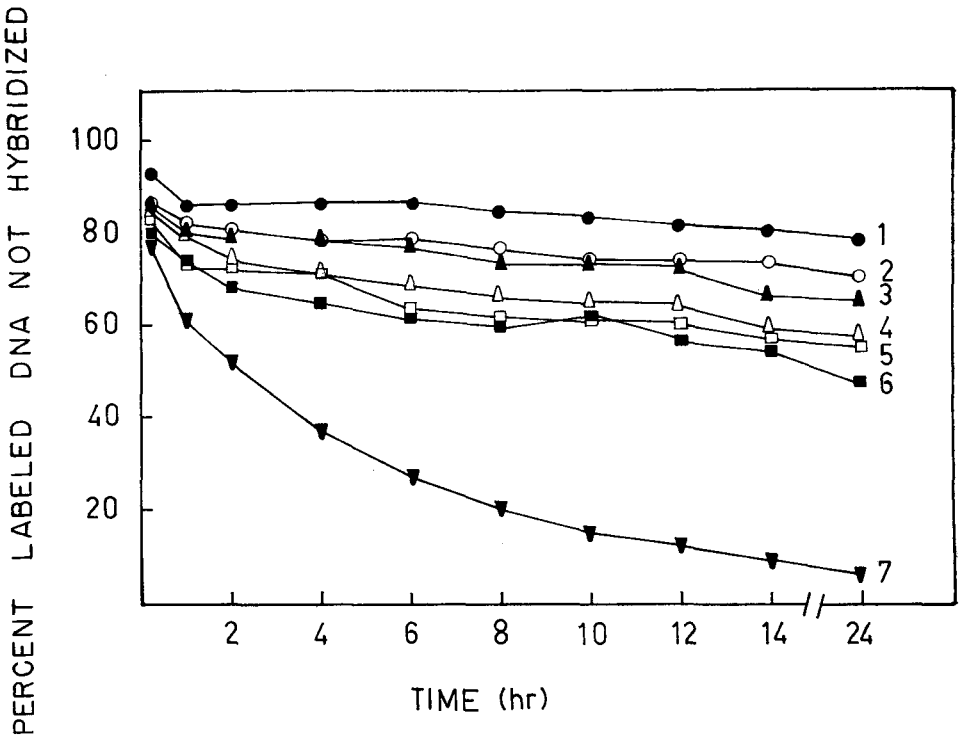


FIGURE 3.—The reassociation of the DNAs of six Vicia species and *E. coli:*

1 = *V. melanops*
2 = *V. sativa*
3 = *V. benghalensis*
4 = *V. atropurpurea*
5 = *V. faba*
6 = *V. narbonensis*
7 = *E. coli*

The reaction of sonicated labeled DNA with homologous filter-bound DNA was determined as a function of time. Each reaction consisted of the interaction of 4μg of sonicated, labeled, denatured DNA with 20 μg of denatured, homologous filter-bound DNA in 1.0 ml of 0.01 M Tris-HCl, 2 × SSC, pH 7.0. Incubations were carried out at 60°C. At specified times (from 10 min to 24 hr) filters were removed, washed as described in MATERIALS AND METHODS, and labeled filter-bound DNA was determined by liquid scintillation spectrometry. The percentage of labeled DNA fragments that was not hybridized to filter-bound DNA is plotted against time.

In the interpretation of the reassociation rates in Figure 3, it was assumed that the DNA that was hybridized before the plateau level probably consisted mainly of DNA that was highly repetitious. In this communication, the amount of "fast DNA" was arbitrarily defined as the percentage of labeled DNA hybridized to filter-bound DNA at 8 hr multiplied by 94%. (94% was the amount of labeled *E. coli* DNA hybridized in 24 hours.) A time interval of 8 hr was chosen because after 8 hr, most of the reassociation rates of the DNAs of the six species appeared to be fairly constant. The percentage of "fast DNA" in the genomes of each of the six species calculated in this way is shown in Table 1. Between 8 and 24 hr,

the rates of reassociation were similar for all six Vicia species, and it is simplest to assume that, had the experiment been continued to completion, they would have remained similar. BRITTEN and KOHNE (1968) showed that in their experiments with higher organisms, nonrepeated nucleotide sequences or nucleotide sequences repeated only a few times did not take a significant part in reassociation before 24 hr; it is probably this fraction that is involved at plateau level in all six species.

*Competition experiments: Interaction of* V. faba *DNA (root and shoot) with* V. faba *(root):* Except for *V. faba,* all Vicia species have very small amounts of root so that if only root tissue was used in DNA extraction, a large number of plants would have to be used. It was decided to determine if there was any difference between the competitive ability of root and shoot DNA, as it is known that plastids and mitochondria in shoot contain DNA (SHIPP, KIERAS and HASELKORN 1965; TEWARI *et al.* 1966).

Only root DNA was immobilized on the filter. Unlabeled root and unlabeled root and shoot DNAs were used to compete with labeled root DNA. The DNAs from root and shoot were indistinguishable as shown by the competition curves (Figure 4). Unlabeled root and unlabeled root and shoot DNAs competed equally well with the labeled DNA. Hence root and shoot DNA was used in all subsequent experiments.

The interaction of the DNA of *V. faba* with the DNAs of *V. atropurpurea, V. benghalensis, V. melanops, V. narbonensis, V. sativa, T. vulgare,* and *E. coli* are shown in Figures 5, 6, 7, 8, 9, and 10. The interaction of *V. benghalensis* DNA with *V. atropurpurea* DNA is shown in Figure 7.

## DISCUSSION

*Reassociation rates; "Fast DNA:"* Figure 3 shows that the DNA of *E. coli* had the fastest rate of reassociation (0.003 to 0.1 μg/min) since it had no repetitious DNA (BRITTEN and KOHNE 1968) and a very small genome (*E. coli* consists of $0.004 \times 10^{-9}$ mg of DNA per cell, CAIRNS 1963).

*V. faba* had approximately 15,000 times more DNA per genome than *E. coli* (*V. faba* contains $6.02 \times 10^{-9}$ mg of DNA per cell, MCLEISH 1963). The DNA of *V. faba* had a reassociation rate that varied from 0.001 to 0.07 μg/min. Table 1 shows that *V. faba* included 35% fast DNA.

Of all the DNAs of the six Vicia species studied, that of *V. narbonensis* had the fastest rate of reassociation (0.002 to 0.08 μg/min). Although *V. narbonensis* (RDC/cell = 54.5 ± 1.8) had only half as much DNA per cell as *V. faba* (RDC/cell = 100), its DNA had reassociation rates that were faster at certain times and similar at other times to those of *V. faba* DNA. This suggested that some of its nucleotide sequences were multiplied to a proportionally larger extent than those in *V. faba* DNA. Table 1 shows that *V. narbonensis* DNA included 38% fast DNA, while *V. faba* DNA included 35% fast DNA.

*V. atropurpurea* DNA had in general a slower reassociation rate (0.001 to 0.06 μg/min) than *V. faba* DNA. This suggested that some of the nucleotide sequences present in *V. atropurpurea* DNA (RDC/cell = 18.2 ± 0.9) were multi-

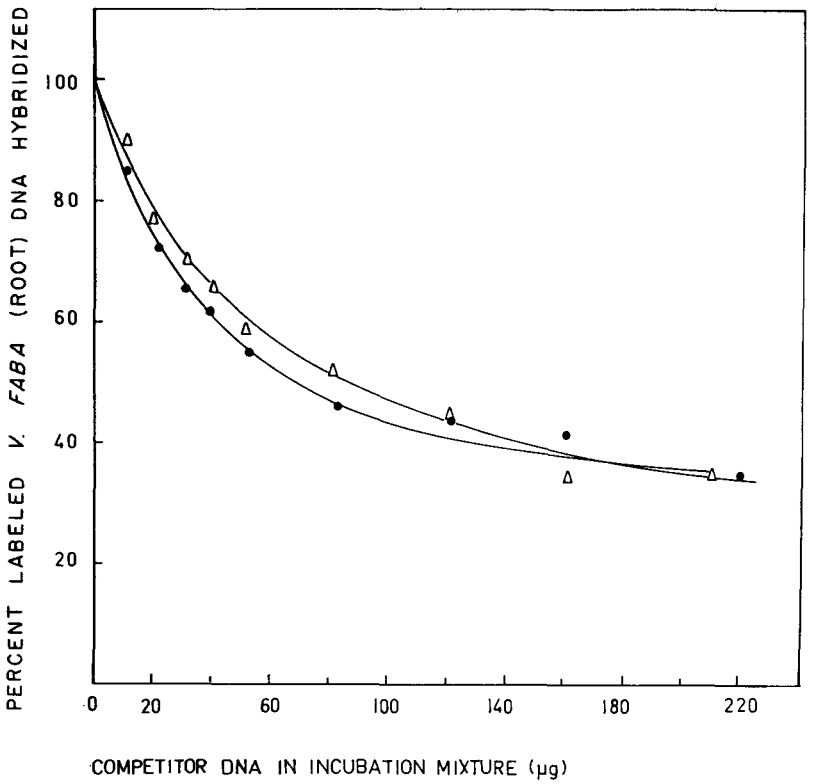


FIGURE 4.—Competition by unlabeled DNA fragments in the reaction between $^{32}$P-labeled *V. faba* (root) DNA fragments and *V. faba* filter-bound (root) DNA. Filters with *V. faba* (root) DNA (20 μg) were each immersed in 1.0 ml incubation mixture containing 4 μg of $^{32}$P-labeled *V. faba* (root) DNA fragments and 10 to 220 μg of *V. faba* (root or root and shoot) unlabeled DNA fragments. The procedure used was as described in MATERIALS AND METHODS. The percentage of $^{32}$P-labeled *V. faba* (root) DNA fragments hybridized to filter-bound *V. faba* DNA is plotted against the amount of competitor DNA in the incubation mixture.

(△—△)—Competition of *V. faba* (root and shoot) DNA with *V. faba* (root) DNA.

(●—●)—Competition of *V. faba* (root) DNA with *V. faba* (root) DNA.

TABLE 1

*The proportions of "fast DNA" in the genomes of six Vicia species*

| Species | Relative DNA content per cell ± standard error | "Fast DNA" | | Remainder of DNA | |
|---|---|---|---|---|---|
| | | Percentage of genome | Amount* | Percentage of genome | Amount* |
| *V. atropurpurea* | 18.2 ± 0.9 | 32(33.8 × .94) | 5.8 | 68 | 12.4 |
| *V. sativa* | 19.8 ± 1.0 | 22(23.3 × .94) | 5.4 | 78 | 14.4 |
| *V. benghalensis* | 26.2 ± 0.2 | 25(27.0 × .94) | 6.6 | 75 | 19.6 |
| *V. narbonensis* | 54.5 ± 1.8 | 38(40.3 × .94) | 20.7 | 62 | 33.8 |
| *V. melanops* | 86.1 ± 1.5 | 15(15.5 × .94) | 12.9 | 85 | 73.2 |
| *V. faba* | 100 | 35(37.8 × .94) | 35.0 | 65 | 65.0 |

* In arbitrary units; obtained by multiplying percentage of genome by relative DNA content per cell.

plied to a proportionally smaller extent than those in *V. faba.* Table 1 shows that *V. atropurpurea* DNA included 32% fast DNA, but *V. faba* DNA included 35% fast DNA.

In general, *V. benghalensis* DNA reassociated at a slower rate (0.001 to 0.06 μg/min) than those of *V. faba* and *V. atropurpurea.* This suggested that some of the nucleotide sequences in *V. benghalensis* DNA were multiplied to a proportionally smaller extent than those in *V. faba* and *V. atropurpurea.* Table 1 shows that *V. benghalensis* DNA included 25% fast DNA, *V. atropurpurea* DNA included 32% fast DNA, and *V. faba* DNA included 35% fast DNA.

The DNA of *V. sativa* had the second slowest rate of reassociation (0.001 to 0.05 μg/min). Table 1 shows that *V. sativa* DNA included 22% fast DNA.

Of all the DNAs of the six Vicia species studied, that of *V. melanops* (RDC/cell = 86.1 ± 1.5) had the slowest rate of reassociation (0.001 to 0.03 μg/min) despite the fact that its DNA content per cell was almost as large as that of *V. faba.* These results implied that unlike *V. faba,* the DNA of *V. melanops* probably included a larger number of nucleotide sequences which were multiplied to a smaller extent. Table 1 shows that *V. melanops* DNA included only 15% fast DNA, while the DNA of *V. faba* included 35% fast DNA.

*Remainder of the genome:* Since the reassociation rates of the DNAs of the six Vicia species were approximately the same as the plateau level, it was concluded that other than the fast DNA, the rest of the genome of each of the six Vicia species consisted of nucleotide sequences that were multiplied to proportionally the same degree. Table 1 shows that approximately 62% to 85% of the genomes of the six species studied, representing a five-fold variation in amount of DNA, was either not multiplied or, particularly in species with higher amounts, was evenly multiplied.

*Competition experiments; Interaction of* E. coli *DNA and* T. vulgare *DNA with* V. faba *DNA:* The DNAs of *E. coli* and *T. vulgare* were used as heterologous competitors. The presence of large quantities of competitor unlabeled *E. coli* DNA did not reduce the amount of $^{32}$P-labeled *V. faba* DNA hybridized. This indicated that *E. coli* DNA had no significant homology with *V. faba* DNA (Figure 5).

Some competition was, however, evident in the interaction of *V. faba* DNA with *T. vulgare* DNA (Figure 5). *T. vulgare* DNA showed 37% homology with *V. faba* DNA at 40 μg of competitor DNA, but this did not increase further with increasing concentrations of competitor DNA. This suggested that the major portion of the DNA of *T. vulgare* was different from the DNA of *V. faba.*

The results of the DNA-DNA competition experiments between the DNAs of the six Vicia species were divided into three groups:

*Group 1.* This group consisted of comparisons in which the species had DNAs which had basically similar nucleotide sequences; these were probably multiplied to proportionally the same (approximately the same) degree. This group included the reciprocal interaction of *V. faba* DNA with *V. atropurpurea* DNA (Figure 6), and of *V. atropurpurea* DNA with *V. benghalensis* DNA (Figure 7).

*Interaction of* V. atropurpurea *DNA with* V. faba *DNA:* Figure 6 shows that

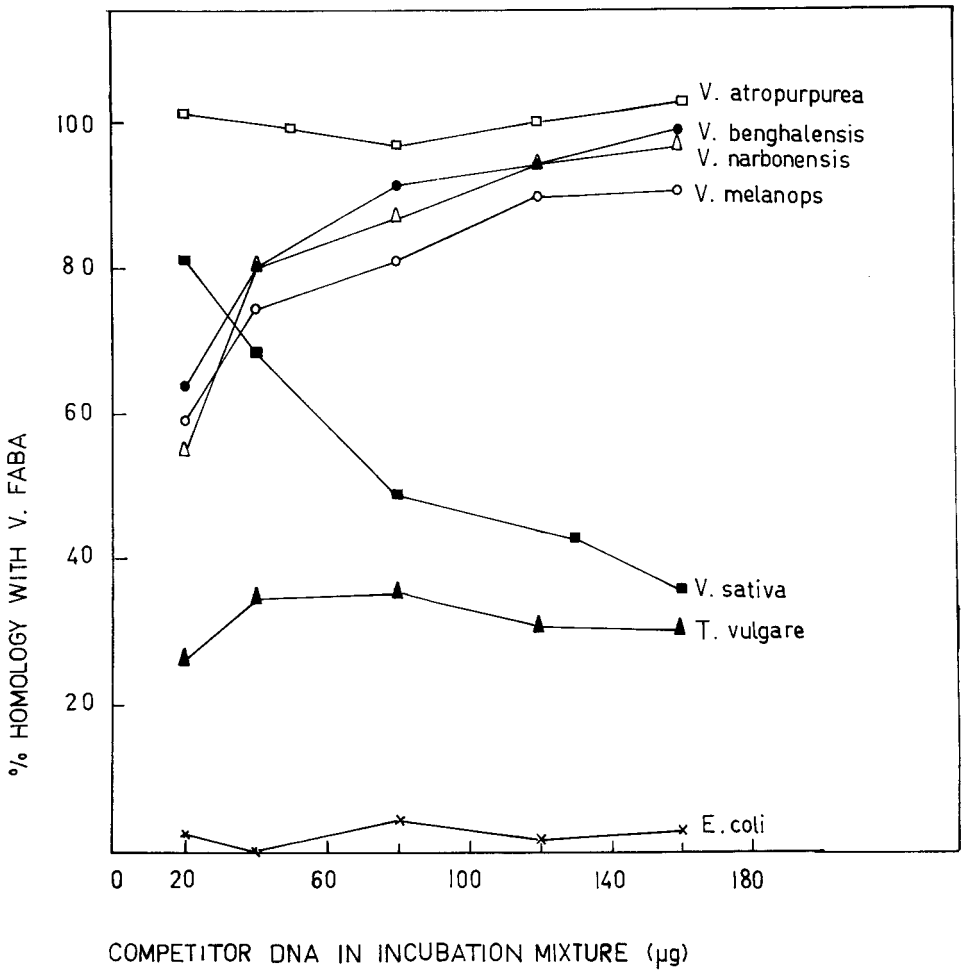


FIGURE 5.—The percentage homologies of the DNAs of five Vicia species, *T. vulgare*, and *E. coli* with the DNA of *V. faba* are plotted against the amount of competitor DNA in the incubation mixture. The percentage homologies of the DNAs of the five *Vicia* species, *E. coli*, and *T. vulgare* with the DNA of *V. faba* were calculated as described in *Interpretation of results* under MATERIALS AND METHODS.

although *V. faba* and *V. atropurpurea* DNAs had similar nucleotide sequences, the majority of which were multiplied proportionally to the same extent, a few could have been multiplied to a smaller extent in *V. faba*. There was some doubt that *V. faba* DNA (RDC/cell = 100) was an exact 5.5-fold multiple of *V. atropurpurea* DNA (RDC/cell = 18.2 ± 0.9). The interaction of *V. atropurpurea* DNA with *V. sativa* DNA reinforced this possibility, since *V. atropurpurea* DNA competed with labeled *V. sativa* DNA significantly better than *V. faba* DNA (Figure 6). Reassociation rate experiments indicated, however, that some nucleotide sequences in *V. faba* DNA were probably multiplied to a proportionally

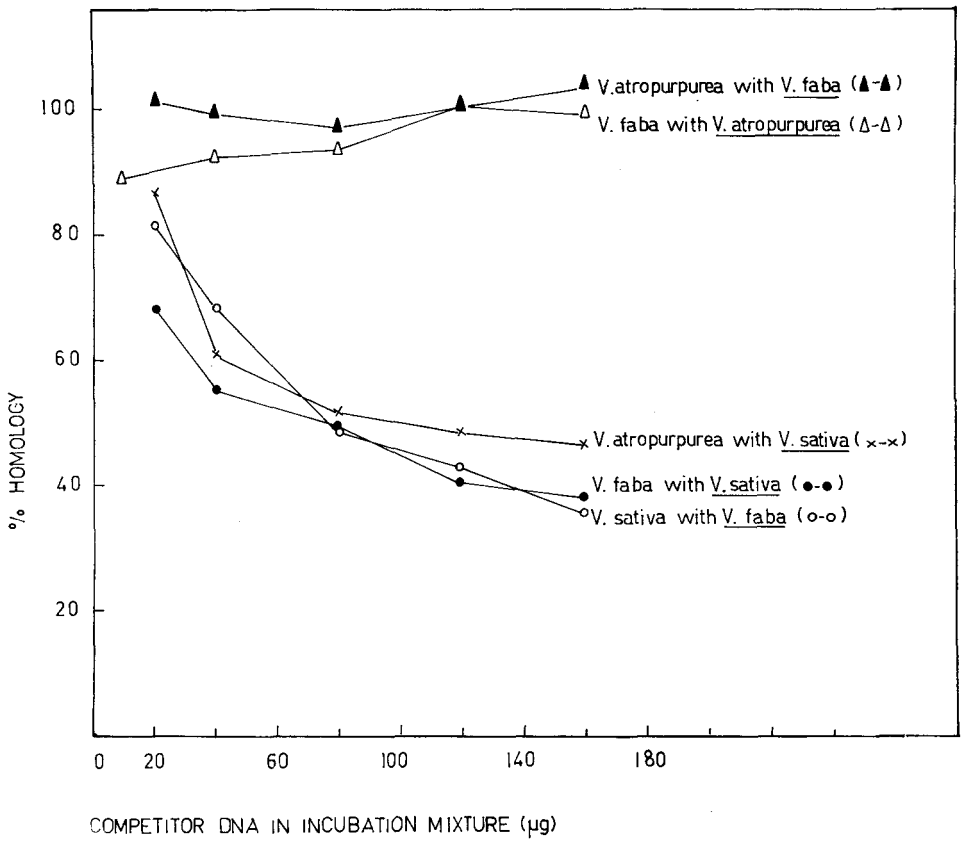


FIGURE 6.—The percentage homologies of the DNA of *V. atropurpurea* with the DNA of *V. faba; V. faba* with *V. atropurpurea*; *V. atropurpurea* with *V. sativa; V. faba* with *V. sativa;* and *V. sativa* with *V. faba* were calculated as described in MATERIALS AND METHODS at 20, 40, 80, 120, and 160 μg of competitor DNA added to the incubation mixture. These percentage homologies are plotted against the amount of competitor DNA in the incubation mixture. The DNA that is filter-bound is underlined.

larger extent than those in *V. atropurpurea.* This inconsistency was attributed to the assumption that *V. atropurpurea* DNA probably included a smaller number of nucleotide sequences that were multiplied to a larger extent than those in *V. faba,* while *V. faba* included a larger number of nucleotide sequences which were multiplied to a smaller degree.

*Interaction of* V. benghalensis *DNA with* V. atropurpurea *DNA:* Figure 7 shows that although the majority of the nucleotide sequences in *V. atropurpurea* DNA was multiplied to proportionally the same degree in *V. benghalensis* DNA, some of the nucleotide sequences could have been multiplied to a higher degree in *V. atropurpurea.* These results were consistent with those obtained from reassociation rates. *V. benghalensis* is regarded as being taxonomically synony-

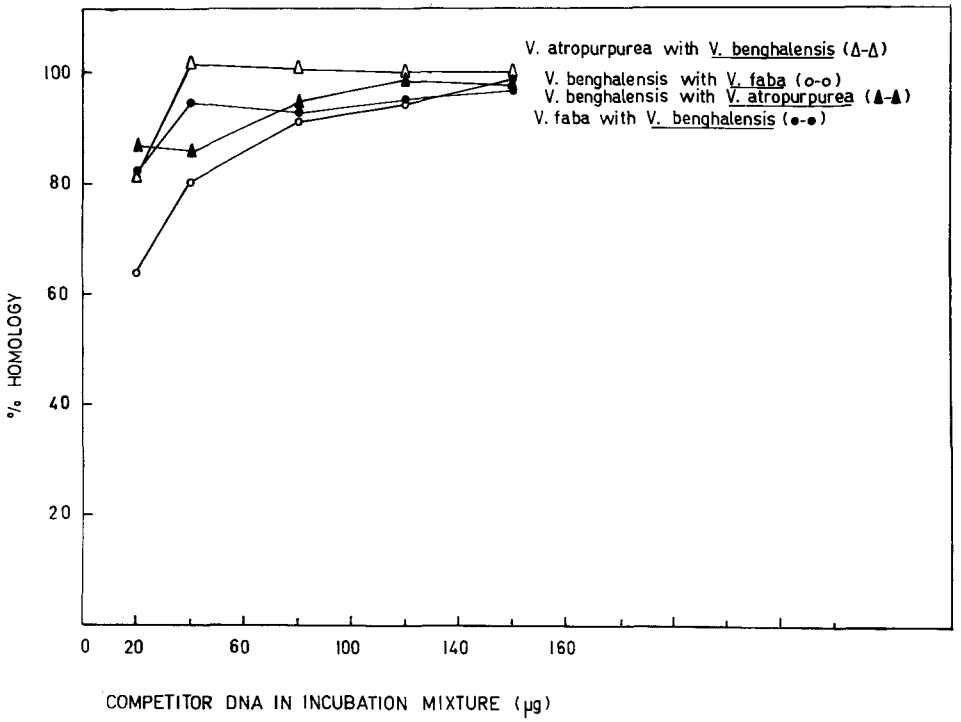


FIGURE 7.—The percentage homologies of *V. faba* DNA with *V. benghalensis* DNA, *V. benghalensis* DNA with *V. faba* DNA, *V. benghalensis* DNA with *V. atropurpurea* DNA, and *V. atropurpurea* DNA with *V. benghalensis* DNA are plotted against the amount of competitor DNA in the incubation mixture. The percentage homologies were calculated as described in MATERIALS AND METHODS at 20, 40, 80, 120, and 160 μg of competitor DNA in the incubation mixture. The DNA that is filter-bound is underlined.

mous with *V. atropurpurea* (METTIN and HANELT 1968). If this is true, *V. benghalensis* DNA (RDC/cell = 26.2 ± 0.2) might include smaller multiplications of a larger number of nucleotide sequences while *V. atropurpurea* DNA (RDC/cell = 18.2 ± 0.9) might include higher multiplications of fewer nucleotide sequences.

*Group 2.* This group of comparisons involved species with DNAs which had basically similar nucleotide sequences. These nucleotide sequences were, however, probably not multiplied to proportionally the same extent. This group included the reciprocal interaction of *V. faba* DNA with the DNAs of *V. melanops*, *V. benghalensis*, and *V. narbonensis*.

*Interaction of* V. melanops *DNA*, V. benghalensis *DNA, and* V. narbonensis *DNA with* V. faba *DNA:* The results (Figures 7, 8, 9, and 10) show that the majority of the nucleotide sequences in the DNA of *V. faba* was probably found in the DNAs of *V. melanops* (RDC/cell = 86.1 ± 1.5), *V. benghalensis* (RDC/cell = 26.2 ± 0.2), and *V. narbonensis* (RDC/cell = 54.5 ± 1.8). Many of these nucleotide sequences were, however, probably multiplied to a proportionally

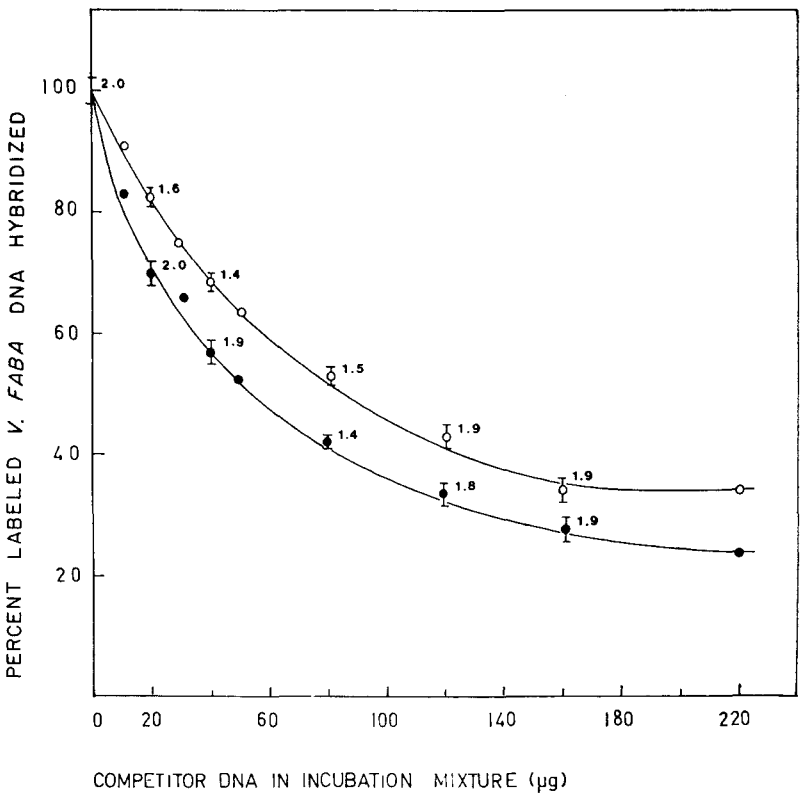


FIGURE 8.—Competition by unlabeled *V. melanops* DNA fragments in the reaction between $^{32}$P-labeled *V. faba* DNA fragments and *V. faba* filter-bound DNA. Filters with 20 μg of *V. faba* DNA were each immersed in 1.0 ml incubation mixture consisting of 4 μg of $^{32}$P-labeled *V. faba* DNA fragments and 10 to 220 μg of heterologous *V. melanops* DNA (O—O) or homologous *V. faba* (●—●) unlabeled DNA fragments. In this experiment 10 filters containing 20 μg of DNA each were used in the hybridization at each concentration of competitor DNA. The rest of the procedure was as described in MATERIALS AND METHODS. The standard errors of the mean percentage homologies at the various concentrations of competitor DNA were calculated as described in MATERIALS AND METHODS. The percentage of $^{32}$P-labeled *V. faba* DNA fragments hybridized to filter-bound *V. faba* DNA is plotted against the amount of competitor DNA in the incubation mixture.

higher degree in *V. faba* (RDC/cell = 100). In the case of *V. melanops* DNA and *V. benghalensis* DNA this was supported by the finding that *V. faba* DNA included a higher proportion of fast DNA than both *V. melanops* DNA and *V. benghalensis* DNA. Reassociation rate experiments showed, however, that *V. narbonensis* DNA included a higher proportion of fast DNA than *V. faba* DNA (Table 1). No satisfactory explanation could be found for this inconsistency.

*Group 3.* In this group of comparisons, the species involved appeared to have a small proportion of their nucleotide sequences similar. The degree of multipli-

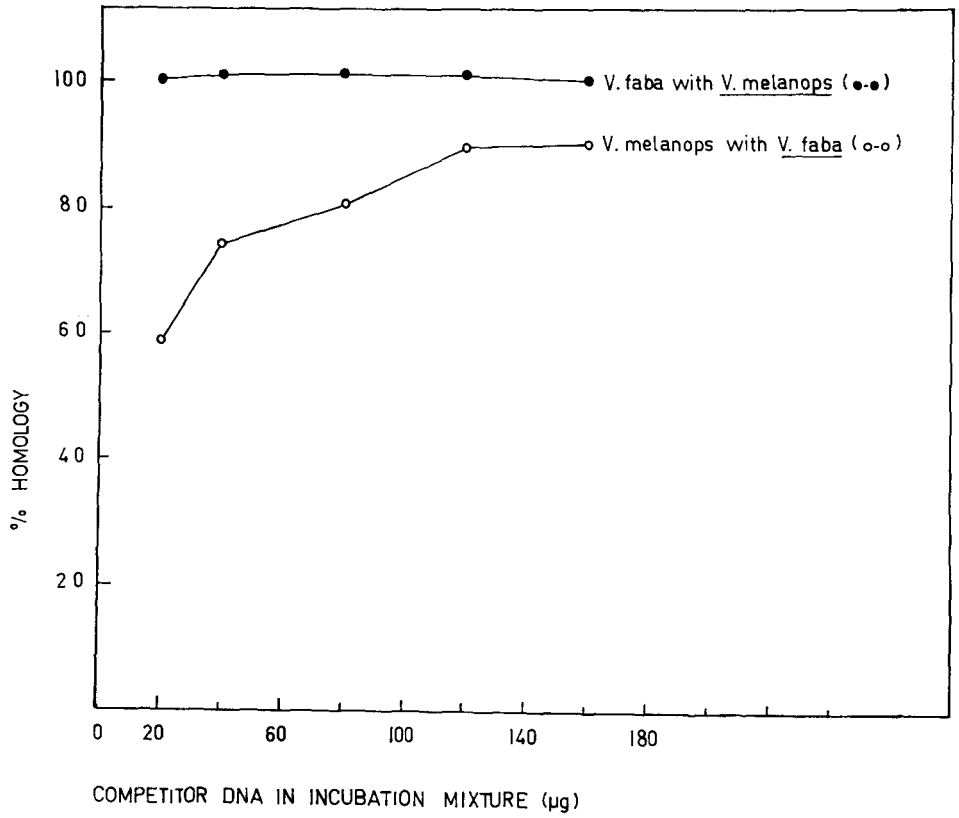


FIGURE 9.—The percentage homologies of *V. faba* DNA with *V. melanops* DNA and those of *V. melanops* DNA with *V. faba* DNA are plotted against the amount of competitor DNA in the incubation mixture. The percentage homologies were calculated as described in MATERIALS AND METHODS at 20, 40, 80, 120, and 160 μg of competitor DNA in the incubation mixture. The DNA that is filter-bound is underlined.

cation of these nucleotide sequences was probably different. The reciprocal interaction of *V. sativa* DNA with *V. faba* DNA (Figure 5) was placed in this group.

*Interaction of* V. sativa *DNA with V. faba DNA:* Figure 5 shows that a large part of the nucleotide sequences in *V. sativa* DNA was different from those of *V. faba* DNA. The high percentage of nucleotide sequence homology shared by the two species at low concentrations of competitor DNA was probably due to the presence of repeated nucleotide sequences. It is not known why these two closely related species should have such a large difference in nucleotide sequences. Since amino acid sequences in the proteins of *V. faba* and *V. sativa* would probably be similar on the average, it was probable that the large difference in homology could be explained by the divergence of the third base of many codons. This could take place without alterations in protein sequence if base substitutions resulted in synonymous codons. Third-position changes are presumably subjected to less direct selection (WALKER 1968; KING and JUKES 1969).

This hypothesis is in accord with two previous suggestions relating to closely related species both of rodents (WALKER 1968) and of Drosophila (LAIRD and MCCARTHY 1968).

As a result of a consideration of the reassociation rates and the degree of homology between the nucleotide sequences of the six Vicia species at the various concentrations of competitor DNA, it is evident that the differences in DNA content per cell between the six Vicia species reside mainly in the degree of repetition of nucleotide sequences. The evidence presented indicated that the increase in DNA per cell could result from an increase in either fast DNA or the remainder of the genome (nonfast DNA), or both. The amount of DNA per cell is no indication of the proportion of these two DNAs in the genome. In other words, species with small relative DNA contents per cell (e.g., *V. atropurpurea*) could have relatively high percentages of fast DNA while species with relatively large DNA contents per cell (e.g., *V. melanops*) could have relatively low percentages of fast DNA. The explanation of this probably lies in the mechanism of increase in DNA per cell in Vicia species. The evidence presented indicated that between 15 and 38% of the nucleotide sequences of the genome involve high and uneven multiplication. The remaining 85 to 62% involves more or less even multiplication. The amounts of DNA (in arbitrary units) involved in even multiplication are shown in the last column of Table 1. Thus in comparison with *V. atropurpurea*, *V. sativa*, and *V. benghalensis*, the degree of even multiplication is in the order of 2- and 3-fold for *V. narbonensis* and 4- to 5-fold for *V. melanops* and *V. faba*.

It has been shown (CHOOI 1971) that species in two sections (Ervum and Cracca) of the genus Vicia had DNA contents per cell which form a continuous distribution. In two other sections (Vicia and Faba), however, larger differences in DNA contents per cell were found between species. Furthermore, these DNA values approximated to a 1:2:4 series. Can a single explanation be given to account for variation in DNA content per cell in Vicia species? If, as has been discussed, the 15 to 38% of "fast DNA" in the six species studied represents highly repeated nucleotide sequences, it seems most probable that these have evolved by local multiplicity; in particular, by the mechanism proposed by KEYL (1965). This mechanism could also account for the 5-fold variation in nonfast DNA; local multiplicity here being in the order of 2-, 4-, or 8-fold. However, it is clear from the uniformity of chromosome size within a species that a very large number of loci must be involved, and these must be distributed throughout the chromosomes. Moreover, to account for the tendency for average chromosome sizes to fall into a 1:2:4 series in the sections Vicia and Faba, one must postulate that natural selection tends to favor evenly multiplied genomes. The alternative to this last explanation is that in these sections of the genus, lateral multiplicity has also been a factor in evolution. Several different lines of evidence have pointed to *Vicia faba* having multistranded chromosomes (PEACOCK 1963; TAYLOR 1957; DEAVEN 1968; HEDDLE 1969). Just as lateral multiplicity cannot yet be excluded, so it is necessary to recognize that local multiplicity may well be supplemented by large segmental duplications such as occur

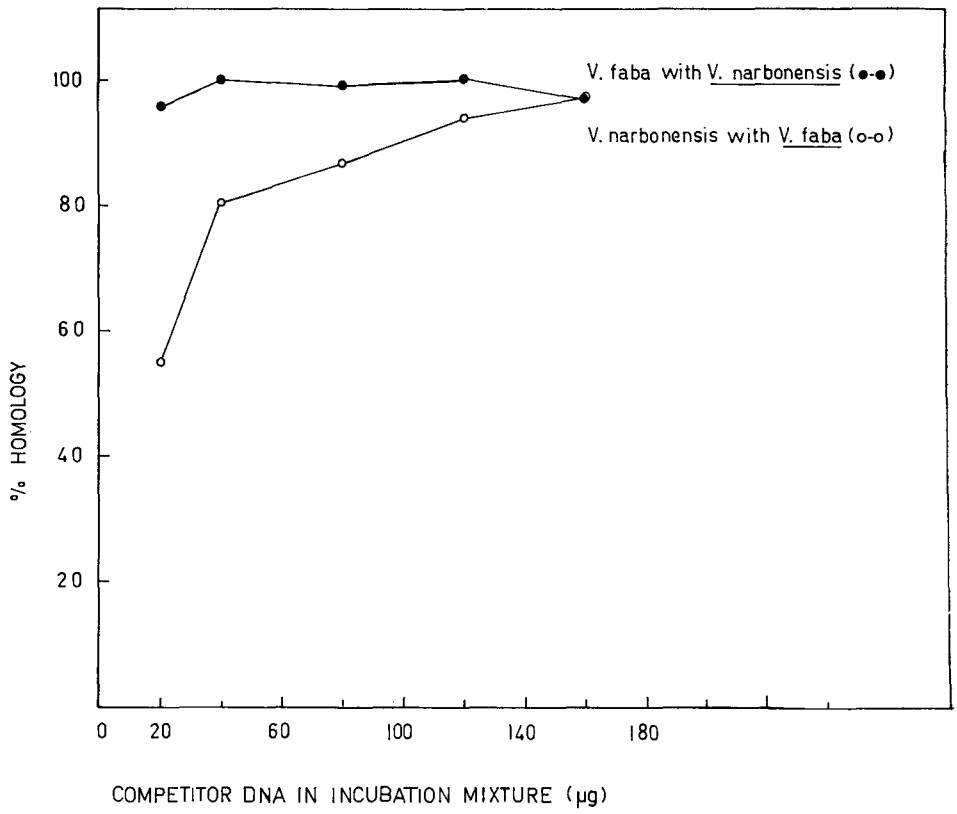


FIGURE 10.—The percentage homologies of *V. faba* DNA with *V. narbonensis* DNA and those of *V. narbonensis* DNA with *V. faba* DNA are plotted against the amount of competitor DNA in the incubation mixture. The percentage homologies were calculated as described in MATERIALS AND METHODS at 20, 40, 80, 120, and 160 µg of competitor DNA in the incubation mixture. The DNA that is filter-bound is underlined.

in both Lolium (REES and JONES 1967) and Allium (JONES and REES 1968). It is probable, therefore, that local multiplicity could probably also account for the small increases in DNA per cell which were found between species in the sections Ervum and Cracca. Similarly, lateral multiplicity could probably also account for the larger increases in DNA per cell which were found between species in the sections Vicia and Faba.

This project was suggested to me by Professor P. G. MARTIN, and I wish to thank him for his criticisms. I am also very grateful to Dr. R. H. SYMONS for criticisms.

## SUMMARY

The DNAs of six species of the genus Vicia which have up to a six-fold variation in DNA content per cell have been compared with respect to nucleotide sequences. Except in *V. sativa*, where some of the nucleotide sequences have

probably undergone nucleotide sequence divergence, all the other five species studied have basically similar nucleotide sequences. The degree of repetition of nucleotide sequences is different in the six species. In general, 15 to 38% of the genomes of the six species has been unevenly multiplied and makes up the "fast DNA" of these species. However, the remainder of the genome of these six species has either not been multiplied at all or, in the species with large chromosomes, has been multiplied evenly and to a small extent. The evidence presented has been discussed in relation to different mechanisms for DNA increase.

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