EVOLUTION IN THE GENUS GOSSYPIUM

WITH SUPPLEMENTARY OBSERVATIONS ON OTHER GENERA

A thesis submitted to the University of Adelaide as a requirement for the Degree of Doctor of Philosophy

by

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DECLARATION.

This thesis, to the best of my knowledge, contains no material previously submitted for a degree in any University either by me or by any other person, except when due reference is made in the text of the thesis.

Z.B.A. KADIR.

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SUMMARY

1. Measurements of relative DNA contents in the genus Gossypium have been carried out in 25 species including three amphiploids. Between the diploid species with the lowest and highest values there is more than a two-fold difference without variation in chromosome number (2n=26).

2. The DNA values within genomes are relatively uniform and continuously distributed. Values between genomes may be partially disjunct. Genome averages are D = 57.7, B = 78.9, E = 87.3, A = 95.3 and C = 114.5.

3. G. longicalyx and G. bickii have DNA values very different from other species of E and C genomes respectively, and their positions in these genomes are discussed.

4. At the genomic level, DNA contents and chromosome lengths are related.

5. The trend in evolution in the diploid species is more likely to be towards increase in DNA content rather than decrease.

6. Statistical tests suggested significant variation between individuals within a cultivated amphiploid species and possibly within two diploid species but not within three other wild diploids.

7. Observed DNA values in three amphiploids are different from each other; they have been compared with those expected from different combinations of diploid parents. If A and D genome species were the diploid progenitors, they must have been different from present species or there have been changes in DNA content since hybridization.

8. The DNA values in two species of Thespesia, closely related to Gossypium and also with 2n=26, showed 3.6-fold variation.

9. DNA estimations made for 12 species of the genus Phalaris reveal a 4-fold variation in DNA content per cell. The genus is divided into four groups, two diploid and two polyploid, and within each group DNA contents vary continuously in contrast to the distribution between the two groups of diploid species. The average DNA content in species with 2n=12 is 257.3 while in species with 2n=14 the average is 127.7.

10. Other observations in the genus Phalaris are:

    (a) No intra-specific variation has been observed.

    (b) The DNA content and chromosome lengths do not reveal a linear relationship; rather it could be reflected in the diameter of the chromosomes.

    (c) The trend in evolution seems to be unidirectional and towards increase in DNA content.

11. Studies of DNA distribution during gametogenesis in Phalaris suggest significant variation between tetrads as against within tetrads.

CHAPTER 1.

INTRODUCTION

Nuclear DNA forms the basis of genotype and therefore any genetic variation should be reflected in nuclear DNA constitution. This variation can be either qualitative or quantitative or both. It appears from a survey of the literature that the evolution of complex forms of life (Eukaryotes) from the simpler forms (Prokaryotes) is achieved or accompanied by quantitative increase in nuclear DNA content per cell. The total DNA content increases from bacterium to mammal by a factor of 10^3 (McCarthy, 1969; Mirsky, 1950-1951; Mirsky and Ris, 1951). This can be understood because there is increased structural and functional complexity from Prokaryotes to Eukaryotes. In Eukaryotes the total information required is greater than in Prokaryotes to synthesise a greater number of different proteins, and hence a greater amount of nuclear DNA is required.

However, when one relates DNA content with the complexity between and within taxonomic groups a systematic increase in DNA content with the increase in complexity or evolutionary advancement is not apparent. It is worth noting that angiosperm species in general have higher DNA content per nucleus than mammals (Sneath, 1964). The values of amphibia are higher

than mammals (Ohno and Atkin, 1966; Mirsky and Ris, 1951). Similar inconsistencies exist between and within other groups of the animal kingdom (Atkin and Ohno, 1967; Mirsky and Ris, 1951; Ohno and Atkin, 1966; Ullerich, 1966, 1967; Hinegardner, 1968).

Examples from plant kingdom where DNA values increase irrespective of the evolutionary advancement are abundant, (Rothfels, Sexmith, Heimburger and Krause, 1966; Rothfels and Heimburger, 1968; Southern, 1967; Halkka, 1964; Sparrow and Evans, 1961; McLeish and Sunderland, 1961; Rees, Cameron, Hazarika and Jones, 1966; Martin, 1966; Baetke, Sparrow, Nauman and Schwemmer, 1967).

Another striking aspect of this evolutionary change in DNA content is that, on the one hand, within taxonomic orders of placental mammals, reptiles and birds the amounts of DNA per cell do not differ more than 10% (Atkin et al. 1965), while on the other, many-fold differences have been recorded between related species within a family or within a genus. These changes may occur irrespective of the change in chromosome number. At the family level, ten-fold differences have been reported within both, the Liliaceae and Papilionaceae (McLeish and Sunderland, 1961; Sunderland and McLeish, 1961), while eighty-fold differences have been reported within the Ranunculaceae (Rothfels et al. 1966) and Droseraceae (Rothfels and Heimburger, 1968).

At the generic level, a number of examples may be quoted: Vicia, Lathyrus and Lolium (Rees et al. 1966; Martin and Shank, 1966); Allium species (Jones and Rees, 1968); Eriocephalus species (Rothfels et al. 1966); Luzula species (Halkka, 1964); Thyanta species (Schrader and Hughes-Schrader, 1958); Enchytraeus species (Christensen, 1966). An intra-specific difference of 1.6 for Picea glauca and 1.5 for Pinus banksiana has been reported (Miksche, 1968). McLaren et al. (1966) has reported a large form of Pseudo-calanus minutus which develops more slowly in nature though otherwise the large forms are indistinguishable morphologically. The chromosome number is the same (n=16). The chromosome size is much larger and there is a ratio of 7:1 in DNA content. These comparisons of nuclear DNA content have provided useful information and have allowed inferences regarding:

1.) Chromosome structure at the macromolecular level and the possible mechanism to account for such quantitative variation in DNA content. (Schrader and Hughes-Schrader, 1958; Rothfels et al. 1966; Keyl, 1965).

2.) Species relationship (Bick and Jackson, 1967),

3.) tracing the ancestry particularly where hybridization and amphiploidy are involved (Rees, 1963).

1.1 Chromosome ultra-structure: Multistranded vs single stranded:

Light microscopists have frequently interpreted the chromosomes as being multistranded. The anaphase split has been observed by a number of workers including Kaufmann, (1948, 1957); Kaufmann, Gay and McDonald (review, 1960); Gay (1956). In favourable material the split can be found even in prophase and metaphase (Maguire, 1966). The sub-chromatid split has been made to appear more clearly in chromosomes treated to decrease the degree of mitotic compaction. Brooke et al. (1962) studied human chromosomes treated with low concentration of potassium chloride and reported subunits of the chromatids. Similar results have been obtained with different treatments in different organisms, (Trosko and Brewen, 1966; Trosko and Wolff, 1965; Wolff, 1965a, 1965b; Martin, 1968). Proponents of single-stranded chromosome structures have often attributed such visible sub-chromatid splits under light microscope to fixation artifacts. Bajer (1965) has however, taken motion pictures of living chromosomes in the endosperm of Haemanthus. These motion pictures of living cells show metaphase chromosomes split into half chromatids. Since these studies are carried on in living cells it cannot be a fixation artifact, though an optical illusion is not excluded.

Lately the study of the chromosomes has shifted

from a purely cytogenetic level to chemical, histochemical and ultra-structure studies. However, evidence from all sources combined has not settled the question of strandedness and no final concept of its structure at the macromolecular level has yet evolved.

As a result of quantitative studies of nuclear DNA two basic types of chromosome models have been proposed; which can accommodate large differences in DNA content:

a.) A single-stranded chromosome model based on large single DNA molecules (Keyl, 1965; Callan, 1963, 1967; Whitehouse, 1967). This model assumes extensive longitudinal repetition of genetic units, producing more-or-less a continuous interspecific variation in DNA values.

b.) A multistranded model which assumes that whole chromosomes become multiplied (Ris, 1961, 1966, 1969; Osgood et al. 1964; Trosko and Wolff, 1965; Sparvoli, Gay and Kaufmann, 1965; Uhl, 1965 and Kaufmann et al. 1956, 1960). A variation of this hypothesis, viz. "linear multiplicity where chromosomes are replicated and joined end to end" has been suggested. Lateral multiplicity or linear multiplicity is more likely to produce multiple classes in the DNA values of related species.

Interpretation of chromosome structure based on quantitative studies of nuclear DNA content was initiated by the Schraders (Hughes-Schrader, 1957; Hughes-Schrader and Schrader, F., 1956; Schrader and Hughes-Schrader, 1956, 1958). These workers reported their studies in insects of the order Homoptera and Hemiptera. Multiple classes in DNA values were explained on the basis of multistrandedness by these workers. In plants in the genus Luzula (Mello-Sampayo, 1961; and Halkka, 1964) a similar interpretation was advanced. The above interpretation of multiple classes of DNA values was criticised by Swift, (1962) because the organisms mentioned above have diffuse centromeres and therefore variation in chromosome number could be brought about by fragmentation. Since then several examples of related species with localised centromeres have been reported to have multiple classes of DNA values. A six-fold difference has been reported in the genus Vicia (Martin and Shank, 1966). An eighty-fold difference in the family Ranunculaceae was reported by Rothfels et al. (1966). Rothfels et al. further recorded that the number of chromosomes is essentially unchanged and the karyotype is sometimes preserved in spite of the large changes in DNA content. Rothfels and his coworkers have explained such a vast change in DNA content mainly on the basis of multistranded-ness; however, it is suggested that values in some species which

do not fit into the proposed progression may be due to the fact that local multiplicity, as suggested by Keyl (1965), may also be operating simultaneously. Approximately 1:2:4:16 distributions of DNA content in sundews (Droseraceae) are compatible with multi-stranded chromosomes (Rothfels and Heimburger,1968).

On the other hand, support for a single-stranded chromosome model comes from continuous variation in DNA content between related species. Variation of 27% in DNA content in the two species of Allium has been interpreted on the basis of length-wise incorporation or loss of chromosomal segments (Rees and Jones, 1967). Apparent non-disjunct DNA values between inbreeding and outbreeding species of Lolium has been interpreted by longitudinal duplication although lateral multiplicity is not ruled out (Rees et al. 1966, 1967). Additional evidence for local multiplicity within a single stranded chromosome comes from the study of Keyl (1965). Keyl and Pelling (1963) and Keyl (1965) reported a ratio of 1.27 in DNA content between two sub-species of Chironomus thummi (i.e. thummi and piger). This difference exists both in polytene nuclei and mitotic cells. Keyl found in hybrids between the two sub-species that salivary gland chromosomes of thummi and piger showed corresponding individual bands with different DNA contents. The difference in DNA content form a geometric series of 1, 2, 4, 8, or 16. No intermediate values were found. In the

absence of intermediate values Keyl (1965) does not believe that increase arose from unequal crossing-over. Unequal crossing-over has however, been reported in Drosophila (Sturtevant, 1925). The observed geometric increase can best be explained by repeated duplication of DNA at individual loci and is evidence for localised multiplicity of genetic information within a single stranded chromosome (local multiplicity). Keyl proposed a mechanism involving mis-replication of a backbone which holds DNA loops. Ullerich (1966, 1967, 1970) has also interpreted the DNA changes among the European toads (Bufo species) in the same way. Support for a single stranded chromosome has come from a number of other experiments. Gall's (1963b) studies of the kinetics of DNase digestion on the lampbrush chromosomes of amphibia, which have high DNA content, support a single Watson-Crick double helix. Further, under the electron microscope the diameter of the strand (60-80A^{o}) including the associated nucleoproteins, does not exceed the theoretical diameter by enough to allow for many double helices arranged side by side.

Callan and MacGregor (1958) distinguished the effects of trypsin and pepsin which digests the proteinaceous surface coat while DNase causes breaks and fragmentation of the axis and lateral loops. This suggests that DNA is responsible for the linear continuity of chromosomes. Gall (1963a) concluded that loops are

composed of two digestible units (one double helix) while the axis contains four units (two double helices). It may, however, be mentioned that lampbrush chromosomes occur only in meiotic prophase and support an active synthesis of RNA and proteins, hence it is not certain how far they represent typical mitotic chromosomes. Miller (1965) reported a core of 34A^{o} in the loops after digesting the RNA and proteins and this supports the idea that there could be only one DNA double helix.

Evidence both for and against the multistranded hypothesis has been obtained from a number of other lines of investigation. Radiation-induced aberrations at different stages of the cell cycle have been interpreted as favouring multistranded chromosomes. Aberrations induced in prophase or early metaphase appear as half-chromatid exchanges. These aberrations take the form of side arm bridges (Swanson, 1947; Crouse, 1954; Wilson et al. 1959; Sax and King, 1955; Davidson, 1957; Mitra, 1958). Peacock, (1961) and Heddle (1969) reported that half-chromatid aberrations following replication appear as full chromatid aberrations. This suggests that the anaphase chromatid not only appears to be multi-stranded but is also functionally multistranded. Comings (1970) however, emphasizes that half-chromatid aberrations may just as easily arise on the basis of a single-stranded chromosome. Aberrations induced early in interphase (The G1 phase) affect both

chromatids of a chromosome at the subsequent metaphase as if it were a single structure. During DNA synthesis and post-synthesis interphase (G2) the chromosome reacts to radiation as if it had already replicated into two chromatids and subsequently chromatid aberration, rather than chromosome aberrations, appear. The semi-conservative distribution of label in successive cell generations reflects the semi-conservative replication of DNA (Taylor et al. 1957; Meselson and Stahl, 1958). This is explained in terms of chromosomes having single DNA double helices. However, the phenomenon of iso-labelling (chromosomes with label on both chromatids at the second mitosis after labelling) reported by LaCour and Pele (1959) and Peacock (1963) supports a model with more than one strand per chromosome. Comings (1971) suggests that iso-labelling is not incompatible with single-stranded models. Models have even been constructed in which semi-conservative distribution of labelled chromatids is possible in a multistranded chromosome (Steffenson, 1961; Cavalier and Rosenberg, 1961; Peacock, 1963; Trosko and Wolff, 1965 and Uhl, 1965).

Electron microscopic studies of thin sections and chromosomes spread on water surface have revealed that chromosomes contain a multitude of fibres and are highly complex structures. There is no major disagreement regarding the

occurrence of 250A° fibres, but the information on its ultimate structure and arrangement within mitotic chromosomes is varied (Ris, 1961, 1962, 1966, 1967, 1969; Ris and Chandler, 1963; S. Wolfe, 1965a, 1965b, 1967, 1969; Wolfe and Martin, 1968; Wolfe and John, 1965a, 1965b; Wolfe and Hewitt, 1966; J. Wolfe, 1967; Gall, 1956, 1963a, 1963b, 1966; DuPraw, 1965a, 1965b, 1965c; Rae, 1966; Kaye and McMaster-Kaye, 1966; Miller, 1965; Kaufmann and McDonald, 1956; Kaufmann et al. 1960; Martin, 1968). In general thin sections of nuclei and chromosomes show thinner fibres (around 100A°) while isolated chromosomes show fibres around 250A°. Wolfe and Grim (1967) suggest that 250A° fibre is an artifact. A number of factors such as side by side packing of 100A° fibres, differential coiling, folding of a single 100A° fibre or chemical combination of a 100A° fibre with the surrounding matrix may be responsible for the increase in diameter. Ris (1969) has shown that 100A° fibres can be reduced to thinner fibres and that the thinner fibres approach the theoretical diameter of the DNA double-helix. The idea of multiple-fibre cores within 100A° fibres or even within 250A° fibres is not supported by others (Abuelo et al. 1969; DuPraw, 1965b; Wolfe, 1965b and Gall, 1966). Barnicot and Huxley (1965) did not record duplex structure in human metaphase chromatids prepared either in whole mount or in thin sections. DuPraw (1966a) has also studied

unsectioned human and honey-bee chromosomes. The chromatin fibres of these chromosomes are not organised into half- or quarter-chromatids. From the above it can be seen that there is no consensus clearly favouring either a multistranded or single-stranded composition of Eukaryotic chromosomes.

1.1.1 Chromosome models and genetic redundancy:

Both lateral multiplicity and local multiplicity point towards genetic redundancy or multiple genetic information. Such multiplicity of genetic information has also been supported by DNA hybridization (Ritossa, Atwood and Spiegelman, 1966; Wallace and Brinstiel, 1966; Britten and Kohne, 1969 and Brown and Webber, 1968).

It has been pointed out earlier that two basic types of chromosome models have been proposed. The advocates of single-stranded chromosome (Taylor, 1957, 1958a, 1958b, 1959; Schwartz, 1958; Freese, 1958; Swift, 1962; McGregor and Callan, 1962; Callan, 1963, 1967; Gall, 1963a, 1963b; Whitehouse, 1967 and DuPraw, 1965a 1965b, 1965c, 1966a, 1966b, 1968) put stress upon the difficulty a multistranded chromosome presents in explaining replication, mutation and recombination in contrast to the simplicity provided by the single stranded model. However, a number of multistranded

models have been proposed which can account for at least replication and crossing over (Trosko and Wolff, 1965; Peacock, 1963; Osgood et al. 1964; Steffensen, 1959, 1961 and Uhl, 1965). Keyl's data (1965, 1966) and DNA hybridization experiments provide convincing proof about the occurrence of genetic redundancy (or multiple copies of all or parts of the genome). Consequently those who advocate a single stranded model often have to postulate control mechanisms which suffer as much from lack of experimental evidence as the postulated control mechanisms in support of multi-stranded model. Callan (1967) in support of single-stranded model has suggested that one copy of the gene is the master copy and all the duplicated segments would be slave genes. Although each slave gene can mutate independently, he postulates that the slaves fold back upon the master and match their base sequences and become corrected if so needed. The corrected slave genes then project from the sides of the chromosome forming the loops of the lampbrush. Whitehouse (1967) has further suggested that, to prevent unequal crossing over during meiosis, all slave genes round up into a circle and become detached by means of intrachromatid crossing over. The master gene remains in the chromatid, which then undergoes crossing over with a homologous chromatid. The slave gene which remains detached until crossing over is completed, then becomes incorporated into the main strand.

1.1.2 Chromosome size and DNA content:

In wide comparisons DNA content per chromosome may not be proportional to chromosome volume. Thus in Droseraceae Rothfels and Heimburger (1968) found a thousand-fold difference in chromosome size, but only an eighty-fold difference in DNA content. A number of other workers have, however, shown a correlation with DNA content and chromosome size (McLaren, Woods and Shea, 1966; Rees, Cameron, Hazarika and Jones, 1966; Rothfels et al. 1966; and Miksche, 1967). Hughes-Schrader and Schrader (1956) reported a marked difference in chromosome size of Acrosternum species and Thyanta species which was parallelled by difference in DNA content. Thus most studies suggest that chromosome size is a good indication of DNA content. Therefore it may be inferred that the many differences in chromosome size described in the cytological literature reflect differences in DNA content. However, chromosome size may not depend merely on the DNA content but also upon other constituents, particularly nuclear proteins (Bennett and Rees, 1969; Bennett, 1970).

1.2 DNA content and species relationship:

Quantitative studies of nuclear DNA content have recently been used as a cytogenetic parameter in conjunction with chromosome number and karyotype studies. Such studies can provide means to elucidate the mode of evolution and species relationship. Inferences regarding evolution and relationship in

the genus Bufo (toad) has been drawn from studies of quantitative variation in DNA content (Ullerich, 1966, 1967, 1970; Goin, Goin and Backman, 1968 and Backman, 1970). DNA estimation in Montremes supported the karyotypic findings that Montremes are closer to mammals than to birds and reptiles (Bick and Jackson, 1967). In the course of vertebrate evolution changes in DNA content (both increase and decrease) have been suggested (Ohno and Atkin, 1966).

Similar studies have been undertaken on placental mammals, reptiles and birds (Atkin et al. 1965; Atkin and Ohno, 1967), Amphibia (Laura, 1968), Tulipa (Southern, 1967), fishes (Ohno and Atkin, 1966), primitive chordates (Bick and Jackson, 1967), Ranunculaceae (Rothfels et al. 1966).

1.3 DNA content and tracing ancestry:

Recently comparison of relative DNA content has been used as a means of investigating phylogenetic relationship and tracing the ancestry particularly where hybridization and amphiploidy are involved. Rees (1963) initiated such an investigation in the genus Triticum. There is some doubt about the origin of the B genome in the tetraploid and hexaploid wheat. Some favour Aegilops speltoides while others support A.bicornis or A.longissima. Rees' comparisons of the DNA content of the diploids and the amphiploid supported A.speltoides as a more likely contributor of the B genome.

A few more reports of such investigations are also available in the literature (Pai and Upadhya, 1961; Upadhya and Swaminathan, 1963; Rees and Walter, 1965; Nishikawa and Furuta, 1969 and Southern, 1967).

1.4 Selection of study material:

Detailed quantitative studies of DNA distributions in the genera Gossypium and Phalaris were undertaken keeping in mind the following points:-

1.) It was hoped that DNA measurements might provide a means to elucidate species relationship and the origin of amphiploids.

2.) Interpretations are possible regarding chromosome ultra structure and the mechanism of DNA increase or decrease between related species through quantitative studies. Such studies so far have been mainly carried out on plant species with large chromosomes, while little information of this nature is available on species with small chromosomes. Of the two genera selected here Gossypium (Chapters 3 and 4) has small chromosomes while Phalaris (Chapter 5) has larger chromosomes. A study of DNA distribution within the two genera could throw light on the pattern of DNA evolution between small chromosome and large chromosome species.

3.) Preliminary studies of quantitative DNA contents were also carried out in two species of Thespesia, closely related to Gossypium, and two strains of maize with a hope that this might throw further light on the pattern of DNA evolution between species and within species.

CHAPTER 2.

MATERIALS AND METHODS

2.1 Materials:

Table 2.1 gives the list of Gossypium species and races used in this study. For each species the genome group, chromosome number, an indication of the geographical distribution and the source of the seeds are given. The species of Phalaris used are listed in Table 2.2. Two species of Thespesia, a genus related to Gossypium with a similar basic chromosome number (n=13) were also used. The two species are T. lampus (cav.) Dalz. ex Dalz. and Gibs. and one unidentified species of Thespesia.

The seeds of Thespesia lampus were obtained from the U.S. Department of Agriculture. The unidentified species was obtained from the West Pakistan Agriculture Department.

TABLE 2.1

List of Gossypium species used in the investigation

Genome	Species	Distribution	Source of seed
	Diploid 2n=26		
A_1	*G. herbaceum* L. var. *africanum* (Walt.) Hutch. & Ghose	S. Africa	U.S. Agric. Dept.
A_2	*G. arboreum* L. race *cernum*	Cultivar	E. Pakistan Agric. Dept.
A_2	*G. arboreum* L. race *bengalense*	"	W. Pakistan Agric. Dept.
B_1	*G. anomalum* Wawra and Peyr.	S. Africa	U.S. Agric. Dept. *
B_2	*G. triphyllum* Hoch.	"	" " *
B_3	*G. barbosanum* Phillips & Clement	Cape Verde Islands	" "
C_1	*G. sturtianum* var. *sturtianum* Will.	Central Aust.	" " *
C_2	*G. robinsonii* F. Muell.	Western Australia	" " *
C_3	*G. australe* F. Muell.	Northern Australia	" " *
D_1	*G. thurberi* Tod.	Arizona U.S.A.	" " *
D_{2-1}	*G. armorianum* Kearn.	Baja California Mexico	" " *

TABLE 2.1 cont.

Genome	Species	Distribution	Source of seed
	Diploid 2n=26		
D_{2-2}	G. harknessii Brandg.	Baja California Mexico	U.S. Agric. Dept. *
D_{3-k}	G. klotzschianum var. klotzschianum Anderss.	Galapagos Islands	" " *
D_{3-d}	G. klotzschianum var. davidsonii (Kell.) Hutch.	Sonora, Mexico Baja California	" " *
D_4	G. aridum (Rose & Standley) Skovs.	Sinaloa, Mexico	" " *
D_5	G. raimondii Ulbr.	Peru	" " *
D_6	G. gossypioides (Ulb.) Standl.	Oaxaca, Mexico	" " *
E_1	G. stocksii Mast. Ex Hook.	Arabia W. Pakistan	Karachi W. Pakistan
E_2	G. somalense (Gurke) Hutch.	North-east Africa	U.S. Agric. Dept.
E_3	G. areysianum (Defl.) Hutch.	Arabia	" "
E_4	G. incanum (Schwartz) Hillc.	Arabia	" "
	Tetraploid 2n=52		
(A_1D_1)	G. hirsutum L.	New world cultigen	Agric. strains (Australia)

TABLE 2.1 cont.

Genome	Species	Distribution	Source of seed
	Tetraploid 2n=52		
(A_2D_2)	G.barbadense L.	New world cultigen	U.S. Agric. Dept.
(A_3D_3)	G.tomentosum Nutt. ex Seem.	Hawaii	" "
C_4**	G.bickii Prokh.	Central Australia	" "
E_5**	G.longicalyx Hutch. & Lee	North-east Africa	" "

* Part of the used seeds were material from plants grown in W. Pakistan from seeds imported from the U.S. Agric. Dept.

** The genomes assigned to these species are uncertain, see text.

TABLE 2.2

List of Phalaris species used in the investigation

Species	Seed source	CPI No.
Diploid 2n=12		
P.truncata Cuss	CSIRO Canberra	19251
P.canariensis L.	" "	14690
P.brachystachys Link.	" "	19135
"	" "	14830
"	" "	19144
"	" "	15589
Diploid 2n=14		
P.coerulescens Desf.	D.L. Hayman*	19193
P.paradoxa L.	CSIRO Canberra	GINH2
P.caroliniana Walt.	" "	No number
P.angusta Nees	" "	25777
Tetraploid 2n=28		
P.tuberosa L.	" "	N 484
P.arundinacea L.	Commercial	
P.californica Hook. & Arn.	CSIRO Canberra	34708
P.minor Retz.	CSIRO Canberra and D.L. Hayman*	32270
"	" "	19197
"	" "	24340
"	" "	19215
"	" "	19203
Hexaploid 2n=42		
P.arundinacea L.	" "	10446

* Dr. D.L. Hayman, Genetics Department, University of Adelaide.

2.2 Methods:

Gossypium seeds were germinated on a moist filter paper in petri dishes kept at $31 \pm 1^{\circ}C$ in an incubator. The seed coat was split open to ensure better germination particularly in the wild species. Where large numbers of root tips were needed from a single plant, individual plants were grown in small plastic pots with perforated bottoms. These pots were suspended in black painted plastic jars full with Hoagland's culture solution. The culture solution was kept aerated.

Actively growing primary root tips of species to be compared for relative DNA content and root tips of G.arboreum L. as the standard were fixed simultaneously in the same vial in 1:3 acetic-alcohol for 5-6 hours. The species to be compared and the standard species were subsequently treated identically for the entire procedure. The fixed root tips were passed through a descending series of alcohol down to distilled water. The root tips were kept in a vial in a drop of water to avoid desiccation and preheated at $60^{\circ}C$ in a water bath for five minutes. The pre-heated root tips were hydrolysed for six minutes in 1N HCl at $60^{\circ}C$. Hydrolysis was stopped immediately by pouring out root tips and HCl in a beaker full with ice cold water.

Root tips of the standard and the species to be compared were squashed in 45% acetic acid and mounted on the same

slide one at each end. The slide was freeze-dried on a block of dry ice, the cover slips flicked off with a scalpel blade and the slide was transferred to absolute ethyl alcohol followed by a descending series of alcohols and two changes of distilled water. The slides were then stained for 2-3 hours in leuco-basic fuchsin (Darlington and LaCour, 1960). The stained slides were washed in SO_2 water for 30 minutes, three changes of 10 minutes each. Finally they were dehydrated in an ascending series of alcohols, washed twice in xylene and mounted in Xam with number 1 cover slips. The procedure is summarized in appendix I.

Relative DNA values were measured at 4C stage, using a Barr and Stroud integrating microdensitometer. For each species an average of 40 prophase nuclei were measured together with the same number of cells of standard species (G.arboreum L.) in 3-4 replicates. DNA values of all species were calculated relative to G.arboreum L. with an arbitrary value of 100 and expressed as the relative DNA content per cell $\pm$ standard error. The method for the calculation of standard error is given in appendix II.

Analysis of variance was carried out to partition variation between the species (i.e. between the species under investigation and the standard species, G.arboreum), between replicates (i.e. slides) and within root tips. The statistical analysis usually showed a significant difference between slides.

This is expected due to variations in staining. It also sometimes revealed a significant interaction between species and slides. This gave rise to the suspicion that there were variable values of DNA between the members of the same species. Therefore a test for comparing DNA between individuals of the same species was designed. For comparing individual variation three primary root tips from different individuals of the same species were fixed and processed together for the entire procedure. The tips were squashed one after the other on the same slide and the successive squashes were marked as positions 1, 2 and 3. This was done in order to measure and eliminate any effect due to order of squashing which might contribute to the variability between individuals. The slides were then processed for staining and making permanent as mentioned before.

Phalaris seeds were germinated on moist filter paper in petri dishes. Treatment for 2-3 days at $4^{o}C$ enhanced germination percentage. Germinating seedlings were transferred individually to pots. Root tips were harvested from actively growing plants in pots. The method used for comparing DNA content of Phalaris species was essentially similar to the one described above. All species were measured against P.coerulescens taken as standard and given a value of 100 arbitrarily. Additional precaution was taken to eliminate any probable effect of variable standard due to individual variation, by using a single plant as standard for comparing all the

species. Enough supply of the root tips from the standard plant could be maintained by multiplying the standard plant vegetatively.

CHAPTER 3.

DNA values in the genus Gossypium

3.1 The genus Gossypium:

The genus Gossypium L. of the tribe Gossypieae of the family Malvaceae (Fryxell, 1968) is widely distributed. Wild species have been reported from all the tropical and sub-tropical regions of the world. However, wild species of Gossypium are confined to small areas, with small populations and with very little variability in contrast with the variability present among the cultivated species (Hutchinson, 1959; Fryxell, 1965).

The genus has received considerable attention both from taxonomists and cytogeneticists. Conservative treatment by Hutchinson et al. (1947) recognizes 20 species while Prokhanov (1947) describes 67 species. Todaro (1877); Mauer (1954) and Fryxell (1965) have moderate approaches. Fryxell (1965) transferred all of Notoxylinon species to Gossypium, thus increasing the number of Gossypium species to approximately 30. Fryxell (1968) also discusses the possibility of elevating the tribe Gossypieae to the rank of family.

Beasley (1942) assigned the diploid species to five cytologically distinct genomes (A, B, C, D and E) and one tetraploid genome (AD). The system of symbols used in this thesis is the same as that used by Saunders (1961) which in itself is a

modification of that used by Beasley (1942) and Knight (1954). The cytogenetic data has been accumulated by a number of workers since the beginning of the present century such as Harland (1923, 1939, 1940); Davie (1934); Arutjunova (1936); Abraham (1940a, 1940b); Beasley (1940a, 1940b); Skovsted (1934a, 1934b, 1935a, 1935b, 1937); Webber (1935, 1939); Gerstel (1953); Hutchinson (1954, 1959, 1962); Saunders (1961); Phillips (1963, 1966); Brown (1954, 1961).

3.2 DNA content per cell and its distribution between species and genomes of Gossypium:

In Table 3.1 mean relative DNA values of the species and their standard errors are listed. These values are relative to G. arboreum.

TABLE 3.1

Mean relative DNA contents of Gossypium species in three replicates, species average, genome average and genome classification.

Genome	Species	Replicates 1	2	3	Rel. DNA values* Mean ± S.E.	Genome Average
D_{3-k}	klotzschianum	56.0	55.3	54.0	54.9 ± 1.2	
D_6	gossypioides	54.1	55.5	57.5	55.7 ± 1.4	
D_{3-d}	davidsonii	56.6	60.0	52.2	56.3 ± 1.1	
D_{2-2}	harknessii	56.5	56.3	56.9	56.4 ± 1.3	
D_{2-1}	armourianum	55.5	63.0	54.7	57.4 ± 1.0	57.7
D_4	aridum	59.8	58.4	57.1	59.4 ± 1.6	
D_1	thurberi	61.2	58.9	52.4	60.0 ± 1.4	
D_5	raimondii	62.6	57.9	64.4	61.9 ± 1.1	
B_2	triphyllum	80.8	74.3	74.5	77.6 ± 1.2	
B_1	anomalum	76.9	83.1	78.0	79.1 ± 0.9	78.9
B_3	barbosanum	78.9	83.1	78.2	80.3 ± 1.4	

TABLE 3.1 cont.

Genome	Species	Replicates 1	2	3	Rel. DNA values* Mean ± S.E.	Genome Average
E_2	somalense	91.0	81.1	80.1	83.7 ± 1.2	
E_1	stocksii	81.0	90.1	91.6	86.0 ± 1.4	
E_4	incanum	87.2	84.0	89.1	87.3 ± 1.3	87.3
E_3	areysianum	89.0	91.9	95.9	92.4 ± 1.4	
A_1	herbaceum var. africanum	91.1	92.1	86.6	90.6 ± 1.1	95.3
A_2	arboreum standard	-	-	-	100.0 ± 0.0**	
C_3	australe	110.0	116.0	112.4	110.7 ± 2.3	114.5
C_1	sturtianum	109.4	109.8	115.5	110.9 ± 1.7	
C_2	robinsonii	114.1	136.2	111.3	121.8 ± 2.7	
$E_5(F_1)$	longicalyx	72.6	68.9	79.5	73.6 ± 1.1)	Genome uncertain excluded from statistical analysis. See text.
C_4	bickii	89.6	88.4	88.5	88.8 ± 1.4)	

TABLE 3.1 cont.

Genome	Species	Rel. DNA values* Mean ± S.E.
$(A_1D_1)_2$	hirsutum	142.6 ± 1.9
$(A_2D_2)_2$	barbadense	137.3 ± 1.9
$(A_3D_3)_2$	tomentosum	125.2 ± 1.7

* Overall average for species and genomes are derived in some cases from more than three replicates.

** Standard excluded from statistical analysis.

TABLE 3.2

Analysis of variance of data in Table 3.1 above

	S.S.	D.F.	Mean Square	F	Probability
Between genomes	24068.3	4	6017.07	279.47	< .001
Between species within genomes	355.21	14	25.37	1.18	> .2
Total between species	24423.51	18	1356.86	63.02	< .001
Between replicates within species	818.18	38	21.53		
Total	25241.69	56	450.74		

It can be seen from the values listed in Table 3.1 that the spread in relative DNA content in diploid species of Gossypium varies from 54.9% to 121.8%. The average DNA values for individual genomes D, B, E, A and C are 57.7, 78.9, 87.3, 95.3 and 114.5 per cent respectively. The mean for each species is derived from at least (and in some cases more than) three replicated experiments; the total number of measured cells ranges from 40 to 50 for each species. The three amphiploids G.hirsutum, G.barbadense and G.tomentosum have average DNA values 142.6, 137.3 and 125.2 per cent of G.arboreum respectively. DNA values in the amphiploids and its origin has been discussed in detail in Chapter 4.

Analysis of variance of the listed data (Table 3.1 species 1 to 20) shows that the variation between species and between genomes is highly significant, probability being less than 0.001 in both cases (Table 3.2). In this analysis variation between species within genomes is not significantly different from variation within species (replicates).

The two species G.longicalyx and G.bickii can be seen from Table 3.1 to have markedly different DNA values from the average values of genomes E and C respectively, to which, as judged by continent of origin, they should belong; they have been excluded from the analysis of variance. The justification for

their exclusion comes from the fact that neither of the species has been cytologically confirmed as belonging to the respective genomes. Phillips (1966) and Saunders (1961) expressed the opinion that G.longicalyx does not belong to E genome and recently it has been treated under a separate genome F (Cherry et al.1970). Similarly the inclusion of G.bickii in C genome may be regarded as tentative. G. bickii was formerly classified in the genus Notoxylinon which has recently been merged with the genus Gossypium (Fryxell,1965). If the merger of Notoxylinon with Gossypium is justified on morphological grounds, it may be that a cytological investigation of these species may establish another diploid genome on the continent of Australia.

3.3 Intraspecific variation:

3.3.1 Individual variation within a species:

The considerable variation between different replicates of the same species, which was of the same magnitude as that between different species of the same genome (Table 3.2), gave rise to the suspicion that there might be variable values between individuals of the same species. Six species, one from each of the five genomes (A,B,C,D and E) and one amphiploid (AD), were selected to test whether there existed a real difference in DNA content between individuals. For comparing individual

variation the procedure used has already been explained in Chapter 2. That is, 12 individuals were studied, 3 being squashed on each of 4 slides. Because hydrolysis and staining conditions are rarely exactly the same for all slides there is usually a significant variance between slides. This "uses up" 3 of the 11 degrees of freedom between the 12 individuals. However it is possible that there could be systematic variation between the 3 positions on slides (i.e. left, centre and right). This variation might arise not so much from position per se as from the time differences arising from squashing in this order. If this were significant it would "use up" 2 degrees of freedom due to "between positions". Moreover it would no longer be possible to ascribe, with any confidence, the remaining 6 degrees of freedom to "between individuals" for, if "between positions" is significant one must allow the possibility of a significant interaction between positions and slides. The 6 degrees of freedom for this interaction would be indistinguishable from "between individuals". Thus the success of this experimental design depends on there being no significant variation between positions.

In Table 3.3 the statistical analyses of variance between individuals are set out for all six species tested in this way. The arbitrary values obtained in measurements have been converted to make them consistent with their DNA values

relative to G.arboreum. Of the six, only one, G.arboreum, had both a non-significant "between positions" variance combined with a significant "between individuals" variance. To arrive at conclusion regarding the variation "between individuals" with confidence therefore requires a more elaborate design of experiment. This is described in Chapter 6 and discussion is deferred until then.

TABLE 3.3

Analyses of variance suggesting individual variations within a species

D.F.*	2	3	11	108	6	119		
Species and DNA values from Table 3.1	Between positions	Between slides	Total between individuals	Within individuals (Error)	Between individuals on same slide	Total variance	Variance ratio column 6 / column 5	Probability
G.arboreum (100)	57.98	1109.58	452.87	59.65	256.16	96.01	4.29	$< .001$
G.australe (110.7)	424.27	15617.85	4604.75	99.33	491.72	516.04	4.95	$< .001$
G.hirsutum (142.6)	1144.63	652.68	565.66	98.79	329.18	141.98	3.33	$.001 < p < .01$
G.stocksii (86)	33.8	3912.28	1112.32	43.16	71.58	141.96	1.65	$> .1$
G.anomalum (79.1)	76.56	1861.05	541.31	51.65	36.12	90.85	0.699	$> .2$
G.raimondii (61.9)	53.36	780.84	229.57	26.26	12.74	45.00	0.485	$> .2$

* D.F. = Degree of freedom

3.32 Variation within individuals:

It was realised that, if the experiments in the last section had given convincing evidence of variation between individuals, this could have arisen from variation between cells in a single plant. It was therefore thought desirable to test if there was any significant variation between root tips of a single plant. The plants tested were grown in Hoagland's culture solution and twelve lateral roots were measured from each plant, three on each of four slides in a manner similar to that of the last section. Individuals from four different species were tested in this way and the results are set out in Table 3.4. For G.hirsutum the "between positions" variance was significant so vitiating the test for the same reasons as those given earlier. For the other three species tested, the "between positions" variance was not significant but in no case was the "between secondary root tips" variance convincingly significant (for two species $.05 > P > .02$ and for one $P > .2$).

TABLE 3.4*

Analysis of variance within individual plants in four species of Gossypium

	D.F.	2	3	11	6	108	119		
Species		Between position	Between slide	Total between secondary root tips	Between secondary root tips	Error variance	Total variance	F	P
G.hirsutum		321.74	1873.79	632.55	115.05	067.31	119.35	1.71	>.1
G.tomentosum		444.03	1622.51	765.74	444.99	179.63	231.16	2.48	.05>p>.02
G.herbaceum race africanum		6.87	71.49	88.45	124.19	52.24	55.91	2.37	.05>p>.02
G.barbosanum		46.95	625.76	205.74	53.78	58.90	72.56	0.91	>.2

* All values converted relative to G.arboreum and therefore comparable directly and also with values in table 3.3.

3.4 CHAPTER DISCUSSION

Interspecific variation:

The DNA values in the diploid species of Gossypium suggest that divergence and evolution has been accompanied by quantitative variations in the nuclear DNA. The highest and lowest values in the diploid species show more than a two-fold difference, and the intermediate values are continuously distributed, particularly within a genome. The statistical analysis for DNA distribution within a genome is non-significant in contrast to the significant value for DNA distribution between genomes. It seems likely that values within a genome tend to occur around a common average, while values between genomes may be partially disjunct in their distribution. Katterman and Ergle (1970) have reported more than three fold difference in DNA content between the highest and lowest values in the diploid species of Gossypium. Their results also suggest that DNA values in C,E and F (G.longicalyx) genomes are even higher than in tetraploid species. Their estimates were obtained by measuring the amount of DNA per gram of de-fatted seed embryos and therefore, if conclusions are to be drawn about amount of DNA per cell, it must be assumed that number of cells per gram of tissue is the same from species to species. This assumption is of doubtful validity (Martin,1966) and probably accounts for the difference between their results

and the present ones.

Where related species show a disjunct distribution of DNA values, and especially when higher values are approximate multiples of the lowest value, this is compatible with the hypothesis that evolution has been accompanied by changes in the degree of strandedness of the chromosomes (Rothfels and Heimburger,1968; and Martin,1968). Although the continuous or partially disjunct distribution of DNA values independent of change in chromosome number does not rule out the possibility of differential polynemy, it does suggest that other factors are more important, e.g. lengthwise increase or loss of chromosome segments.

Apparently non-disjunct DNA values between inbreeding and outbreeding species of Lolium have been interpreted in terms of longitudinal duplication although lateral multiplicity is not ruled out (Rees et al. 1966; Rees and Jones, 1967a.). Variation of 27% DNA content in the species of Allium has been interpreted solely on the basis of lengthwise incorporation or loss of chromosomal segments (Rees and Jones, 1967b.). The much reduced chromosome pairing and chiasma frequency, particularly in intergenomic hybrids, suggests that speciation in this genus has been accompanied by chromosomal aberration (Phillips, 1966).

Katterman and Ergle (1970) advance differential

polynemy as the explanation for the increase in nuclear volume (taking nuclear volume and DNA content to be directly related) in Gossypium since in hybrids between species of Gossypium with large and small chromosomes, the pachytene chromosomes pair closely and are equal in length with no evidence of loops (Brown, 1954). However, the continuous DNA distribution, particularly within genomes, in the present investigation, along with the evidence that speciation in the genus has been accompanied by chromosomal aberration (Phillips,1966), suggest that length-wise increase or loss of segments, or local multiplicity in the sense of Keyl (1965) are more important in the evolution of DNA variation in the genus Gossypium.

Chromosome length and DNA value:

Small size and large number of chromosomes in Gossypium make it difficult to make a direct comparison between chromosome and DNA value. However, the meagre information that is available about the chromosome length between different genome fits well with the comparison of DNA value assuming them to be linearly related. The minimum DNA value is found in D genome and the maximum in C genome. The chromosomes length likewise are smallest in D and largest in C genome (Webber, 1939; Skovsted,1935, 1937). The chromosomes lengths in A and B genomes are

usually considered to be similar though Skovsted (1935) has suggested that A chromosomes are larger than B. The DNA value in A genome species is greater than in B genome species.

Recently Endrizi and Brown (1968) have reported that B genome chromosomes are larger than A genome chromosomes. The average DNA value in A genome species is greater than in B genome species and lends support to the earlier finding. The contradiction in the two findings may be due to the fact that Endrizi and Brown (1968) compared A genome chromosome lengths indirectly from derivatives of G.anomalum (B_1) x G.hirsutum (AD) assuming A genome complement of (AD) amphiploids to be exactly the same as those of A genome diploid species. While working on the chromosomes lengths in G.herbaceum (A) and G.hirsutum (AD), Davie (1934) and Arutjunova (1936) recorded a significant decrease in chromosome size in G.hirsutum as compared to G.herbaceum. From the foregoing observations it is possible that conclusions reached about the size of chromosome lengths between A and B genomes indirectly from 2(AD) x 2(B) derivatives may be different.

A similar contradiction has been created about the size of C genome chromosomes due to the recent revision of the genus Gossypium-(Fryxell,1965) which resulted

in the transfer of the genus Notoxylinon to the genus Gossypium. G. bickii, now a member of C genome species as a result of this revision, has recently been reported to have chromosome size smaller than A genome species by Wilson and Fryxell (1970), in contrast to the previous report about the size of C genome chromosome cited above (Skovsted,1937). The smaller chromosome size reported in G.bickii is also supported by the present DNA estimation. The DNA content in G.bickii is much less than the other C genome species and is in fact lower than the A genome species (Table 3.1). Thus both DNA estimation and chromosome size point towards the conclusion that G.bickii is different from C genome species and should be assigned to a separate genome (G).

Very little work has been done on the measurement of E genome chromosomes. Recently Katterman and Ergle (1970) have quoted Brown (private communication 1969) that E genome chromosomes are larger than A and B genome chromosomes. The average DNA value in E genome species is greater than B genome species; however, the average DNA content of A genome species is greater than E genome species (Table 3.1). The inconsistency regarding the size of A genome chromosomes has already been mentioned above. It may be pointed out here that the two A genome species differ in their DNA content; the DNA content

of G.herbaceum (A_1) is similar to some of the E genome species. Possibly the two A genome species differ in chromosome length as well. There is no apparent correlation between DNA value and chromosome length in G.longicalyx if its chromosome length is regarded to be similar to those of E genome species to which it was grouped previously.

The data discussed above suggest a reasonable degree of correlation between chromosome length and DNA value in Gossypium and is comparable with those in Lolium (Rees and Jones, 1967). Hughes Schrader and Franz Schrader (1956) recorded a marked distinction in size between chromosomes of Acrosternum species and species of Thyanta and this in turn was paralleled by the DNA values of the species. In Lathyrus chromosome volume is linearly related to both nuclear dry mass and nuclear DNA (Rees and Jones, 1972). However, it has also been reported that chromosome volume (size) depends not merely on the DNA content but also upon other constituents, particularly nuclear proteins (Bennett and Rees,1969; Bennett, 1970). Obviously the degree of coiling is important though it is possible that this depends to some extent on protein content.

Trend in evolution:

The trend in evolution, whether towards increase

or decrease in DNA content is difficult to ascertain. In the A genome diploid species it is apparent that evolution has been accompanied by increase in DNA content. G.arboreum has evolved under cultivation from G.herbaceum (Hutchinson,1962) and is therefore more recent than G.herbaceum. Likewise Gerstel (1953) has demonstrated that G.herbaceum is cytologically closer to G.anomalum than G.arboreum is to G.anomalum, and therefore G.herbaceum is more primitive than G.arboreum. The DNA value in G.arboreum is higher than in G.herbaceum (Table 3.1). However, there is no consistent relation between average DNA content in the different genomes and their primitiveness as postulated by Phillips (1966).

CHAPTER 4

DNA content and the origin of amphiploids

4.1 Introduction:

Three of the new world species of Gossypium are allopolyploids. Two of them are cultivated cottons, namely G.hirsutum and G.barbadense, while the third, G.tomentosum, is a wild species endemic to the Hawaian Islands. It is generally agreed that all three were derived from hybrids between diploid new world species (D genome) and diploid old world species (A genome), (Skovsted, 1934a, 1934b, 1937; Beasley,1942; Gerstel, 1953; Phillips,1963). The origins and evolution of these species have been under investigation for a long time, an important field for such investigation being cytogenetics. Recently comparison of the relative DNA content has been used as a means of tracing ancestry in the genus Triticum by a number of workers (Pai and Upadhya,1961; Upadhya and Swaminathan,1963; Rees,1963; Rees and Walter,1965 and Nishikawa and Furuta,1969). A similar attempt has been made in the genus Gossypium in the present investigation.

Quantitative measurements of nuclear DNA content of the diploid species of Gossypium belonging to five genomes (A, B, C, D and E) and the three amphiploids (AD) have been reported in Chapter 3. For these the standard was

G.arboreum but since there was some possibility that this species has variable DNA values (Table 3.3), another more obviously constant species, G.stocksii, was used as standard in another series of experiments. The observed DNA content of an amphiploid can be compared with the expected value of an amphiploid derived by combining the DNA values of its putative diploid parents. It can thus be seen whether the predicted value agrees with the one actually observed or not.

4.2 Material and method:

In Chapter 3 relative DNA contents in the genus Gossypium were measured against G.arboreum taken as standard. Measurements were repeated for six species using G.stocksii, (a relatively constant species), as standard. The six species measured were G.arboreum, G.herbaceum, G.anomalum, G.raimondii, G.hirsutum and G.tomentosum.

The method used was essentially the same as explained before. However, root tips of the standard and the species to be compared were placed in 45% acetic acid together. The standard was squashed always at position 1 on the slide and the species to be compared at position 2. The observed values in the three amphiploids (Table 3.1 Chapter 3) show that the three species have different DNA contents. Since those values are derived indirectly from a common standard it was therefore thought proper to compare

the amphiploids directly. G.barbadense and G.tomentosum were compared against G.hirsutum and the results were statistically analysed.

4.3 Results:

In Table 4.1, row 4, relative average DNA content for the six species measured against G.stocksii as standard (100) are listed. These values are compared with values in row 5 derived from Table 3.1, Chapter 3. The original values in Table 3.1, Chapter 3 are relative to G.arboreum as standard (100) which has been converted relative to G.stocksii as 100 (from the same table) so as to make possible a direct comparison with values obtained directly with G.stocksii as standard (Table 4.1, row 4). The observed values for the three tetraploid species (all three species were measured against G.arboreum as standard and two species were measured against G.stocksii (as standard) are different although they are regarded as having a single origin combining A and D genomes. Direct tests were therefore carried out to confirm this difference. G.tomentosum and G.barbadense were measured against G.hirsutum as standard. Statistical analysis (Table 4.2) revealed a significant difference between G.hirsutum and G.tomentosum ($P < .001$) while G.barbadense was not significantly different from G.hirsutum ($P > .05$), although the observed value was between those of G.hirsutum and G.tomentosum.

Tables 4.3a and 4.3b are constructed to facilitate comparison between the observed values in tetraploids and the expected values. The expected values are derived by combining various diploid genome values in Table 4.1.

TABLE 4.1

Mean relative DNA contents of six Gossypium species measured against G.stocksii as standard (row 4) and compared with mean DNA contents measured against G.arboreum as standard and converted relative to G.stocksii from chapter 3 table 3.1 (row 5)

	stocksii	raimondii	herbaceum var. africanum	arboreum	anomalum	barbosanum	hirsutum	tomentosum	barbadense
Genome	E1	D5	A1	A2	B1	B3	AD	AD	AD
No. of replicates	standard	5	5	5	4	-	6	4	-
No. of cells measured	standard	50	50	50	40	-	60	40	-
Mean rel. DNA values	100 (standard)	72.4	112.3	122.8	90.9	-	170.2	150.6	-
Rel DNA values from table 3.1 chapter 3.	100	71.9	105.3	116.2	91.9	93.3	165.3	144.0	159.5

TABLE 4.2

Analysis of variance of DNA values between tetraploid species.

Item	Mean square	Error mean square (variance within + interaction)	F	Probability
G.barbadense Vs G.hirsutum	10.2001 (D.F. = 1)	2.92 (D.F.68)	3.51	>.05
G.tomentosum Vs G.hirsutum	230.414 (D.F. = 1)	2.0865(D.F.67)	110.45	<.001

TABLE 4.3a

Predicted and observed values for amphiploids.
Data taken from table 4.1 row 5

Possible genome combination	Expected	Observed
(A1 D5)	177.2	G.hirsutum = 165.3
(A2 D5)	188.1	G.barbadense = 159.5
(B1 D5)	163.8	G.tomentosum = 144.0
(B3 D5)	165.2	
(E1 D5)	171.9	
(D5 D5)	143.8	

TABLE 4.3b

Predicted and observed values for amphiploids. Data taken from Table 4.1 row 4. Measured directly against G.stocksii.

Possible genome combination	Expected	Observed
(A_1 D_5)	184.7	G.hirsutum = 170.2
(A_2 D_5)	195.2	G.barbadense = not measured
(B_1 D_5)	163.3	G.tomentosum = 150.6
(E_1 D_5)	172.4	
(D_5 D_5)	144.8	

4.4 CHAPTER DISCUSSION

Table 4.1, rows 4 and 5 show that DNA content measured against G.stocksii and G.arboreum as standards respectively are the same for species belonging to B (G.anomalum) and D (G.raimondii) genome species. The DNA values of A genome species however, measured against the two standards, are slightly different. Tables 4.3a and 4.3b constructed from values obtained against the two standards, however, lead to similar conclusions about the expected and the observed values for the amphiploids.

The three amphiploids have different DNA contents although they are regarded as having a single origin combining A and D genomes. It can further be seen from Table 4.3a and 4.3b that even the highest observed value among the amphiploids (i.e. in G.hirsutum) is lower than the expected value assuming the constitution as 2 (AD). These observations suggest two possibilities.

First, the amphiploidy arose before the present day species of A genome were established while the D genome species might have had a DNA value close to the present genome average. The A genome parent which was involved in the origin of the amphiploids might have had a lower DNA content. In the course of evolution, increase in the DNA content and changes in the genetical makeup led to the establishment of G.hirsutum

with the largest DNA value while G.tomentosum represents the value closest to the original ones; G.barbadense occupies a middle position. The trend in evolution within A genome discussed earlier (Chapter 3) suggests that evolution has been accompanied by increase in DNA content. Perhaps a parallel situation may exist in amphiploid cottons, and G.tomentosum with the smallest DNA value is the most primitive one, while G.hirsutum with the largest DNA value is the most advanced.

The second possibility is that hybridization took place after the establishment of A and D genome with their present DNA contents. Decrease in DNA content later gave rise to G.tomentosum with the smallest DNA value; G.hirsutum retained a value closest to the original level, G.barbadense again occupying the middle position.

A few cases are on record of reduction in DNA content per chromosome following polyploidy. Pai et al.(1961) recorded chromosome lengths and DNA contents in tetraploid and hexaploid wheat which were smaller than those expected on the basis of additive values of the respective genome donors. Upadhya and Swaminathan (1963) recorded less chromatin diminution in the above wheats than reported earlier (Pai et al.1961). Rees (1963), Rees and Walter (1965) and Nishikawa and Furuta (1969) do not confirm the above reports of chromatin diminution. Southern

(1967) recorded in the tetraploid Tulipa whittalli a DNA value less than twice the value of the proposed diploid parent. Likewise Grant (1969) reports decrease in DNA content in Betula chromosomes at higher ploidy. Davie (1934) and Arutjunova (1936) recorded a marked tendency towards decrease in chromosome length in tetraploid cotton G.hirsutum as compared with diploid G.herbaceum. Rather both authors, working independently on the size and morphology of chromosomes, concluded that amphiploid cotton (G.hirsutum) does not have two distinct groups of chromosomes differing in size. They believed that each type of chromosome in G.hirsutum is represented four times. This strongly suggests reduction in chromosome size in tetraploid cottons if they originated combining A and D genomes which differ markedly in their chromosome size. If this reduction in chromosome length is reflected in DNA content of the amphiploids, it will mean that the G.hirsutum value is closest to the primitive amphiploids while G.tomentosum value represents the greatest divergence from the primitive amphiploids.

From the foregoing discussions it becomes difficult to reconcile different DNA values in the three amphiploids assuming A and D genomes as the donors for all the three species and also assuming a undirectional trend in

evolution regarding the change in DNA content. Perhaps the trend in amphiploids is towards reduction in DNA content if left to nature (as might be the case with G.tomentosum) while the two cultivated amphiploids (G.barbadense and G.hirsutum) under selection pressure have not lost or even might have increased in their DNA content and remain close to the original level. On this view, the trend in DNA evolution is not undirectional.

The different DNA values in the three amphiploids also show that if all the species have a single origin, significant changes have taken place in these species since their origin. This may add to the evidence (Phillips, 1963) which indicates that the origin of the new world amphiploid was not in very recent times, as suggested by Hutchinson et al. (1947). However, it can also be argued granting the possibility of "individual variation" in DNA content, that, under selection, the variability in DNA content provided an excellent basis for bringing about a rapid change in the DNA content. This rapid change in nuclear DNA might have been accompanied by qualitative changes as well. This may explain how the three amphiploids could evolve in a relatively short time a

genetic barrier between themselves, which has been taken as an evidence against recent origin (Phillips, 1963).

An attempt can be made to explain the apparent inconsistencies in the rates of chromosomal evolution in the B, A and A_{nw} genomes following amphiploidy. 11 to 13 bivalents are formed between A x B hybrids, while B x A_{nw} hybrids rarely form bivalents. Two sources of structural differentiation in the A_{nw} can be visualised in contrast to the A old world. One is diploidization within amphiploids (Phillips, 1963) and the other changes under selection for an economically useful phenotype. The latter source of differentiation, if connected with the quantitative variability in DNA content, may be relatively a quicker one and probably common to A (old world) and A_{nw} and thus bringing the two closer. The B genome on the contrary has remained relatively unchanged, retaining more homology to A old world than to A_{nw}.

A_1 or A_2 as the diploid A genome parent:

Comparing the theoretical value based on 2(AD) constitution and the observed value in the amphiploids (Table 4.3a and 4.3b) it can be seen that G.hirsutum value comes closer to 2(A1D5) than 2(A2D5). Thus G.herbaceum (A1) is closer to the probable A genome parent. This observation is consistent with

cytological findings (Gerstel, 1953).

Other possible genome combinations:

Theoretical expectations other than that based on 2(AD) constitution have also been included in Table 4.3a and 4.3b. These are 2(ED), 2(BD) and 2(DD). The estimated value in G.tomentosum is almost twice the value of D genome species. Thus an autotetraploid or an allo-tetraploid within the genome D can account for the DNA value in G.tomentosum in contrast to the DNA value in G.hirsutum which can roughly be accounted for by combining A and D genome species. Cytological evidence, however, is against the possibility of more than one line of origin for the three amphiploids since they are interfertile and 26 bivalents are formed in their hybrids (Webber 1935, 1939; Gerstel and Sarvella, 1956). Alternatively a single origin for all the three amphiploids with a constitution of 2(DD) instead of 2(AD) followed by increase in DNA content under cultivation seems a remote possibility. Likewise there is no cytological affinity to suggest that the E genome might have played a part in the origin of the amphiploids. However, it may be interesting to note that earlier workers such as Longley (1933); Davie (1934); Gates (1934); Arutjunova (1936) and Webber (1939) regarded the tetraploid new world cottons

either a true tetraploid or allopolyploid derived from two very closely related species.

While no direct affinity between the B genome and the A_{nw} has yet been discovered, the close homology between G.herbaceum (A1) and G.anomalum (B1) (Gerstel, 1953) and the suggested phylogenetic pathway from B to A and then to amphiploid (Gerstel, 1953) suggests much closer homology between B and A_{nw} genomes than so far detected cytologically. The predicted and observed values in Table 4.3a and 4.3b also show that the 2(BD) value comes closer to G.hirsutum observed values than 2(AD) values. That none of the amphiploids shows homology with B genome, speaks against B genome involvement. Gerstel and Phillips (1958) have shown that G.anomalum could not have been involved in the origin of new world amphiploids. No such studies have been carried out with other species of B genome, particularly the recently reported G.barbosanum and G.capitis viridis (Phillips, 1963). From the present evidence it seems possible that the original A parent had a DNA value nearer to species of B genome.

CHAPTER 5.

DNA values in the genera Phalaris and Thespesia

5.1 Introduction:

Results in the genus Gossypium suggested that the variation in DNA content might be continuously distributed between species. Gossypium is a genus with small chromosomes and the pattern of evolution may be different in genera with large chromosomes. Measurements were therefore carried out in the genera Phalaris and Thespesia. Although the initial choice of these two genera was based on qualitative judgements, subsequent comparisons have shown that the Phalaris species with the largest chromosomes (P. canariensis) has about 5-6 times as much DNA per chromosomes as the Gossypium species with the largest chromosome (G. robinsonii). The corresponding figure for Thespesia is 3-4 times.

5.2 The genus Phalaris:

The genus Phalaris belongs to the tribe Phalarideae of the family Gramineae (Hitchcock, 1951). Species of this genus are chiefly native of Mediterranean countries (Klages, 1947), though four species i.e. P. californica Hook and Arn., P. caroliniana Walt., P. angusta Nees and P. platensis Henrard., have been reported from the new world. Willis (1966) in

"Dictionary of flowering plants and ferns" lists 20 species in this genus, though several names may be synonyms.

Little cytological investigation has been carried out in the genus to elucidate phylogenetic relationship. The species of Phalaris can be divided into three groups with chromosome numbers n=6, 7 and 14. The basic chromosome number is 7 (Ambastha, 1956). Considering the growth habit and chromosome morphology, P. coerulescens is the most primitive among the seven-chromosome group of this genus (Ambastha, 1956). Reduction in chromosome number is regarded as having originated as a result of fusion of two chromosomes. The species having 6 as their haploid number are presumed to be more advanced than those having 7 as their haploid number. Doubling of the basic chromosome number 7, into 2n=28 and 2n=42 can either originate through auto-ploidy or allo-ploidy.

5.3 RESULTS AND DISCUSSION

5.3.1 Relative DNA contents of species of Phalaris:

Mean relative DNA values of the species studied and their standard errors are shown in Table 5.1. All values are relative to P.coerulescens as standard with an arbitrary value of 100. In the diploid species the number is either 2n=12 or 2n=14 while all the tetraploids have 2n=28 and one hexaploid species has 2n=42.

If the genus Phalaris is considered as a whole the relative DNA content per cell varies approximately 4-fold, the range being from 100 to 431. The analysis of variance of the data in Table 5.1 is set out in Table 5.2 which shows that the variation between species as a whole and also within each of the three groups (i.e. with 2n=12, 2n=14, 2n=28) are highly significant. It appears from the table that the DNA contents form continuous series within each group. However, the distribution between the two groups of diploid species (2n=12 and 2n=14) appears to be disjunct. It is interesting to note that, on the whole, diploid species with 2n=12 have twice the DNA content of that in species with 2n=14. The overall average DNA content in species with 2n=12 is 257.3 while in species with 2n=14 the average is 127.7. Average DNA content per chromosome ranges from 7.1 in P.coerulescens to 23.1 in P.canariensis. However, if the

2n=12 species are derived from species with 2n=14 by the fusion of two chromosomes as postulated by Adulov (1931) and Ambastha (1956), the DNA content per chromosome in 2n=12 species can be obtained by dividing the mean DNA content per cell by 14 rather than by 12. The DNA per chromosome in this 2n=12 group would then average 18.4 (range 16.1 to 19.8) which is approximately double that in the 2n=14 group (mean 9.1, range 7.1 to 10.4).

TABLE 5.1

Mean relative DNA values of Phalaris species in three to four replicates, species average and average DNA per chromosomes

Species	Annual (A) / Perennial (P)	Replicates 1	2	3	4	Rel.DNA Mean ± S.E.	Average DNA per chromosome
2n=14.							
P. coerulescens (standard)	p	-	-	-	-	100.0	7.1
P. paradoxa	a	118.9	121.6	122.1	-	120.9±1.8	8.6
P. caroliniana	a	137.4	145.4	145.9	147.7	144.1±1.6	10.2
P. angusta	a	138.0	146.3	149.2	150.2	145.9±1.9	10.4
2n=12.							
P. truncata	p	219.0	223.9	225.8	235.6	226.1±3.7	18.8* or 16.1*
P. brachystachys	a	263.9	265.2	270.1	274.3	268.4±3.2	22.3 or 19.1*
P. canariensis	a	269.8	272.6	289.6	-	277.3±3.1	23.1 or 19.8*
2n=28.							
P. tuberosa	p	281.6	286.5	290.9	297.0	289.0±4.2	10.3
P. arundinacea	p	288.1	289.2	301.6	311.8	297.7±3.4	10.6
P. californica	p	297.9	312.4	313.7	316.7	310.2±3.4	11.0
P. minor	a	311.9	325.1	327.3	331.9	324.0±4.6	11.5

TABLE 5.1 cont.

Species	Annual (A) / Perennial (P)	Replicates 1	2	3	4	Rel.DNA Mean ± S.E.	Average DNA per chromosome
2n=42.							
P. arundinacea	p	410.1	441.1	442.0	-	431.0±6.4	10.2

* Average per chromosome obtained by dividing DNA content per cell by 14. See text.

TABLE 5.2

Analysis of variance of data in Table 5.1

Item	S.S.	D.F.	Variance	Ratio	P
1. Between groups (2n=12, 2n=14, 2n=28 and 2n=42)	282770.6	3	94256.9	2504.2	< .001
2. Between species within groups	10704.9	7	1529.3	40.63	< .001
3. Total between species	293475.5	10	29347.5	779.7	< .001
4. Between replicates within species (5-3 error)	1129.2	30	37.64		
5. Total	294604.7	40	7365.1		

5.3.2 Relative DNA content of different geographical races:

Five different collections of P.minor and four different collections of P.brachystachys were compared (Table 5.3 and 5.5) for their DNA content to ascertain whether any intraspecific variation in nuclear DNA content due to geographical isolation could be detected in Phalaris. This sort of variation has been reported in Pinus and Luzula species by Miksche (1968) and Halkka (1964) respectively. Statistical analyses gave non-significant results for both P.minor and P.brachystachys (Tables 5.4 and 5.6).

TABLE 5.3

Relative DNA content in geographical races of P.minor measured against P.corulescens

CPI No.	Locality	Replicates 1	2	3	Relative Mean DNA
32270	India	300.9	313.5	321.2	311.9
19197	Egypt	320.5	331.0	343.5	331.7
24340	Italy	329.7	330.4	335.5	331.9
19215	Morrocco	313.2	331.9	337.0	327.3
19203	Algeria	319.1	321.3	334.9	325.1

TABLE 5.4

Analysis of variance of data in Table 5.3

Item	S.S.	D.F.	Variance	F	P
Between races of P. minor	804.2	4	201.05	2.1	>.1
Between replicates within race (error)	955.8	10	95.58	-	
Total	1760.00	14	125.71		

TABLE 5.5

Relative DNA content in four different collections of P. brachystachys measured against P. coerulescens as standard

CPI No.	Replicates 1	2	3	4	Mean DNA content
19135	251.3	260.5	270.4	278.6	265.2
14830	258.4	264.3	277.6	280.2	270.1
19144	259.5	259.7	267.5	269.1	263.9
15589	262.3	266.6	281.9	286.3	274.3

TABLE 5.6

Analysis of variance of data in Table 5.5

Item	S.S.	D.F.	Variance	F	P
Between collections of *brachystachys*	270.1	3	90.3	.879	>.2
Between replicates within collections error	1232.8	12	102.7		
Total	1502.9	15	100.2		

5.3.3 Chromosome length and DNA value:

Total haploid chromosome lengths in different species of *Phalaris* have been reported by Ambastha (1956). A comparison of the total lengths and DNA values is made in Table 5.7 and there appears to be some correlation between the two in diploid species with 2n=14, the main exception being *P. paradoxa* (Table 5.7). It has been suggested already that average DNA content in species in the group with 2n=12 are approximately double those in species in the group with 2n=14. A similar relation is not held by chromosome lengths in the two groups. On the contrary it can be seen that almost similar lengths in *P. truncata* (29.7) and *P. caroliniana* (29.1) correspond to DNA contents of 226.1 and 144.1 respectively.

TABLE 5.7

Total chromosome lengths and relative DNA value in the genus Phalaris. Chromosome measurements are taken from Ambastha (1956)

Species	Relative DNA content	Total chromosome lengths in micron	DNA content per unit length
Diploid 2n=14			
P.coerulescens	100	22.3	4.48
P.paradoxa	120.9	31.8	3.80
P.caroliniana	144.1	29.1	4.95
P.angusta	145.9	30.0	4.86
Diploid 2n=12			
P.truncata	226.1	29.7	7.61
P.brachystachys	268.4	35.9	7.49
P.canariensis	277.3	37.0	7.49
Tetraploid 2n=28			
P.tuberosa	289.0	96.0	3.01
P.arundinacea	297.7	69.1	4.30
P.californica	310.2	74.6	4.15
P.minor	324.0	65.8	4.92

A comparison of DNA content per unit length in the two diploid groups (Table 5.7 column 4) suggests that approximately twice the amount of DNA per unit length is present in the group with 2n=12 as compared with the group with 2n=14. It means that most of the additional DNA in the group with 2n=12 is reflected in the diameter or density of the chromosomes rather than in length alone.

Within the group of four tetraploid species there is not a direct relation between DNA content and chromosome lengths. It may be that changes in nuclear DNA content associated with the chromosome lengths might have followed polyploidy. However it is striking to note that the DNA content per unit length is not very different in 2n=14 and 2n=28 groups. It can further be noted that within the tetraploid species the maximum DNA content is found in P.minor which has the minimum total chromosome length as reported by Ambastha (1956). However the measurement of chromosome lengths as recorded by Ambastha and also the view held by him that P.minor is an autotetraploid with doubling of chromosome sets identical in lengths is not supported by a subsequent report. It has been reported through interspecific hybridization that P.minor is an allotetraploid combining two sets of chromosomes, differing markedly in chromosome lengths. Seven pairs of small chromosomes pair perfectly in the hybrids with chromosomes of P.coerulescens

(Hayman, 1955). The DNA value in P.minor also supports its origin through amphiploidy rather than by autoploidy and has been discussed in the next section.

5.3.4 DNA content and polyploidy in the genus Phalaris:

P.minor is known to have 7 pairs of chromosomes homologous to the 7 pairs of chromosomes in P.coerulescens (Hayman, 1955) while there is little known about the source of the other 7 pairs of chromosomes. A survey of the DNA content suggests that P.minor might have originated by combining genomes from the two diploid groups (i.e. 2n=12 and 2n=14). Combining DNA values of P.coerulescens and P.truncata fits well with the observed DNA value in P.minor, but the morphological affinities and growth habits do not support this idea (P.minor is annual while P.truncata and P.coerulescens are both perennials). Combining either P.brachystachys or P.canariensis with P.coerulescens might result in an amphiploid closer to the morphology and growth habit to P.minor (P.minor, P.brachystachys, P.canariensis-all three species are annual), but the combined DNA values of these species are slightly higher than the observed DNA value in P.minor. However, reduction in chromatin material following polyploidy is common and has been discussed in Chapter 4. The DNA values in other tetraploids can also be arrived at either by combining appropriate

species from the two diploid groups (alloploidy) or by doubling DNA contents within the group 2n=14 (autoploidy). One of the difficulties in combining the two groups of diploid species is the difference in the chromosome number, since combining 2n=12 and 2n=14 will result in amphiploids with 2n=26 while all the tetraploids have 2n=28. The origin of 2n=12 has already been suggested by fusion of two chromosomes in the 2n=14 group. This suggests that the reduction in chromosome number to 2n=12 might have been brought about after the origin of amphiploids like P.minor. The DNA value in the hexaploid P.arundinacea involves an addition of 133 units to the tetraploid P.arundinacea DNA value (298 Table 5.1). The relative DNA values in Table 5.1 also suggests that both the tetraploid and hexaploid P.arundinacea can be derived from a diploid with a DNA value like that in P.caroliniana (2x=144.1, 4x=288.2, 6x=432.3) or P.angusta (2x=145.9, 4x=290.8, 6x=437.3) either through straight doubling of the chromosome sets or after hybridization between the two.

5.3.5 Trend in the evolution of DNA content:

There appears to be an evolutionary trend towards increase in DNA content per cell correlated with the trend in the genus towards reduced life cycle. P.coerulescens, a perennial species and probably the most primitive one (Ambastha,

1956) in the genus has the lowest DNA content. If the primitive plants are mostly perennials as has been postulated by Stebbins (1950) it is evident that the trend in evolution in this genus is towards increase in DNA content. A unidirectional trend of increase in DNA content in each group can also be seen since perennials in each group have lower DNA contents than the corresponding annuals in the same group. The diploids with 2n=12 are regarded as having been derived from diploids with 2n=14 and are therefore more advanced than diploids with 2n=14. The DNA values in group 2n=12 are approximately twice those in group 2n=14.

5.4 Relative DNA content between Thespesia species:

Results obtained in the genus Phalaris and Gossypium suggested the possibility that related species with large chromosomes might have disjunct DNA values (Phalaris species) in contrast to the small chromosome species (Gossypium species) which are more likely to have a continuous DNA distribution. The genus Thespesia is a close relative of Gossypium with the same chromosome number (2n=26) (Fryxell, 1969), but having larger chromosomes than the Gossypium species. A study of DNA distribution in this genus might provide additional information about the pattern of DNA evolution in large chromosome

species in contrast to the small chromosome species. A preliminary study of DNA content in two species of Thespesia (Thespesia lampus and one unidentified species of Thespesia) is reported here; more species have not been included because it is difficult to procure seeds. The DNA values are measured against G.stocksii as standard and are given in Table 5.8.

TABLE 5.8

Relative DNA content in two species of Thespesia measured against G.stocksii as standard (100).

Species	Replicates 1	2	3	4	Average
G.stocksii (standard)					100.0
T.lampus	91.2	89.9	90.9	-	90.6
T.species unidentified	322.8	322.9	323.9	341.5	327.7

$$\frac{\text{T.species unidentified}}{\text{T.lampus}} = \frac{327.7}{90.6} = 3.6$$

5.5 CHAPTER CONCLUSION

DNA distribution between the diploid species of *Phalaris* belonging to 2n=12 and 2n=14, was found to be disjunct and approximately two fold. A 1: 2 ratio in the average DNA content per chromosome in the two groups and also the amount of DNA per unit length, suggests that the method for DNA increase is more likely to be through polynemy rather than longitudinal addition or subtraction between the two groups of diploid species. However, longitudinal addition or subtraction may best explain the continuous DNA distribution within each group of diploid and tetraploid species. It is not possible in the genus *Thespesia* to conclude whether the DNA distribution is continuous or disjunct until DNA values are measured in all the species. The DNA values in the two species measured show that in this genus it varies at least 3.6 fold, and that the lower value is close to the smallest values in *Gossypium*.

CHAPTER 6.

Observations relevant to the possibility of intra-specific variation in DNA content.

6.1 Introduction:

Boivin, Vendrely and Vendrely (1948) and Mirsky and Ris (1949) formulated the DNA constancy hypothesis, which states that the DNA content per cell is constant for the somatic cells of an organism and this amount is twice that found in the gametes; a consequence of this is that, in general, all individuals of a species have the same DNA value. The importance of the constancy in DNA content lies in maintaining genetic uniformity in all the cells of an organism. Allowances must be made for variation during the time of DNA replication in the normal mitotic cycle and for polyploidy which accompanies differentiation in some tissues of both plants and animals.

There are some reports in the literature, which will be discussed at the end of this chapter, which cast doubt on the DNA constancy hypothesis. As has been reported in section 3.3.1, similar doubts arose during the investigation of Gossypium species although convincing evidence was not obtained for variation between individuals of the same species. A new experiment was therefore designed with the help of Dr. G.M. Tallis and Mr. P.I. Leppard of the Statistics Department

of the University of Adelaide. This is reported in section 6.2.1. In the three succeeding sections further investigations relevant to variation between individuals are reported.

6.2 Results:

6.2.1 Investigation of variation between individuals of Gossypium hirsutum

The basic design of an experiment was that of a latin square with samples of three individuals being measured in each of the three positions (left, centre, right) on three slides. Five such experiments were carried out so that altogether fifteen individuals were measured.

There are twelve ways in which an experiment (i.e. involving three individuals and three slides) can be performed. These are shown in fig. 1. The particular way used was chosen from the twelve possibilities by a random process. The three slides within an experiment were then prepared in that order and, later, measured in the same order. However, all five sets of slides were made before any measurements were taken and my supervisor gave me slides so that I had no idea which slide in which experiment I was using. This procedure was designed to eliminate any possibility of bias.

The method for preparing slides was basically the same as described earlier. The samples were secondary

root tips chosen from seedling which had a total of about ten secondary roots growing from the primary root. Three such primary roots were fixed together and all solution changing and hydrolysis was done together. Thus as near as possible the three individuals in an experiment were treated identically. Fixed root tips if not processed immediately for hydrolysis were stored in 70% alcohol. Squashing was carried out, using two secondary root tips from the same individual under each cover slip, in the way dictated by the "latin square". After removing coverslips, staining and all subsequent treatment was carried out as given in appendix I.

1	2	3	1	2	3
2	3	1	3	1	2
3	1	2	2	3	1
1	3	2	1	3	2
2	1	3	3	2	1
3	2	1	2	1	3
2	1	3	2	1	3
3	2	1	1	3	2
1	3	2	3	2	1
2	3	1	2	3	1
3	1	2	1	2	3
1	2	3	3	1	2
3	2	1	3	2	1
2	1	3	1	3	2
1	3	2	2	1	3
3	1	2	3	1	2
2	3	1	1	2	3
1	2	3	2	3	1

Fig. 1

Latin squares showing the twelve possible ways that three individuals (1,2,3) can be distributed between the three positions on three slides.

Neutral formalin in phosphate buffer was tried as fixative since formaldehyde fixation is reported as giving more consistent results than acetic acid fixation (McLeish and Sunderland, 1961). Formaldehyde fixed material requires longer hydrolysis and harder tapping on the slide and it was found that this results in fragmentation of nuclei. Early stages of mitosis, particularly prophases, were preferentially lost and fragmented. On the contrary, acetic-alcohol fixation gave well-spread and intact prophases. Therefore acetic-alcohol fication was used. All treatments were as shown in appendix I except that squashing was carried out in distilled water (instead of 45% acetic acid) in the belief that this would reduce variation between positions. Measurements were made at 4C stage using prophases only. Ten nuclei were measured under each coverslips. Thus there were five experiments, each involving three individuals, from each of which there were three samples (2 secondary root tips each), in each of which ten nuclei were measured. The analysis of variance is shown in table 6.1(table 6.1a,6.1b and 6.1c).

TABLE 6.1a.

Analysis of variance of experiment testing variation between individuals of G.hirsutum

Source	SS	DF	MS	VR
Experiments	1706.21	4	426.55	41.29***
Slides	105.66	10	10.57	3.93***
Positions	55.76	10	5.58	2.07*
Plants	147.45	10	14.75	5.48***
Slides x Positions	53.55)	10)	5.36)	2.04*
	)1116.75	)415	)2.69	
Within samples	1063.20)	405)	2.63)	
Total	3131.84	449		

* = sig at 5% level.
** = sig at 1% level.
*** = sig at .1% level.

) Indicates that "slides x positions" has been pooled with "within".

In table 6.1a partitioning out variation due to experiments, slides and positions, variation due to plants is highly significant ($P < .001$). This is so whether or not "slides x positions" is pooled with "within samples". This analysis, experiment by experiment, is further detailed in Table 6.1b.

TABLE 6.1b

Analysis of data in Table 6.1a set out experiment by experiment

	Expt.1 SS	Expt.2 SS	Expt.3 SS	Expt.4 SS	Expt.5 SS	Total SS	DF	MS	VR
Slides	0.64	3.0	39.03	24.78	38.21	105.66	10	10.57	3.93
Plants	2.04	10.66	15.56	71.47	47.72	147.45	10	14.75	5.48
Positions	12.82	26.11	8.78	6.59	1.47	55.76	10	5.58	2.07
Slides x Positions	.080	3.94	41.09	3.02	5.42	53.55)	10)	5.36)	2.04
						)1116.75	)415		)2.69
Within samples	330.18	234.45	175.21	188.88	134.48	1063.20)	405)	2.63)	-
Total (within exp.)	345.76	278.16	279.67	294.74	227.29	1425.62	445		
Between Expt.						1706.21	4	426.55	41.29
Total						3131.84	449		

It can be seen that the great majority of the "slides x positions" variance is contributed by Experiment 3, which is therefore regarded as a "statistical outlier". In Table 6.1c below the analysis is carried out omitting Experiment 3.

TABLE 6.1c.

Re-analysis of Table 6.1a omitting Experiment 3 from Table 6.1b.

Source	SS	DF	MS	VR
Experiments	1694.23	3	564.74	55.25***
Slides	66.63	8	8.32	3.03**
Plants	131.89	8	16.48	6.01***
Positions	46.99	8	5.87	2.14*
Slides x Positions	12.46	8	1.55	0.56
Within samples	887.99	324	2.74	
Total	2840.19	359		

* = sig at 5% level. ** = sig at 1% level. *** = sig at .1% level.

This experiment suggests that there is indeed significant variation between individuals of Gossypium hirsutum. However, the design of experiments assumes that the samples (secondary root tips) are drawn from the population without any true variation. If this were not so, e.g. if due to some developmental event true variation in DNA content exists between

root tips of the same individual, then variation between plants should be tested against variation between root tips within individuals. In the above experiments this cannot be done since "between samples within individuals" variation is confounded with "slides x positions". If the analysis in Table 6.1c is accepted, then the non-significance of "slides x positions" variance strongly suggests that variation between root tips within plants can be ignored.

6.2.2 Quantitative DNA measurements in the two strains of maize:

It seemed possible that, if two varieties of the same species had, for many generations, been subjected to selection in diverging directions with respect to some character, then DNA value might have been affected also. Seeds of the high and low seed protein lines of Zea mays, subjected to selection for 50 generations, were obtained from the United States*. Although the result revealed no difference in a t-test it seems worth recording the data. In this test a pair of high and low protein plants were processed together and tested on the same slide. Eleven pairs of such plants were compared and for each pair a number of replicates (2-4) were measured. The result of these measurements are tabulated below (Table 6.2).

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TABLE 6.2

Relative DNA content between low (L) protein and high (H) protein strains of maize

		L	H
$L9/_{H2}$		18.90	21.43
		18.63	19.48
		18.89	15.90
		17.27	15.68
$L7/_{H8}$		19.42	20.35
		14.63	14.97
		19.38	19.34
$L6/_{H6}$		18.33	19.41
		20.32	21.03
		19.68	19.48
$L5/_{H5}$		19.21	19.70
		19.81	20.51
		16.11	15.64
$L4/_{H4}$		20.77	20.38
		18.43	20.98
		17.38	16.81
$L10/_{H13}$		18.40	18.97
		17.35	15.5
$L14/_{H15}$		21.90	20.77
		21.60	19.80
$L16/_{H7}$		17.90	17.15
		17.79	17.96
$L11/_{H9}$		17.97	17.19
		18.06	16.52
$L12/_{H11}$		17.08	16.56
		18.90	19.43
$L2/_{H1}$		15.01	14.88
		15.24	14.97
	average	514.36	510.79

D.F. = 27
t ratio = .17
P = $>.8$

6.2.3 Quantitative DNA distribution during gametogenesis in Phalaris:

It seemed possible that an investigation of the distribution of DNA during meiosis might throw light on the origin of the variability which appears to occur between individuals of a species. Because Gossypium species have lower DNA values than Phalaris species, the latter were chosen for investigation. The reason for this was that in tetrads the amount being measured (1C) would be a quarter the quantity normally measured in somatic prophase cells (4C). Therefore a diploid species, in which meiosis is regular, and with a high DNA content, was required. P.brachystachys was the species selected.

Anthers were squashed in 45% acetic acid on a subbed slide, slides were freeze-dried and the cover slips flicked off. The slides were brought down to distilled water, hydrolysed at 60^{o}C for ten minutes, washed in cold distilled water and finally stained in leuco-basic fuchsin for two to three hours. The stained slides were made permanent according to the schedule in Appendix I. Measurements were taken at different stages of meiotic division, namely, late pachytene, anaphase I (whole dyads), anaphase II (measuring tetrads as a whole and also individual spores within a tetrad) and young spores from different tetrads. The average densitometer readings are shown in Table 6.3. These

averages have no real meaning since they are obtained combining values from different slides stained separately but nevertheless the ratios expected from C values are approximately correct. Components having 4C value (pachytene, whole dyads and whole tetrads) gave similar variances and these were roughly four times the variance observed in 1C component (individual spores). The results are tabulated in Table 6.3.

TABLE 6.3

Mean DNA content and variances between pachytene, between dyads, between tetrads and between spores.

Stage	Mean densitometer reading	Number of Cells	Variance
Pachytene 4C	29.5	60	5.4
Dyads 4C	27.2	10	4.1
Tetrads 4C	31.2	29	4.6
Spores 1C	7.2	60	.9

Analysis of variance of 10 complete tetrads from the one slide in which each spore was measured separately, are shown in Table 6.4. The variability between tetrads is much higher than that within tetrads ($P < .01$).

TABLE 6.4

Variances between and within tetrads of Phalaris brachystachys

Item	SS	DF	Variance	F	Probability
Between tetrads	24.28	9	2.69	3.34	$<.01$
Within tetrads	24.26	30	0.81		
Total	48.54	39	1.24		

Significant variability between tetrads (Table 6.4) is expected to lead to spores with variable DNA contents. This in turn will lead to individual variation provided these variations are heritable. To test this, DNA contents between individuals of Phalaris canariensis were compared in an experiment identical to that designed for testing individual variation in Gossypium species (Chapter 3, section 3.3.1). The statistical analysis of this test is given in Table 6.5 below. It may be pointed out here that it would have been more informative to test individual variation in P.brachystachys where DNA distribution during gametogenesis has been estimated quantitatively. However, because P.brachystachys plants were not available this test was carried out in P.canariensis.

TABLE 6.5

Analysis of variance for individual variation within P.canariensis

	Between positions	Between slides	Total between individuals	Within individuals (Error)	Between individuals on same slide	Total variance	Variance ratio F	P
Degree of freedom	2	3	11	108	6	119		
	2.08	52.16	20.39	4.8	10.62	6.29	2.21	.05

The probability near .05 for individual variation within P.canariensis is on the border line of significance but does not prove conclusively individual variation within P.canariensis.

6.3 CHAPTER DISCUSSION

References were made in the introduction to examples that appear to be exceptions to the constancy rule. Such exceptions, as suggested by Fox (1969a), could be either ontogenetic or phylogenetic. Ontogenetic exceptions to the constancy hypothesis originate from the differential replication of chromosomal DNA and are usually confined to the regions of genomes which are active. Further, ontogenetic changes in the genotype do not permanently affect the structure of the genotype and are not heritable. Differential replications have been reported by a number of workers in a number of organisms (Perkowska, McGregor and Brinstiel, 1968; Rudkin and Crottele, 1957; Rudkin, 1963 and Berendes and Keyl, 1967).

Several phylogenetic exceptions to the DNA constancy hypothesis have been reported. A few reports of differences in DNA content between sub-species or varieties are on record (Mello-Sampayo, 1961; Nishikawa and Furuta, 1969). Mello-Sampayo associates this difference with geographical separation. Intra-specific variations in species of gymnosperms have recently been reported by Miksche (1968 and 1971) and variation was found to be related to latitude. The variation between sympatric individuals within species presumably has the same origin whatever that may be. Dowrick and Elbayoumi (1969) reported significant variation

in DNA content, not only between different individuals of Chrysanthemum but also between different roots of the same individual.

Fox (1969a, 1969b, 1970) has reported naturally occurring significant differences in DNA content between individuals within species. He has investigated spermatid nuclei of seven species of beetles of the genus Dermestes. However, in his experiments, each individual is represented by one slide so that slides are completely confounded with individuals. In my experiment (Table 6.1) significant "between slides" variance is recorded. Although Fox's technique was different, nevertheless his work would bear repetition with more elaborate experimental design.

Although the possibility of variation of the DNA content per chromosome cannot be denied, any significant variation in DNA content between individuals can also be regarded as arising from irregularities like aneuploidy and B chromosomes. In the absence of evidence that such abnormalities are not present, which presumably is the case in Gossypium though it has not been demonstrated, any attempt to explain the mode and nature of these variations will be speculative. Dowrick and Elbayoumi do not discuss the possibility of any cytological abnormality in Chrysanthemum.

Another source of individual variation may be attributed to the extra-nuclear DNA since chloroplasts and mitochondria are known to have their own DNA (Edelman et al., 1964; Kirk, 1963; Luck and Reich, 1964), and estimations of DNA from whole cells may include some amount of DNA from these organelles. However, Suyama and Bonner (1966) and Rothfels et al. (1966) made a quantitative estimation of mitochondrial DNA and plastid DNA respectively and reported it to be extremely small when compared with nuclear DNA contents. Thus the DNA from these organelles should not contribute materially to alter the conclusion regarding the intra-specific variation.

The work of Evans et al. (1966) and Evans (1968a, 1968b) with flax plants regarding evironmentally induced heritable variation is one clear example of exceptions to the DNA constancy hypothesis. The variation was induced in the variety Stormont Cirrus treating plants of the original variety (plastic genotroph) with high nitrogen and high phosphate and so producing plants of the large stable genotroph (L) and plants of the small stable genotroph (S) respectively. These changes are stable once induced and are inherited. These changes are also accompanied by an increase or decrease in DNA content when compared with the plastic genotroph. The plants of the large genotroph contain 16 per cent more nuclear DNA than the plants of the small genotroph. The

increase or decrease is induced progessively at the early stage o f development and is completed prior to gametogenesis. However, once the induction process is complete DNA constancy again operates in the following generation.

Intra-specific variation in DNA content might be expressed in phenotypic variation and vice versa. With this assumption DNA content in two strains of maize differing in their protein content were compared. No significant difference was observed in the average DNA content between the two strains. However, this was only a single test and only shows that this particular selection experiment (for high and low seed protein over fifty generations) did not involve detectable change in DNA content.

The possibility that intra-specific variation might originate some time during gametogenesis was suggested by the possibility of DNA synthesis throughout meiosis as has recently been reported by Riley and Bennett (1971) in Triticum aestivum. Their auto-radiographic results suggested identical labelling in the two homologues of a bivalent, which mean an equal distribution of DNA content between the two halves of a dyad. However, if DNA synthesis continues further in each half-dyad it is possible that it is not identical which may lead to spores different in DNA content.

The statistical analyses of data for DNA distribution during gametogenesis in Phalaris in Table 6.3 do not suggest any increase in variability from pachytene to tetrads since all 4C

values have similar variability. The variability between tetrads being significantly higher than variability within tetrads (Table 6.4) suggested the possibility of true variation between PMC but a relatively precise distribution of DNA content within each PMC. If true, this would lead to spores differing in their DNA content.

However, the significant variation between tetrads may, alternatively, be attributed to variation in staining from one part of a slide to another. This possibility was investigated by plotting the densitometer DNA readings against the densitometer stage readings which will be called the x and y co-ordinates. With the help of one of my colleagues (Mr. I.R. Noble), the following tests were applied: 1) Visual examination did not suggest any obvious pattern. 2) Multiple regression analyses of readings on x and y co-ordinates were non-significant. 3) Applying the hypothesis that there is an unknown centre of concentration (point of highest reading in this case) from which densitometer readings decrease with distance. The data does not fit the hypothesis. 4) "t" tests were used by dividing the slide into two equal groups (i.e. left and right; top and bottom; centre and edge). Non-significant results were obtained in each case. Thus there do not appear to be any reason for doubting a basic assumption of the method, that there is not systematic variation of readings over the surface of a slide.

Although spores with different DNA contents should result in individual variation, tests so far in P.canariensis do not lead to this conclusion. The test of significance gave probability just less than .05 (Table 6.5). This may imply that the variation in the spores is eliminated due to strong selection either at the gametic level or even zygotic level. Another explanation of this phenomenon may be that since the variability is present right from pachytene cell and does not originate during gametogenesis, it may be ontogenetic. Any attempt to explain the mechanism at this stage regarding the origin of variability within species will be hardly more than speculation. The possibility of "between individuals" variation within species however, suggests that intraspecific variation might give rise to variation between species progressively. This points to changes in the longitudinal structure of the chromosomes, rather than supporting the multistranded hypothesis. It is quite likely that different trends may be present in different taxonomic groups.

CHAPTER 7.

CONCLUSION:

Relative DNA distributions within the genera Gossypium and Phalaris have been studied in detail. Among the diploid species in the genus Gossypium there is more than a two-fold difference in nuclear DNA content. This variation occurs without difference in chromosome number (2n=26) and appears to be continuously distributed. The diploid species of Phalaris also revealed a two-fold difference; however, they can be divided into two groups according to whether the chromosome number is 2n=12 or 2n=14. Within each group, DNA contents vary continuously in contrast to the distribution between the two groups which is disjunct and in a ratio of 1:2. The average DNA content in species with 2n=12 is 257.3 while in species with 2n=14 the average is 127.7. The average DNA per chromosome in group 2n=12 is 18.4 and is apparently double that in the 2n=14 group (average 9.1).

Continuous distribution in DNA contents between diploid species of Gossypium suggest that the data are more compatible with a single-stranded chromosome with extensive longitudinal repetitions, brought about either by well known methods of chromosomal segment duplication or by local multiplicity as postulated by Keyl (1965).

Variation between individuals within a species as has been reported in the genus Gossypium, is one exception to the DNA constancy rule. Evans and his colleagues (1966) reported induced DNA variation in flax which was heritable. Fox (1969a and 1969b) has reported naturally occurring individual variation within species of Dermestes, though his evidence is not conclusive (see page92). Although investigation to find out whether this intraspecific variation is heritable have not been reported yet, Fox (1969a) suggests such a possibility.

It is hard to explain the mechanism which is responsible for the variation between individuals within a species; however, either Keyl's mechanism of local multiplicity or segmental duplications, which can account for the continuous variation in DNA content per cell between related species, can also account for the individual variation within a species. It is conceivable that intraspecific variation might gradually lead to interspecific variation.

The possibility of DNA synthesis throughout the meiotic division has recently been reported by Riley and Bennett (1971). It is not unlikely that continuous DNA synthesis at all stages of meiosis may lead to spores with unequal contents of DNA. This may finally result in

individuals with variable DNA contents within a species. Although variability may occur early in meiosis in Phalaris there is nothing in the results to indicate that this increases as meiosis progresses since all 4C values have similar variances (Table 6.3). The significant variation between tetrads (Table 6.4) as against within tetrads suggests the possibility of spores being formed with different DNA content, which may lead to individual variation. This has not in fact been demonstrated in Phalaris (Table 6.5). It is hard to reconcile the two facts, though if real the possibility of selection both at the gametic level and zygotic level might be invoked to account for this.

The pattern of DNA evolution in the genera Gossypium and Phalaris do not parallel in every respect. In Phalaris the continuous variation within each group (two diploid and two polyploid) can be explained on the basis of local multiplicity or segmental duplications but the disjunct distribution of 1:2 between the two diploid groups stands in contrast to the continuous distribution in the genus Gossypium. Disjunct distribution can best be explained assuming the mechanism of DNA increase through lateral multiplicity. It appears that the large chromosome species may have their DNA increase through local multiplicity, segmental duplications

or by lateral multiplicity while species with small chromosomes have their DNA increase through local multiplicity or segmental duplications. 3.6-fold variation between two species of Thespesia, a closely related genus to Gossypium with 2n=26, also suggests lateral multiplicity as the mechanism of DNA increase.

Whether the trend in evolution in the genus Gossypium is towards increase or decrease in DNA content is difficult to ascertain convincingly. It seems that in the diploid species, particularly (A) genome species, that the trend is towards increase. The DNA content in G.herbaceum var. africanum, the only wild representative in (A) genome, has lower DNA content than G.arboreum (also from A genome). If the cultivated species can be regarded as being more advanced than its wild counterpart, it seems that there has been increase in DNA content in the course of evolution. However, there is no consistent relationship between average DNA content in different genomes and their primitiveness. In the tetraploids it is still hard to reconcile a unidirectional increase or decrease in DNA content with other aspects of evolution. On the other hand in the genus Phalaris the trend in evolution seems to be unidirectional and towards increase in DNA content. Further, no change in the average

DNA contents in different races of P.minor and P.brachystachys could be recorded.

The roles the repetitious DNA or multiple copies of genes play in evolution is hard to explain and can be only speculative so far. Britten and Kohne (1968, 1969) suggest that these might provide higher rates of synthesis. The repetitious DNA, either through mutation or by translocation to different parts of the chromosomal complement, may yield a range of genetic activity which eventually leads to nucleotide sequences being able to code for new proteins. This will finally result in divergence and evolution (Britten and Kohne, 1967). It is quite likely that the increase or decrease in DNA content per cell during evolution is adaptive. The variation in DNA content can be expressed as differences in cell size (Martin, 1966) and this may act as an isolating mechanism leading to speciation (Martin, 1968). The induced changes in DNA content in flax plants (Evans et al. 1966), naturally occurring intraspecific variation in Dermestes (Fox, 1969a, 1969b) and in Gossypium (in the present report) may gradually lead to interspecific differences in DNA content, finally providing one of the means for speciation.

To arrive at a definite conclusion regarding the origin of tetraploid cottons was hard because of the variation in DNA contents between individuals of the same

species and the possibility of changes in DNA content either in the diploid progenitors or the tetraploids following amphiploidy. Nevertheless a comparison between the observed value and the expected one combining diploid parents and the fact that the DNA values in the three amphiploids are different suggests that if A and D genome species were the diploid progenitors for three tetraploids they must have been different from present day species or there have been changes in DNA contents since hybridization. It may be pointed out here that the three amphiploids hybridize easily showing thereby that their chromosomes are still sufficiently homologous in spite of the significant changes in their DNA contents.

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APPENDIX I.

Time Schedule for Feulgen-staining

After the slides had been freeze-dried and the cover slips were removed the following treatment was given.

Treatment	Time (min.)
90% alcohol	2
70% alcohol	2
50% alcohol	2
30% alcohol	2
Distilled water 1	2
Distilled water 2	2
Feulgen-stain	120-180
SO_2 water 1	10
SO_2 water 2	10
SO_2 water 3	10
Distilled water 1	2
Distilled water 2	2
30% alcohol	2
50% alcohol	2
70% alcohol	2
90% alcohol	2
Absolute Alcohol 1	2
Absolute Alcohol 2	2
Xylene I	2
Xylene II	2

Mounted in Xam.

APPENDIX II

Method for calculating DNA value of species X relative to species Y, analysis of variance and standard error.

The standard error of a DNA value has been estimated using the method of N.G. Martin (in press). The mathematical expressions for calculating the ratio of the DNA content of species X to species Y, its variance and standard error are reproduced below. Weighting factors are used when combining the data from different slides to give the mean and its standard error. The x values are measurements of species X and y values of species Y. The method is general for the case where there are l slides and the number of cells under one coverslip is not necessarily the same.

Slide 1	Slide i	Slide l
$x_{1,1}$	$x_{1,i}$	$x_{1,l}$
$\vdots$	$\vdots$	$\vdots$
$x_{m_1,1}$	$x_{m_i,i}$	$x_{m_l,l}$
$y_{1,1}$	$y_{1,i}$	$y_{1,l}$
$\vdots$	$\vdots$	$\vdots$
$y_{n_1,1}$	$y_{n_i,i}$	$y_{n_l,l}$
No. cells n_1 n_1	m_i n_i	m_l n_l
Mean $\bar{x}_1$ $\bar{y}_1$	$\bar{x}_i$ $\bar{y}_i$	$\bar{x}_l$ $\bar{y}_l$
s.d. s_{x_1} s_{y_1}	s_{x_i} s_{y_i}	s_{x_l} s_{y_l}

Let

$$k_x = \frac{1}{l} \sum_{i=1}^{l} \frac{s_{x_i}}{x_i}$$

$$k_y = \frac{1}{l} \sum_{i=1}^{l} \frac{s_{y_i}}{y_i}$$

and

$$c_i = 1 + \frac{k_y^2}{n_i}$$

Then an estimate of the ratio, $R = \frac{\text{Mean species X DNA content}}{\text{Mean species Y DNA content}}$

for the i^{th} slide, $\hat{R}_i$, and its variance, $V(\hat{R}_i)$, are given

by $$\hat{R}_i = \frac{\bar{x}_i}{\bar{y}_i} \quad (\text{where } E(\hat{R}_i) = Rc_i)$$

and $$V(\hat{R}_i) = R^2 \left(\frac{k_x^2}{m_i} + \frac{k_y^2}{n_i} \right)$$

Combining the data from all slides

$$\hat{R} = \sum_{i=1}^{l} w_i \hat{R}_i$$

and $$V(\hat{R}) = \sum_{i=1}^{l} w_i^2 V(\hat{R}_i)$$

where w_i are weights chosen such that $\hat{R}$ is the best linear unbiased estimate of R. These are given by

$$w_i = \frac{c_i}{V(\hat{R}_i) \sum_{i=1}^{l} \frac{c_i^2}{V(\hat{R}_i)}}$$

$$= \frac{c_i}{\frac{k_x^2}{m_i} + \frac{k_y^2}{n_i} \sum_{i=1}^{l} \frac{c_i^2}{\frac{k_x^2}{m_i} + \frac{k_y^2}{n_i}}}$$

Having obtained w_i, $\hat{R}$ can be obtained and used to calculate $V(\hat{R}_i)$ and hence $V(\hat{R})$. If $m_i = n_i$ for all i and each m_i is sufficiently large, then $c_i = 1$ and $w_i = \frac{m_i}{\sum_{i=1}^{l} m_i}$.

In addition, if $n_i = n_{+1}$ for all , then $w_i = \frac{1}{l}$