RESTRICTION ENDONUCLEASE ANALYSIS OF PEANUT MITOCHONDRIAL DNA

By

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RESTRICTION ENDONUCLEASE ANALYSIS

OF PEANUT MITOCHONDRIAL DNA

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CHAPTER I

INTRODUCTION

Cytoplasmic, or non-Medelian inheritance was first described in the early 1900's, but not widely accepted for many years (16, 42). Interest grew with the discovery of cytoplasmic male sterility in the early 1920's, and mushroomed when it was found in maize in 1933 (16). Cytoplasmic diversity in peanuts has been identified in growth habit (2-5), nitrogen fixation characteristics (19), and pod morphology (11). Ashri (2-5), has done exhaustive studies on growth habit, and identified three plasmons which he named [V4], [O], and [G].

The elucidation of the structure of DNA opened the modern era of cell and molecular biology, and methods were developed to investigate the nature of cytoplasmic genes and organelle function (42, 50), but these methods have only lately been adapted to the study of plants (6, 13, 27). A rather elegant technique for identifying cytoplasmic diversity involves agarose gel electrophoresis of DNA fragments which have been produced by various restriction endonuclease enzymes (7, 12, 13, 18, 25, 26, 28, 29, 32, 33, 36-40, 43-49, 51). This method was used by Pring et al. (38), working with maize, to identify three different

mitochondrial plasmons in lines which showed no variance in their chloroplast DNA.

The objective of this study was to investigate the practicality of using restriction endonuclease analysis to characterize cytoplasmic differences in peanut (*Arachis hypogaea* L.). As other studies have found more variance present in mitochondrial DNA (mtDNA) than in chloroplast DNA, work was limited to mtDNA. Because Ashri's (2-5, 17, 30) work was considered to be the most authoritative in peanut cytoplasmic inheritance, seed was requested for his three plasmons while procedural work began on locally available cultivars. There was a problem in obtaining seed for Ashri's plasmons, but seed was eventually received for two of them, V4 [V4] and VSM [O].

Difficulties were encountered initially in producing healthy peanut seedlings. Many trials had to be destroyed due to bacterial and/or fungal contamination. Attempts to overcome this included surface sterilization of seed with sodium hypochlorite, hydrogen peroxide, ethanol and eventually, ultra-violet (UV) light, all to no avail. The difficulties were finally controlled with Captan fungicide, and judicious moisture control. The germinator which was used for most of the early trials was abandoned due to excess moisture and internal contamination midway through the study, and subsequent trials were grown in a constant temperature chamber at 30° C.

CHAPTER II

REVIEW OF LITERATURE

Plant Breeding Studies

Cytoplasmic inheritance has been an object of study for 75 years. First reported in 1908, it was identified by two separate researchers (Carl Correns and Erwin Baur), as affecting chloroplast development in two different plant species, and labelled non-Mendelian inheritance (16, 42). Cytoplasmic genes are fairly easy to identify in higher plants, as any difference between the respective F_1 progenies of a reciprocal cross, followed by somatic segregation of the two parental phenotypes during growth of the progeny, can be attributed to cytoplasmic inheritance. This ease of identification produced a wealth of information over the next 50 years as plant breeders vied to find new cytoplasmic genes.

Perhaps the most significant discovery in this area was that of Rhoades, who found cytoplasmic male sterility (CMS) in maize in 1933. This facilitated the production of hybrid maize seed to such a degree that it quickly became the preferred mechanism among seed producers, while the very existence of cytoplasmic genes was still being bitterly debated among geneticists (42). Grun (16) calculates that

there were 3×10^9 maize plants with Texas cytoplasm growing in the United States when the extreme susceptibility of this cytoplasm to *Helminthosporium* leaf blight was discovered. The rapid spread of this fungus in 1970 caused great loss to American corn growers, and resulted in the immediate reversion to the more expensive process of detasseling by hybrid maize seed producers, followed by a search for new sources of cytoplasmic diversity to incorporate into their cultivars.

The first authoritative cytoplasmic inheritance work in peanuts was done by Ashri (2-5), in the area of growth habit. He first proposed two plasmons [V4], and [O] (2), and then added a third, [G], after a subsequent study (3). He then tested the plasmon constitution of 68 different accessions of cultivated peanuts in crosses with three testers having [V4], [O], and [G] plasmons, and concluded that only the [O] cytoplasm was common in the cultivars tested, the other two occurring only in the testers (5).

Halevy et al. (17) tested the [V4] and [O] plasmons for response to gibberellic acid and its inhibitors. They concluded that the differences in growth habit could be attributed to a gibberellin antagonist such as abscisic acid. This theory was later supported by Ziv, Halevy and Ashri (54), when they showed that the balance between gibberellic acid and its inhibitors is affected by light conditions. Levy and Ashri (30) were successful in inducing plasmon mutation in [V4] and bunch cultivars, but somatic

segregation of heteroplasmons continued beyond the time of their report.

Resslar and Emery (41) disagreed with Ashri as to the existence of different plasmons in peanuts. They tested [V4] and [O] cytoplasms using larger F_1 populations, and reported the dissipation of reciprocal differences after the F_1 generation. They suggested that the differences between the two F_1 groups may be due to dissipating maternal effects of the [V4] parent, rather than to cytoplasmic inheritance.

Coffelt and Hammons (11), proposed a cytoplasmic factor in the inheritance of pod constriction in peanuts. This gene exhibits complementary-duplicate action with three unlinked nuclear loci, in which the presence of dominant alleles at any three of the four loci would produce constriction, while any two of the four loci being homozygous recessive would produce unconstricted pods.

Perhaps the most intriguing evidence of maternal effect in peanuts comes from Isleib et al. (19). In their study on the quantitative genetics of nitrogen fixation, they found significant reciprocal differences in nodule number, nodule mass, shoot weight, and total nitrogen content, which they attributed to interaction between nuclear and extranuclear factors.

Molecular Studies

The delineation of the Structure of DNA by Watson and Crick in 1953 commenced a new era in genetics, because it

facilitated the study of genes at the molecular level. Scientists from many disciplines began studying the basis of life and its workings. Enzymes were isolated which could polymerize, ligate, or digest DNA and the mechanisms of recombination and reproduction were delineated. The discovery of DNA restricting enzymes gave birth to modern recombinant DNA technology and the applied science of genetic engineering was born. Watson (50, p. 716) gives the following definition:

> Restriction Enzymes - Components of the restriction-modification cellular defense system against foreign nucleic acids. These enzymes cut unmodified (e.g., methylated) double-stranded DNA at specific sequences which exhibit twofold symmetry about a point.

In addition to facilitating recombinant DNA work, these enzymes can be used to characterize small and relatively simple DNA molecules, such as those present in viruses, prokaryotic organisms, and eukaryotic organelles.

According to Bendich (6), plant mitochondrial DNA is the last frontier in this area, due to the unusually large sizes of plant mtDNA molecules. He suggests that

> ...the genic sequences in the mitochondria of all organisms are located on a single linkage group, and most of the plant mitochondrial DNA molecules are composed of noncoding sequences. Rather than providing the information we seek concerning genes, an analysis of the sequence arrangement for most mitochondrial DNA molecules may actually lead us to detailed relationships among complements of ... 'junk' DNA (p. 480).

While he admits that the single linkage group of this theory has yet to be isolated, he is convinced that it does exist,

and will be found when the proper method is developed. Leaver et al. (27, p. 458) also recognize the size of plant mtDNA as a source of difficulty in its analysis, but they offer an alternate explanation for its large size, which might be due to the inclusion of additional "regulation sequences" that are involved in some form of communication with the chloroplast and nuclear genomes during the coordinated changes in cellular function associated with the development and differentiation of the plant cell. They also point out that mtDNA is strongly implicated as the source of CMS, and therefore worthy of investigation regardless of obstacle.

In spite of these difficulties, work is proceeding in the characterization of the plant mitochondrial genome. Boutry and Briquet (7) have used restriction endonuclease analysis to differentiate among CMS and normal lines of faba beans, and Conde et al. (12) have used it to characterize extrachromosomal inheritance in interspecific crosses of *Zea*. Levings and Pring (28) have used this method to characterize differences between normal and Texas cytoplasms in maize, and to identify five plasmons among the mitochondria present in normal cytoplasms of maize (29). Pring has been involved in several studies wherein this method has been used to characterize diversity in male-sterile cytoplasms of maize (38, 40) and in sorghum (39). Sisson worked with Brim and Levings (48) to characterize cytoplasmic diversity in soybeans by the same

type of technique. Though methods vary among researchers and plant species, they generally include a mitochondrial isolation by differential centrifugation, digestion of extramitochondrial DNA, isolation and purification of mtDNA, restriction with one or more enzymes, and agarose gel electrophoresis followed by visualization of the banding patterns. Of particular interest was the finding of Pring, Conde, and Levings (38) that the C group of male-sterile maize cytoplasms included three different plasmons of mtDNA, while no variation was found in its chloroplast DNA.

Kemble and Bedbrook (23), and Kemble et al. (24) electrophoreses whole mtDNA from various maize cytoplasms, and found differing patterns of low molecular weight bands in each. Kemble and Bedbrook (23, p. 565) suggested that these might be "autonomously replicated" plasmids.

Other Works Useful in Method Evolution

Breidenbach and others (8, 9) used a food mill to chop peanuts prior to homogenization, and established the buoyant density of peanut mtDNA as 1.716. Their proposition that the numbers of mitochondria in peanut cotyledons increase during germination was of special interest. This idea was confirmed by Cherry (10), whose work in peanuts delineates the seedling age which will produce maximum yields of mtDNA as 8 days. Though he showed maximum DNA content at 10 days, his electron microscope studies showed mitochondrial deterioration beginning after 8 days. Further evidence of

the increase in mitochondrial numbers during germination was found in Morohashi et al. (34, 35).

Dhillon et al. (14), ignored mtDNA in thèir characterization of peanut nuclear DNA, and Dhillon and Miksche (15) showed that peanut cotyledons contain several times as much nuclear DNA as do other growing tissues. They theorized that the heterochromatin content of the cotyledons is a nucleoside and phosphate source for growing tissue, as the content decreases with age.

Ikuma and Bonner (18) stressed the importance of removing starch early in mung bean routines, while Jacks et al. (20) delineated procedures for the removal of fats from peanut preparations. Koldner and Tewari (25) described buffer formulas, and Parenti and Margulies (36) established the need for a high pH, and also delineated several ways to avoid contamination during processing. Yarbrough's studies of peanut seedling morphology (52, 53) confirmed the presence of tough, woody tissue in roots and hypocotyls, necessitating chopping prior to homogenization.

Organelle Evolution

The discovery of cytoplasmic inheritance naturally led scientists to theorize on the evolution of the eukaryotic organism. There are two major theories which have survived to date, the first holding that a nucleated prokaryote somehow pinched off pieces of its nucleus and DNA to form the organelles, while the more generally accepted hypothesis

proposes that free-living organelle precursors were ingested by a larger autotroph, which then evolved an endosymbiotic relationship. Good reviews in this area can be found in the following references: Dale (13), Grun (16), Keeton (22), Sager (42), and Watson (50). The controversy stems from the fact that many organelle proteins are coded in nuclear DNA.

The endosymbiont theory has gained new support from Anderson et al. (1, p. 458), who found that the human mitochondrial code has basic differences from what was once thought to be the "universal code", and Lewin (31), who cites several recent reports which unequivocably identify mtDNA fragments present in nuclear DNA, and one which has found chloroplast genes in mtDNA. While several of these authors cite transposition as the method of organelle gene transfer, none has yet been so bold as to propose a particular method for DNA movement between cellular organelles. It is possible that the organelles may engage in something very much like bacterial conjugation. Though this would be hard to prove, it does seem more plausible than transposons leaping about between cellular constituents much as frogs between lily pads.

CHAPTER III

MATERIALS AND METHODS

Genetic Materials

The genotypes tested and their sources are listed in Table I. The first four entries are related lines, and were expected to have identical cytoplasms, while P-1192 and US98y were suspected of having unusual plasmons. Seeds were obtained for two of Ashri's (2-5) plasmons, PI-315616 V4 [V4], and PI-315618 VSM [O], and grown for increase at the Perkins Research Station during 1982. Prior to the availability of known cytoplasmic materials, several locally available cultivars were used to establish procedures.

Several methods were tried for producing plant tissue, including various seed surface sterilization and growth techniques. Initial trials were planted in autoclaved sand and dark-grown for 7 to 12 days, in a germinating chamber set for 30° C for 14 hours/day alternating to 25° C for 10 hours/day, using approximately 50 g of surface sterilized seed. One trial was light-grown at room temperature for 15 days, and another used seed which had been imbibed for only 1 day. Later trials used 10 to 38 g of seed (depending on germination and contamination rates), treated with Captan fungicide and dark-grown for 8 days at 30° C, in sterile

sand, or in moist paper towels wrapped in waxed paper and placed inside a 5 gallon bucket. Throughout the study, contaminated seedlings were removed prior to processing.

TABLE I

SOURCES OF PEANUT GENOTYPES USED IN STUDIES

GENOTYPE	P. I. NO.	PLASMON[1]	COUNTRY OF ORIGIN	SOURCE[2]
CHICO		[O]?	RUSSIA	OAES
COMET		[O]?	USA	OAES
EM-12		[O]?	USA	OAES
EM-13		[O]?	USA	OAES
P-1192	298853	[G]?	S. AFRICA	OAES
US98Y[3]	468295	[?]	BOLIVIA	D.J.BANKS
V4	315616	[V4]	ISRAEL	SRPIS
VSM	315618	[VSM]	ISRAEL	SRPIS

1 - According to Ashri's classification (5).
2 - OAES - Oklahoma Agricultural Experiment Station.
SRPIS - Southern Regional Plant Introduction Station, USDA-ARS, Experiment, GA.
3 - Yellow flowered mutant.

Two different techniques, plus various combinations of the two, were used for the isolation of mtDNA. This was done in an attempt to minimize the amount of plant tissue

needed to produce a fair quantity of restrictable DNA, so that, in the future, some wild genotypes might be tested, for which seed is extremely rare.

Method 1

This method is an adaptation of one which has been used by several researchers on various plants, including Kolodner and Tewari (25) on peas, Levings and Pring and several others on corn and sorghum (12, 28, 29, 37, 38, 39, 40, 44, 45, 48) and Sisson (47) on soybeans.

Isolation of Mitochondria

Cotyledons and shoots were processed separately from hypocotyls and roots during initial steps to isolate fatty tissues and facilitate resuspension of non-fatty materials. Throughout the differential centrifugation described below, fatty segregates were removed or excluded from cotyledonary preparations, which were then combined with non-cotyledon fractions for further processing at the discretion of the experimenter. Such combinations were limited to samples of the same genotype, and did not generally involve isolates from different plantings. Processing took place at 0 to 4° C except where noted.

Tissue was weighed, rinsed in glass-distilled water, coarsely chopped, added to 4 ml/g fresh tissue weight of buffer A [0.3 *M* mannitol, 0.5 *M* tris adjusted to pH 8.0 with HCl, and 0.1% (w/v) of bovine serum albumin (BSA), 1 m*M*

2-mercaptoethanol], and homogenized for 4 seconds at low speed and for 4 seconds at high speed in a 10 speed pulse-matic Osterizer. Some trials were homogenized in a mortar and pestle. The homogenate was filtered through four layers of cheesecloth and one layer of miracloth (Calbiochem) before centrifugation for 10 minutes at 3,000 rpm (1,500 x g) in a Sorvall GSA rotor. The supernatant was then centrifuged for 20 minutes at 9,500 rpm (15,000 x g) in the same rotor to produce crude mitochondrial pellets which were resuspended in 0.2 ml/g fresh tissue weight of buffer B [0.3 *M* mannitol, 0.05 *M* tris, adjusted to pH 8.0 with HCl, 0.1% (w/v) BSA, 5 m*M* $MgCl_2$] and repelleted by centrifugation for 10 minutes at 12,000 rpm (17,000 x g) in a Sorvall SS-34 rotor. Pellets were twice resuspended, as above, and the suspensions centrifuged for 10 minutes at 3,500 rpm (1,500 x g) to remove large cellular debris. The supernatant was then centrifuged for 15 minutes at 12,000 rpm (17,000 x g) to provide a fairly pure mitochondrial pellet, which was resuspended in 0.04 ml/g fresh tissue weight of buffer B.

Extramitochondrial DNA was digested with deoxyribonuclease I (DNase) (Sigma, DN-CL) (60 µg/ml) for 1 hour at room temperature. After incubation, buffer C [0.3 *M* sucrose, 0.1 *M* EDTA, adjusted to pH 8.0 with NaOh] was added to 0.12 ml/g tissue fresh weight, and the mitochondria pelleted at 10,000 rpm (12,000 x g) for 10 minutes. Mitochondria were twice resuspended in buffer C, as above, and repelleted to eliminate the DNase, the final pellet

being resuspended in a minimum volume (1 to 5 ml) of buffer C and frozen or immediately processed for DNA extraction.

MtDNA Preparation

The volume of the mitochondrial isolates was adjusted to 5.1 ml by the addition of lysis medium [0.1 *M* tris, 0.1 *M* EDTA, adjusted to pH 8.0 with HCl], to which 0.3 ml proteinase K (Sigma) (2 mg/ml) and 0.25 ml 10% (w/v) Sarkosyl (sodium lauryl sarcosinate) were added, and the reaction mixture gently swirled, occasionally, during a 1 hour incubation at room temperature. The lysate was added to 6.5 g of CsCl in polyallomer centrifuge tubes. In dim light, 1 ml of ethidium bromide (700 µg/ml) was added, the tube was filled with paraffin oil, capped, air-bubbles excluded, and the preparation throughly mixed to dissolve the CsCl prior to centrifugation in a Beckman Ti-75 rotor for 42 to 50 hours at 44,000 rpm (126,400 x g) and 20° C. The banded mtDNA was visualized with short-wave UV light, and removed with a disposable syringe and 18 guage needle. The ethidium bromide was removed from the sample by 3 to 5 extractions with equal volumes of 1-butanol, and the CsCl was removed by dialysis against several changes of 0.3 *M* NaCl.

Samples were transferred to siliconized COREX centrifuge tubes, 2 volumes of ethanol were added, and the mtDNA was allowed to precipitate at -20° C overnight or at -70° C for 20 minutes (43). The precipitate was pelleted at

12,500 rpm (19,000 x g) for 30 minutes in a Sorvall SS-34 rotor, and the pellet was washed with 70% ethanol at 12,500 rpm (19,000 x g) for 10 minutes in the same rotor to remove NaCl. The tubes were carefully drained and dried for about 15 minutes at 37° C, before pellet resuspension in 1 ml of glass-distilled water. Purity and amount of mtDNA were estimated by UV absorbance at 320, 280, 260, and 230 nm respectively (43). Samples were adjusted to 0.3 *M* NaCl and again ethanol precipitated, as above, before being resuspended in glass-distilled water to a concentration of 0.2 µg/µl. Final samples were frozen for future restriction endonuclease analysis.

Restriction Endonuclease Analysis of MtDNA

The restriction endonucleases, *Bam* HI from *Bacillus amyloliquefaciens* H., *Eco* RI from *Escherichia coli* RY13, *Pst* I from *Providencia stuartii*, (Bethesda Research Labs) and *Sal* I from *Streptomyces albus* G., (New England Bio Labs) were used to digest the mtDNA. Recognition sites for these enzymes are (5' to 3'): GGATCC, GAATTC, CTGCAG, and GTCGAC, respectively. Digestion took place in sterile plastic tubes in 50 µl reaction volumes consisting of 15 µl of glass-distilled H_2O, 10 µl of 5X restriction buffer (as recommended by supplier for each enzyme), 5 µl of restriction enzyme (10 units of activity/µl), and 20 µl of mtDNA sample, for at least 3 hours at 37° C. Reactions were terminated by adding SUEB [50% (w/v) sucrose, 4 *M* urea,

50 m*M* Na_2EDTA, and 0.1% bromophenol blue].

Agarose gel electrophoresis was done with 0.7% agarose in a 278 x 152 x 4.5 mm slab with ten 70 µl wells, for about 640 volt hours (1.4 v/cm x 16 hours) at 4° C in tris-phospahte-EDTA buffer [36 m*M* tris, 10 m*M* Na_2EDTA, 30 m*M* NaH_2PO_4, pH 7.7-7.8] (32). Gels were then incubated in 0.5 µg/ml ethidium bromide for at least 1 hour before being placed over short-wave UV light and photographed with Kodak Panatomic-X film through a Wratten 23 A filter.

Method 2

This technique was adapted from Kemble, Gunn and Flavell (24), and Kemble and Bedbrook (23). Though neither of these papers report restriction endonuclease analysis, Roger Kemble gave personal assurances, during a visit to our campus, that his method would, indeed, produce restrictable mtDNA from only 20 g of maize tissue. Since this was significantly less than the 100 g required by Method 1, its adaptation to peanuts was attempted. Other significant differences include fewer centrifugations, lower pH in the buffers, and mtDNA isolation without the time-consuming and expensive CsCl density gradient centrifugation. Another attraction of this method was the speed of the process, which could be completed in 1 day.

Isolation of Mitochondria

Mitochondria were again isolated at 0 to 4° C.

Initally, about 7 g of tissue was homogenized in 21 ml of homogenization buffer [0.5 *M* mannitol, 10 m*M* N-tris (hydroxymethyl) methyl-2-amino ethane-sulphonic acid (TES), 1 m*M* EGTA, adjusted to pH 7.2 with NaOH, 0.2% (w/v) BSA, and 0.05% (w/v) cysteine], using a mortar and pestle. Later, it was decided that 20 g of tissue could be homogenized at once, in 60 ml of homogenization buffer. The brei was then filtered through 4 layers of cheesecloth and one layer of miracloth, prior to centrifugation at 3,000 rpm (1,000 x g) for 10 minutes in a Sorvall SS-34 rotor. The supernatant was then centrifuged at 10,000 rpm (12,000 x g) for 10 minutes, and the pellet resuspended in 6 ml of homogenization buffer. This suspension was then recentrifuged at 3,000 rpm (1,000 x g) for 10 minutes, and the resulting supernatant was adjusted to 10 m*M* $MgCl_2$. DNase I was then added to a concentration of 10 µg/g tissue fresh weight, and allowed to incubate for 1 hour at room temperature. Mitochondria were centrifuged through a layer of 0.6 *M* sucrose, 10 m*M* TES, 20 m*M* EDTA, adjusted to pH 7.2 with NaOH, at 9,000 rpm (10,000 x g) for 20 minutes, and washed twice in the same solution before extraction of mtDNA.

MtDNA Preparation

Pellets from the final wash, above, were resuspended in 5.4 ml lysis medium [50 m*M* tris, 10 m*M* EDTA, adjusted to pH 8.0 with HCl], to which 0.6 ml proteinase K (2 mg/ml) and 2 ml 10% (w/v) sarkosyl were added carefully, to avoid

bubbling the enzyme. This mixture was incubated at 37° C for 1 hour, before the addition of 2 ml of 1 *M* ammonium acetate and the purification of the mtDNA by three extractions with 1:1 chloroform-phenol. Samples were ethanol precipitated and their quantities estimated, as in Method 1, before being resuspended in glass-distilled water to a concentration of 0.2 µg/µl, and frozen for future analysis.

Restriction Endonuclease Analysis of MtDNA

See Method 1, above.

Electrophoretic Analysis of MtDNA

Two to 10 µg of unrestricted mtDNA was electrophoresed for about 640 volt hours (1.4 v/cm x 16 hours) through 1 to 1.4% agarose as described above. The gels were then stained in 1 µg/ml ethidium bromide prior to visualization and photography as previously described.

CHAPTER IV

RESULTS AND DISCUSSION

In the comparison of the two processes, Method 2 seemed to produce more mtDNA from less plant tissue than did Method 1, but these samples would not restrict. This failure may have been due to the presence of phenol or ammonium acetate, either of which can interfere with restriction endonucleases, and both of which absorb in the UV spectrum. As the presence of any chemical which absorbs UV would have skewed the DNA yield estimates, mtDNA yields from Method 2 were questioned. Other contaminants which cound have caused the restriction problems are heavy metals and charged oligosaccharides, which can adhere to DNA. In spite of apparently lower yields, Method 1 was judged superior for peanuts, as only four out of 17 attempted restrictions failed. It should be pointed out that this researcher's lack of success with Method 2 does not negate Dr. Kemble's claim to have found a superior method in maize, but merely shows that she could not adapt his protocol to the study of peanuts. The technique used by Jackson et al. (21) was also considered for use, but it was rejected as being unnecessarily complex for this study.

MtDNA from each method was electrophoresed without restriction to ascertain the presence of small plasmid-like

pieces of DNA in peanut mitochondria, and none were found. Electrophoresis of unrestricted mtDNA, isolated by either method, produced only one band for all genotypes tested.

According to Schleif and Wensink (43), the quantity of DNA present in a sample can be estimated by its absorbance at 260 nm (A_{260}), and the accuracy of the estimate judged by the ratios of that reading to its A_{320} and A_{280} readings respectively. These readings were taken regularly, but they seemed to have little bearing on the restrictability of the samples tested. Other chemicals which may have confounded the estimates include RNA, incompletely digested extramitochondrial DNA, and a number of plant pigments, in addition to the phenol and ammonium acetate previously noted. Tables II and III show absorbance readings from the two methods used. The amount of DNA was estimated by dividing the A_{260} reading by 0.02, and multiplying the result by the volume of the sample at the time of the reading. Some samples shown in Table II were homogenized with mortar and pestle rather than blender. This method produced far lower yields of mtDNA, probably because of the thickness of the tissues involved. Test 1-5 was an attempt to process imbibed seed. Yield was extremely low, and restriction was not attempted.

Sample 1-6 was isolated from green tissue. Its mitochondrial isolate remained frozen for 10 months before the DNA was removed, yet it still produced 39 µg of restrictable mtDNA, enough for 10 attempted restrictions.

TABLE II

ABSORBANCE READINGS AND ESTIMATED DNA ISOLATED BY METHOD 1

TEST[1] I.D.	TISSUE[2] TYPE-WT.(g)		ABSORBANCE AT WAVELENGTH (nm) 320	280	260	230	VOL. (µl)	ESTIMATED[3] DNA (µg)	
1-1	ALL	127	-	.456	.718	.551	2.2	79.0	R
1-2	HYP	95	-	.128	.224	.096	1.2	13.4	
1-3	TOP	133	-	.527	.834	.572	1.5	62.6	R
1-4	ALL	94	-	.209	.353	.210	1.2	21.2	R
1-5	SEED	148	.013	.023	.030	.028	1.05	1.6	*
1-6	GRN	116	.204	.505	.749	.590	1.05	39.3	R
1-7	COT	33	.083	.162	.218	.220	1.05	11.4	
1-8	ALL	103	.041	.066	.086	.073	2.1	9.0	
1-9	ALL	59	.065	.117	.155	.148	1.05	8.1	
1-10	ALL	175	.021	.063	.104	.066	2.1	10.9	R
1-11	ALL	156	.024	.046	.073	.064	2.1	7.7	R
1-12	ALL	135	.066	.124	.149	.257	1.05	7.8	R
1-13	BOT	68	.050	.092	.123	.150	1.05	6.5	R
1-14	TOP	61	.051	.078	.095	.115	1.05	5.0	R
1-15	ALL	105	.045	.108	.145	.162	1.05	7.9	R
1-16	ALL	119	.086	.161	.219	.213	1.05	11.5	R
1-17	ALL	138	.193	.685	1.057	.655	1.05	55.5	R
1-18	ALL	131	.450	.890	1.199	.944	1.05	62.9	R

1-Tests 1-7 to 1-16 homogenized with mortar and pestle.
2-ALL-Whole plant, BOT-Hypocotyl + Roots, COT-Cotyledons, GRN-Green Shoots, HYP-Hypocotyl, TOP-Cotyledons + Shoot.
3-R indicates restricted, *-Restriction not attempted.

TABLE III

ABSORBANCE READINGS AND ESTIMATED DNA ISOLATED BY METHOD 2

TEST I.D.	TISSUE[1] TYPE-WT.(g)		ABSORBANCE AT WAVELENGTH (nm) 320	280	260	230	VOL. (µl)	ESTIMATED DNA (µg)
2-1	HYP	30	-	.448	.812	.319	1.0	40.6
2-2	H+S	60	-	.907	1.735	.778	1.0	86.8
2-3	SHO	29	-	.975	1.822	.780	1.0	91.1
2-4	H+S	31	-	.250	.499	.277	1.0	25.0
2-5	HYP	30	-	.038	.053	.036	1.0	2.7
2-6	SHO	14	-	.082	.142	.091	1.0	7.1
2-7	SHO	13	-	.047	.066	.039	1.0	3.3
2-8	HYP	25	.031	.165	.308	-	1.0	15.4
2-9	HYP	26	.044	.247	.438	-	1.0	21.9
2-10	SHO	30	.040	.145	.233	-	1.0	11.7
2-11	H+S	11	.019	.073	.129	-	1.13	7.3
2-12	H+S	11	.023	.132	.248	-	1.13	14.0
2-13	H+S	11	.012	.048	.087	-	1.13	4.9
2-14	H+S	11	.018	.051	.086	-	1.13	4.9
2-15	H+S	11	.027	.110	.194	-	1.13	11.0
2-16	H+S	11	.021	.052	.087	-	1.13	4.9

1-HYP-Hypocotyl, H+S-Hypocotyl + Stem, SHO-Shoots.

A_{320} readings were not taken for the first year of the study. Missing A_{230} readings could not be taken due to problems with the spectrophotometer which may have been due to the presence of contaminating materials. Ratios and yield percentages are shown in Table IV. $A_{320}:A_{260}$ varied from 0.2 to 0.5 in restrictable samples, and from 0.1 to 0.2 in non-restrictable ones. This does not agree with Schleif and Wensink (43), who suggest that a ratio of these readings above a few percent indicates the presence of undesireable foreign material, though it can be as high as 10% in preparations from higher organisms. They also state that the $A_{260}:A_{280}$ ratio should "be between 1.65 and 1.85 unless the DNA has a very bizarre (G + C)%" (p. 90). Higher ratios can be due to RNA, and lower ones to phenol or protein contamination (43). This ratio ranges between 1.2 and 2.0 for all samples shown, and from 1.2 to 1.7 among those which restricted. For a pure DNA sample, the absorbance in the UV spectrum should be highest at 260 nm, while proteins would absorb most at 230 nm. Nevertheless, the $A_{260}:A_{230}$ ratios varied from 0.6 to 1.7 among restrictable samples.

As mentioned earlier, difficulty was encountered in producing uncontaminated plant trials. This was due to the presence of mold spores on the seed being used, and was eventually overcome by treating seed with Captan fungicide, plus prompt removal of molded seedlings and addition of Captan to contaminated growth medium. Seedling growth at 30° C constant temperature was satisfactory.

TABLE IV

ABSORBANCE RATIOS AND YIELDS OF RESTRICTION TESTED SAMPLES

TEST	I.D.	TISSUE[1] TYPE-WT.(g)		ABSORBANCE RATIOS 320/260	260/280	260/230	ESTIMATED[2] DNA (µg)	YIELD[3] (%)
1-1	EM-13	ALL	127	-	1.6	1.3	79.0 R	62
1-2	EM-13	HYP	95	-	1.8	2.3	13.4	14
1-3	EM-13	TOP	133	-	1.6	1.5	62.6 R	47
1-4	EM-13	ALL	94	-	1.7	1.7	21.2 R	23
1-6	EM-13	GRN	116	.3	1.5	1.3	39.3 R	34
1-7	EM-13	COT	33	.4	1.4	1.0	11.4	35
1-8	VSM	ALL	103	.5	1.3	1.2	9.0	9
1-9	V4	ALL	59	.4	1.3	1.1	8.1	14
1-10	CHICO	ALL	175	.2	1.7	1.6	10.9 R	6
1-11	COMET	ALL	156	.3	1.6	1.2	7.7 R	5
1-12	COMET	ALL	135	.4	1.2	.6	7.8 R	6
1-13	V4	BOT	68	.4	1.3	.8	6.5 R	10
1-14	V4	TOP	61	.5	1.2	.8	5.0 R	8
1-15	P-1192	ALL	105	.3	1.3	.9	7.9 R	8
1-16	US98Y	ALL	119	.3	1.4	1.0	11.5 R	10
1-17	VSM	ALL	138	.2	1.5	1.6	55.5 R	40
1-18	V4	ALL	131	.4	1.4	1.3	62.9 R	48
2-1	EM-13	HYP	30	-	1.8	2.5	40.6	135
2-2	EM-13	H+S	60	-	1.9	2.2	86.8	145
2-3	EM-13	SHO	29	-	1.9	2.3	91.1	314
2-4	EM-13	H+S	31	-	2.0	1.8	25.0	81
2-8	EM-13	HYP	25	.1	1.9	-	15.4	62

TABLE IV (Continued)

TEST	I.D.	TISSUE[1] TYPE-WT.(g)		ABSORBANCE RATIOS 320/260	260/280	260/230	ESTIMATED[2] DNA (µg)	YIELD[3] (%)
2-9	EM-13	HYP	26	.1	1.8	-	21.9	84
2-10	EM-13	SHO	30	.2	1.6	-	11.7	39
2-11	EM-13	H+S	11	.1	1.8	-	7.3	66
2-12	EM-13	H+S	11	.1	1.9	-	14.0	127
2-13	EM-13	H+S	11	.1	1.8	-	4.9	45
2-14	EM-13	H+S	11	.2	1.7	-	4.9	45
2-15	EM-13	H+S	11	.1	1.8	-	11.0	100
2-16	EM-13	H+S	11	.2	1.7	-	4.9	45

1-ALL-Whole plant, BOT-Hypocotyl + Roots, COT-Cotyledons, GRN-Green Shoots, HYP-Hypocotyl, TOP-Cotyledons + Shoot, H+S-Hypocotyl + Stem, SHO-Shoots.
2-R indicates restricted.
3-Figures should be multiplied by 10^{-6}.

An attempt was made to determine the amount of seed necessary to produce minimal weights for processing, but this varied between genotypes and with the amount of contamination encountered, so no valid conclusion could be reached. Because plantings were very small, a wide variation in germination percentage was found in seed from the same source.

All restriction results are from Method 1. Restriction endonuclease analysis of the mtDNA examined in this study

revealed no difference between any of the genotypes tested. Figures 1 - 4 are pictures of restriction banding patterns, and have been reproduced from photographs by a technique involving screened negatives, which unfortunately does not produce clarity comparable to that on the originals. The author assures all readers that the original photographs did appear to show identical banding patterns for all genotypes studied, although some samples did not stain as well as others. Figure 1 shows an *Eco* RI digest of seven different samples, and the banding patterns appear to be similar. Figure 2 is the result of an *Eco* RI digest of the VSM [O] and V4 [V4] plasmons, and it shows the same duplicate patterns. Figures 3 and 4 are banding patterns produced by VSM and V4 digested with *Bam* HI and *Sal* I, and *Pst* I, respectively, and again, no differences can be seen between the two plasmons. Figure 5 is a graphic representation of the DNA banding patterns produced by the enzymes used. It was traced from enlargements of selected negatives of the gel photographs.

This research does not settle the dispute between Ashri, and Ressler and Emery, regarding the presence of different plasmons in V4 and VSM. It is possible that a cytoplasmic difference exists in their chloroplast DNA. There could even be a difference in their mtDNA which is too small to be characterized with the method used here, which should show differences on the order of 100 or more base-pairs (as in insertions or deletions), but is not

sensitive enough to illustrate smaller changes (such as point mutations) unless they happen to occur in an enzyme recognition site.

Figure 1. Agarose gel electrophoretic patterns of *Eco* RI digests. Sources of mtDNA were A. Chico, B. EM-13, C. Comet, D. EM-13, E. V4, F. P-1192, and G. US98y.

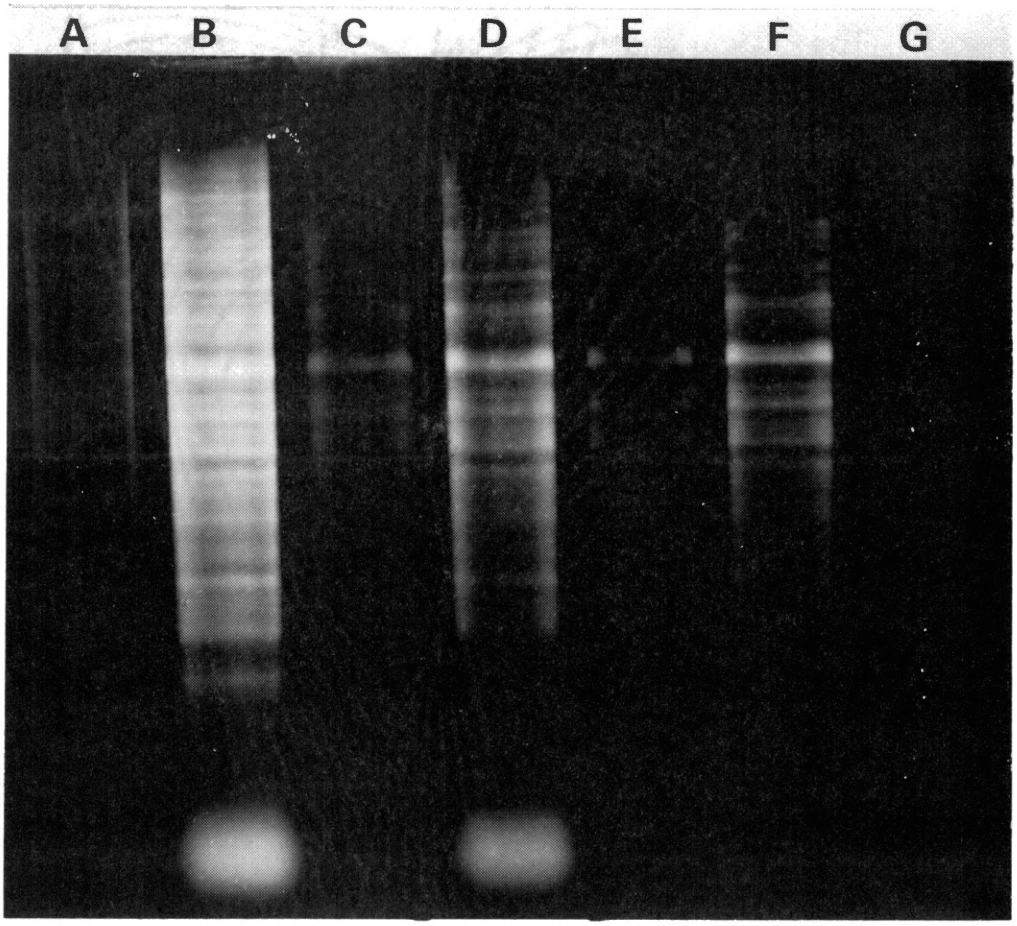

A
B
C
D
E
F
G

Figure 2. Agarose gel electrophoretic patterns of *Eco* RI digests. Sources of mtDNA were A. VSM, and B. V4.

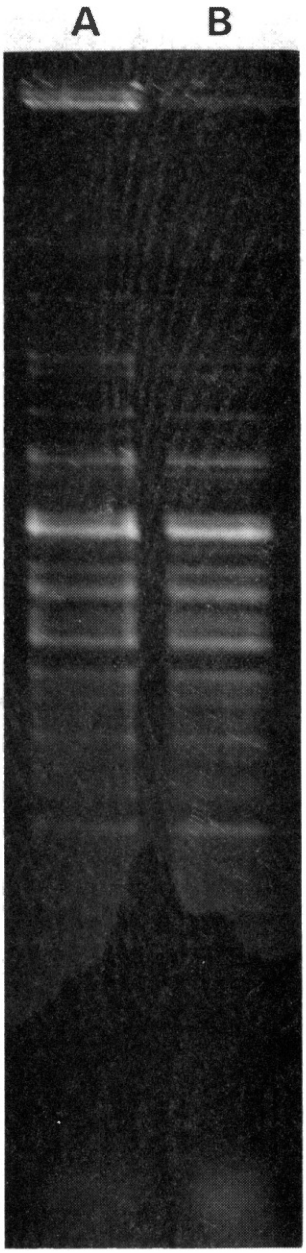

A
B

Figure 3. Agarose gel electrophoretic patterns of *Bam* HI digests of A. VSM, and B. V4, and *Sal* I digests of C. VSM, and D. V4.

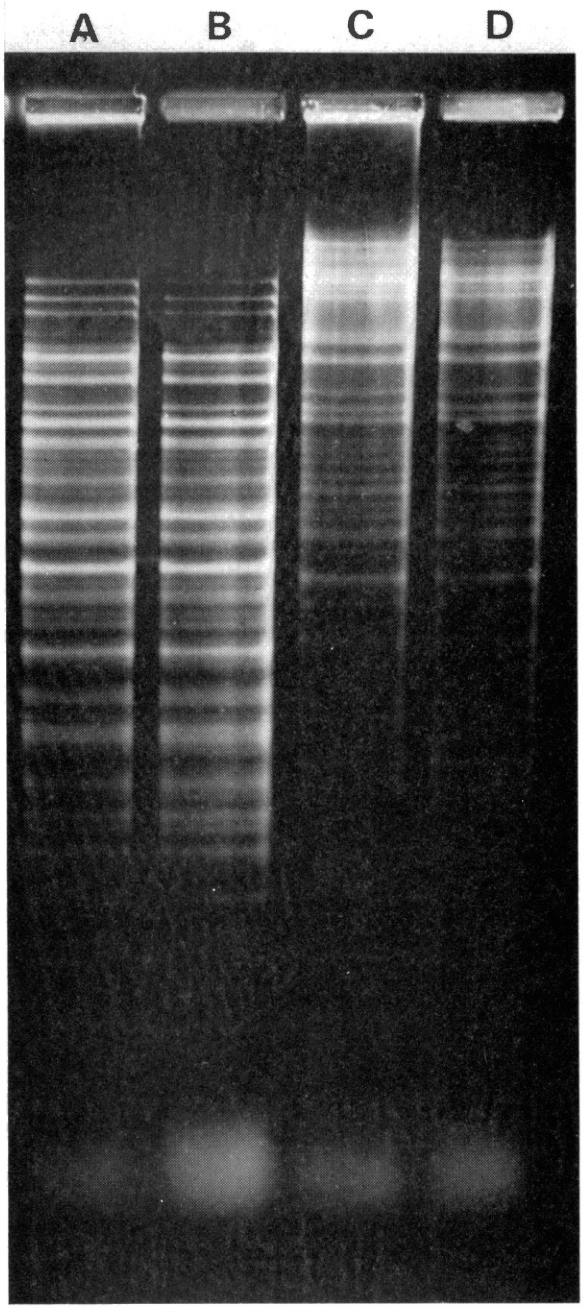

A
B
C
D

Figure 4. Agarose gel electrophoretic patterns of *Pst* I digests. Sources of mtDNA were A. VSM, and B. V4.

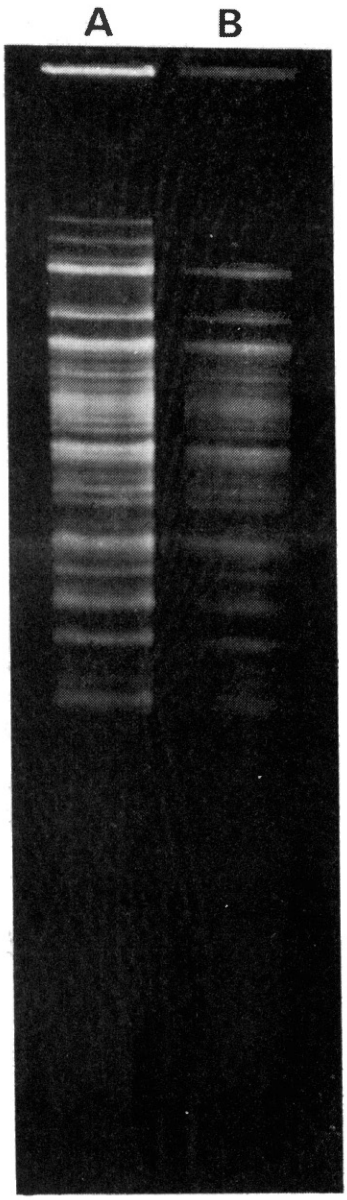
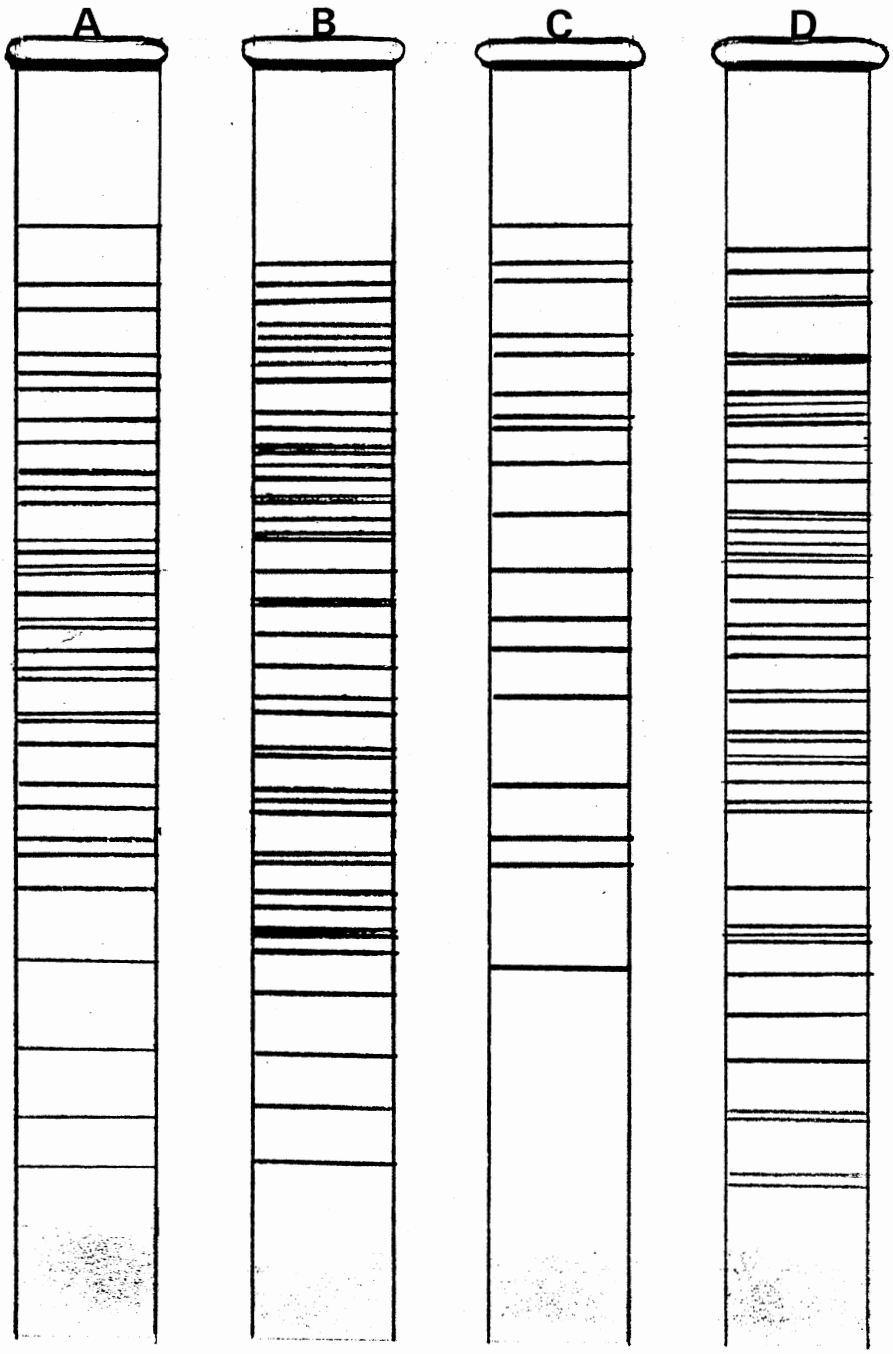

A
B

Figure 5. Schematic representation of agarose gel electrophoretic patterns produced by restriction endonuclease digestion of peanut mtDNA. Enzymes were A. *Eco* RI, B. *Bam* HI, C. *Sal* I, and D. *Pst* I.


A
B
C
D

CHAPTER V

SUMMARY AND CONCLUSIONS

This research produced no evidence of variance in the peanut ntDNA of the genotypes tested. Because no differences were found in banding patterns of restricted DNA from the genotypes tested, the practicality of using restriction endonuclease analysis to characterize peanut mtDNA variance cannot be judged by this study. It is questionable whether or not the materials tested do, in fact, differ cytoplasmically. Further testing with other materials is suggested.

It is suggested that Method 1 be tried on plant trimmings from the greenhouse in future experiments. This procedure should provide good results for two reasons: first, because a relatively small (100 g) sample of peanut tissue can produce enough mtDNA for several restriction runs; and second, because the success of the trial using green plants (test 1-6, Table II) suggests that it might be feasible. This material should produce good isolates provided that the following precautions are observed: a) plant trimmings are immediately immersed in ice water, b) trimmings are well-rinsed to remove residual chemicals, and c) trimmings are taken straight to the lab and well-chopped

before homogenization. Such material should also be limited to healthy, unblemished shoot tips, and may need to have tough stems removed prior to processing. Some rarer genotypes may be tested in this manner without the necessity of using seed.

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