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Non-Mendelian Segregation Of The Maize Embryo-Specific Mutation UND-9

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NON-MENDELIAN SEGREGATION OF THE MAIZE EMBRYO-SPECIFIC MUTATION UND-9

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Historical Background

The story of maize begins with its ancient ancestor, teosinte (more precisely, Zea mays subspecies parviglumis) (Doebley, 2004). Teosinte is a very different plant from the maize we know today. The ear lacks any sort of solid cob, instead consisting of 8-14 individually-husked kernels in one of two rows with many of these units on multiple branches (Doebley and Stec, 1991). As teosinte was domesticated into maize, selective breeding resulted in the primary stalk becoming stronger and more prominent, while the lateral branches shrunk, combining their many smaller ears into a much larger and heavier ear, with 18-20 rows of kernels on a cob approximately a foot in length, though different modern stocks can vary considerably in these characteristics. The kernels also lost their individual husks, trading it instead for a single husk covering the entire ear. Archeological evidence suggests that this ancestor to modern maize was first cultivated by humans in what is now southwestern Mexico, in the valley of the Balsas River, approximately 8700 years ago (Ranere et al., 2009). At the time, the kernels required extensive preparation for consumption, necessitating stone grinding tools that provide the base of evidence for its history. Cultivation eventually became domestication a couple millennia later, when teosinte gave way to maize (Benz, 2001).

This early maize was quite unlike the maize of today, but that early maize had undergone several significant changes. In particular, the overall body plan of the plant and the rearrangement of its reproductive structures changed dramatically. Teosinte consisted of a central stem with many lateral branches. Each branch had multiple small ears and were tipped with a pollen-producing tassel. As teosinte became maize, these branches shrank in length, the many ears disappearing and being replaced with a feminized tassel, resulting in a sudden shift from hard, individually husked kernels to softer, edible kernels on an ear covered by a single husk (Iltis, 1983).

This resulted in a very different type of ear from the one originally grown on teosinte. For one, while the kernels of the teosinte ear were evolved to break off easily in order to facilitate spreading in the wild, the pollen-producing flowers of the teosinte tassel had no need to do the same, and kept a rigid attachment to the plant when they became the maize kernels. This follows a pattern seen in other plants domesticated for the use of their seeds; seeds that

leave the plant on their own in order to spread are replaced by seeds that necessitate human intervention in order to be propagated into the next generation. Further, the number of flowers on the tassel (now kernels on an ear) is significantly more variable than the number of ears on the teosinte branch (Iltis, 1983). However, this new ear only had four rows of kernels at this point and is only a few centimeters in length. Shortly afterwards, it began to spread across North America, becoming a cultural staple of many different tribes of Native Americans. Maize split into different breeds as it spread, making incremental improvements under the Indigenous People's cultivation. Over time, domestication would slow as it reached a form capable of meeting the Indigenous People's needs, and by the time European settlers began to arrive en masse, most individual tribes were using a single line of maize (Wallace and Brown, 1956; Browne, 1837). Two of these types, known as Flint and Dent, would eventually become progenitors to the most widely studied inbred strains used today.

The northern flints were originally grown across the eastern portion of what would become the United States. These were known for their wide and smooth white or yellow kernels and longer, narrower ears (Anderson and Brown, 1952). The southern dents, on the other hand, originated in Mexico and were characterized by thicker ears and small, white kernels with a pronounced dent in the tip. Crosses of these two types of corn marked the beginning of the intentional husbandry of maize, creating vastly more productive new lines such as Reid's Yellow Dent, Lancaster's Surecrop, and many others. In fact, a survey of farmers in 1850 indicated that the most valued maize in their areas were crosses between gourdseed (a type of dent) and flint corn (Anderson and Brown, 1952).

While some of the original crosses of Dent and Flint corns may have been a fortunate accident, their success certainly was not. It would not be long before the concepts of hybrid vigor and hybrid maize began to take over the corn farming world, despite the opposite being found in many other plants, such as tobacco, where hybridization would produce weaker, less productive plants (Shull, 1908). At around the same time, Mendel's work was being rediscovered and the task of the continued improvement of maize would pass from the hands of farmers to those of dedicated researchers. At Cold Spring Harbor in New York, a series of experiments were begun to examine hybrid vigor and how best to take advantage of it. Aside

from Mendel's work with maize (Mendel, 1950), the theories arising from these experiments would be some of the first steps of maize research into the new field of genetics. While today's inbreds are strong enough that a single level of hybridization is sufficient for commercial farming, a century ago double-hybrid plants (resulting from the cross of two single hybrids) were necessary for the practice to be economically viable (Paterniani, 2001; Sheridan, Personal correspondence). Several important studies were made around this time that helped to flesh out early genetics. Alongside the rediscovery of Mendel's work came the discovery of meiosis (Weismann, 1889) and the chromosome theory of heredity (Sutton, 1903).

Maize Research and Embryo-Specific Mutations

One of the earlier studies of the maize kernel examined what were then known as 'germless seeds' (Wentz 1930). Now known in our lab as *embryo-specific* mutations, or *embs*, these were described as having an endosperm with a healthy appearance, while the germ (embryo) had a sunken or wrinkled appearance. The study examined and mapped two of these mutations, the genes *gm-1* and *gm2*, but was unable to identify the actual causes underlying the resulting phenotype. These early studies were limited to naturally occurring mutations, but the use of ethyl methanesulfonate (EMS)-treated pollen would later allow researchers to generate and study a much greater number of mutations. In the 1970s, researchers at the University of Missouri examined a large number of *defective-kernel* mutants, in which both the embryo and the endosperm of kernels would fail to properly develop (Neuffer and Sheridan 1980). While many of these mutant kernels could still be grown with proper treatment, some were found to be entirely non-viable due to a failure of the embryo to develop. These sorts of embryo-affecting mutations would go on to become a focus of later studies (Clark and Sheridan, 1991; Sheridan and Clark, 1993).

In 2010, Professor William Sheridan's lab began a long-term study of *embryo-specific* mutations in maize (Brunelle, Clark and Sheridan 2017). Now more properly defined, these are recessive mutant alleles in the maize genome that prevent the full development of the maize embryo while allowing endosperm development to proceed essentially as normal. We believe these gene products are involved in the signaling pathways directly involved in the

development of the embryo. These mutations were created by exposing pollen from the W22 inbred of maize to EMS before crossing it onto the silks of the B73 inbred. The B73 inbred is considered the main reference line in maize, and is therefore the best annotated genome for use in future molecular genetic analysis. The resulting mutations were then repeatedly backcrossed into B73 in order to transfer the target mutation into the B73 genome. This backcrossing would also assist in later DNA analysis by removing W22 SNPs from the genome, except for the area immediately surrounding the targeted mutant allele, allowing the allele to be identified more quickly. In each generation, plants possibly carrying the mutant allele were self-pollinated to detect the presence of the recessive mutant allele, and also crossed onto a B73 plant in order to transfer the allele further into the B73 lineage.

UND-9

Earlier studies in our lab revealed that the UND-9 mutation did not behave as would be expected of a single-locus, recessive allele, instead segregating its mutant phenotype in a distribution significantly divergent from the expected Mendelian ratio of 3:1 caused by the self-pollination of a plant heterozygous for the gene controlling that phenotype. While most self-pollinated plants heterozygous for an embryo-specific mutant would be expected to produce an ear with an average of 25% mutant embryos, approximately half of such UND-9 ears have less than 10% of their embryos segregate for this phenotype. It would be normal to expect such ears to appear rarely by pure chance across the hundreds of ears we examine, but not nearly as many as we have found. An alternate hypothesis might be that these rare kernels might be the result of additional spontaneous mutations that occur during cellular division, but I believe this is not the case. This is supported by the fact that normal phenotype kernels on those ears are able to pass along the mutant allele, something that would not be possible if the mutations in question were spontaneous and dominant, as necessitated by the almost non-existent probability of a spontaneous mutation affecting both alleles at a single locus. If a spontaneous mutation were recessive, it would not appear for an additional generation and would then be expected to have a 25% segregation ratio. If it were dominant, it would only appear in a single kernel, and would, by its nature, be unable to be propagated (This latter interpretation does not rule out the possibility of multiple dominant mutations affecting multiple kernels). By

eliminating this other possibility, we are left with the conclusion that the mutation within the low segregating ears is likely to still be UND-9.

Research Goals

The goal of the research was to understand the mechanisms behind the non-Mendelian segregation of the UND-9 mutant allele. This study examined whether the mutant phenotype in question is more or less likely to be passed through the male gametophyte (pollen) or through the female gametophyte (embryo sac), or through both. Further, it examined whether past segregation ratios can be used to predict future ratios. In other words, whether a kernel from an ear with a low segregation ratio of kernels with mutant embryos is more or less likely to produce a self-pollinated ear with a similarly low ratio, or to segregate at all.

Methods

In order to pursue this analysis, a selection of five ears from the 2011 winter season were chosen to be the starting point for this study (Figure 1, Row A). Each of the five ears was a self-pollinated ear from a plant grown from a kernel taken from an ear grown in the previous season, GG22-5. The segregation frequencies of kernels with mutant embryos of four of the five ears were outside the Mendelian range of segregation values. Each of the five self-pollinated ears segregated for the mutant phenotype caused by UND-9. However, the segregation percentages of four of the ears spanned a much greater range than would normally be expected to result from Mendelian genetics, ranging from 5% to 44.5% of their kernels having mutant embryos. In order to examine the frequency of transmission through the two gametophytes (The male gametophyte, pollen, and the female gametophyte, the embryo sac), we aimed for a large sample population of 160 plants. In order to reach this number, thirty-two kernels were taken from each of the five ears and planted in the summer of 2017. Each resulting plant was double pollinated (described in a later section) by itself (m/+ x m/+) and by crossing by B73 Rscm2 (m/+ x +/+) and also had their pollen crossed out onto wild-type B73 Rscm2 ears (+/+ x m/+), creating three sets of kernels on a pair of two ears. The three sets of kernels represent the mutant allele being passed through the male gametophyte (pollen, cross

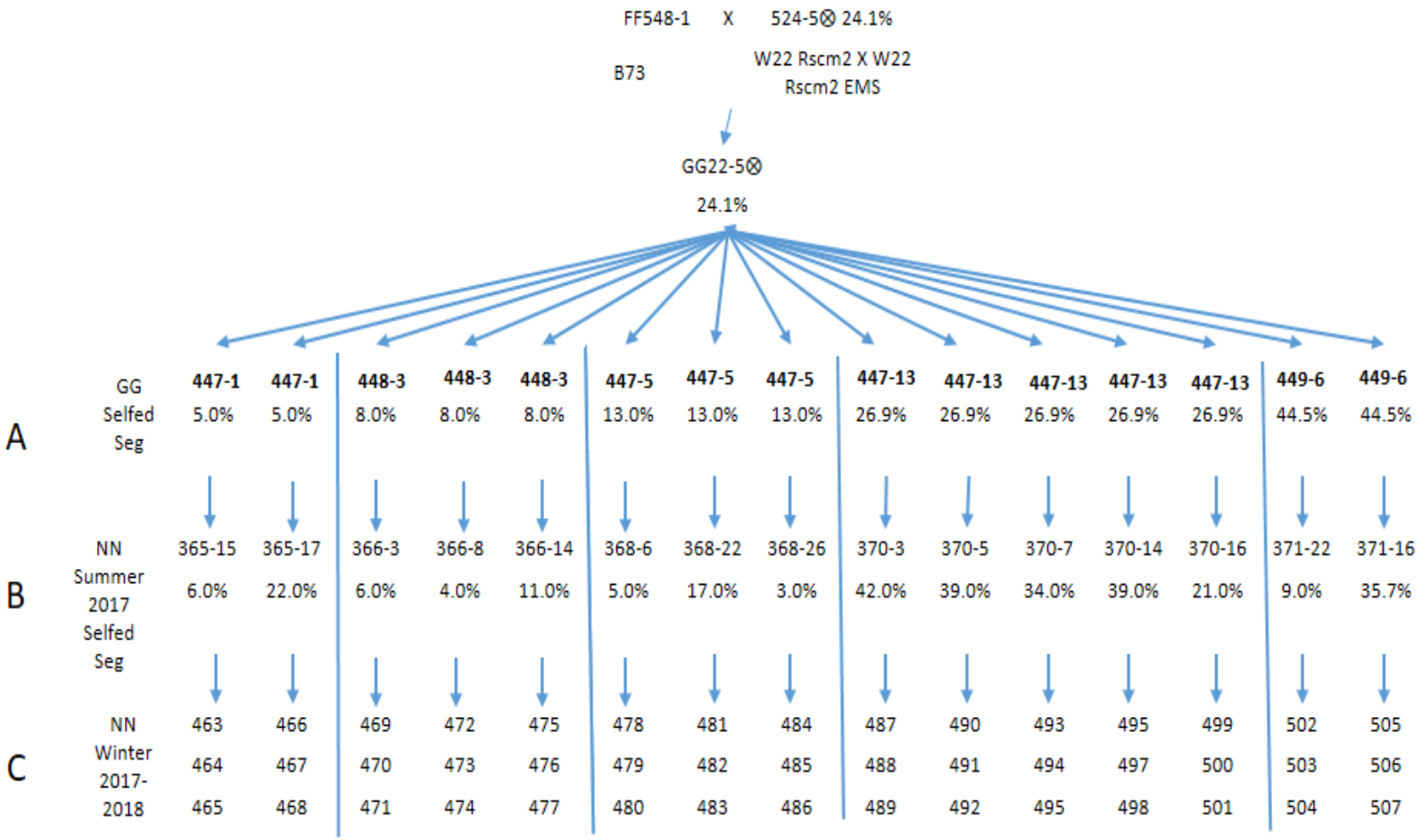

Figure 1 depicts the pedigree of the UND-9 plants and families described in the methods section of this paper. The vertical blue lines delineate the five ears in row A. The numbers formatted ###-## in rows A and B represent the unique identifiers of the plants used (and the ears from those plants), while the percentages represent the percentage of kernels on the ear with defective embryos. The first set of numbers in the identifier represent a family of ears, while the second set represent a single ear within that family. The numbers in row C each represent a family of ideally 10 or more ears. The three numbers in each column of row C represent the three sets of kernels (cross-on, cross-out, and self-pollination). The top row (e.g. 463) represents the self-pollinated kernels of the double pollinated ear. The middle row (e.g. 464) represents the cross-onto kernels of the double-pollinated ear, and the bottom row (e.g. 465) represents kernels from the cross-out ear.

out), the female gametophyte (embryo sac, cross-on), or both (self-pollination). Of these, 50 double-pollinated ears were harvested, of which 31 were able to be scored. Fifteen of these ears and their matching cross-out ear (Figure 1, Row B) were selected to have their combined 45 sets (15 self-pollinated sets of kernels, and their matching cross-out and cross-on sets) of kernels grown in the next season. The self-pollinated kernels of the fifteen plants had segregation scores ranging from 4% to 42%, with twelve of the fifteen having non-Mendelian ratios. These twelve ears enabled us to focus on those abnormal percentages. In the next season, sixty kernels from each of the 15 plants were planted as three families of twenty kernels each in Molokai, Hawaii in the winter of 2017, and each resulting plant was then self-pollinated. Of the 900 expected plants (15 plants x 60 kernels per plant), almost half produced usable ears (Figure 1, row C). The low ratio of useable ears is the result of ears grown in Hawaii being more susceptible to mold and fungal infection. While these conditions may result in ears being discarded, it is highly unlikely that this or other environmental differences will affect individual kernels in such a way as to cause us confusion regarding the phenotype of these kernels with regards to the appearance of lack thereof of mutant embryos, due to the rather distinct appearance of mutant embryos. Extra kernels from the same source ears (Figure 1, Row B) were planted in the next season in order to fill out the goal of having at least 10 self-pollinated ears from each of the 45 families. In total, 440 self-pollinated ears were produced (Figure 1, Row C). Each of these 440 ears was then scored and tabulated for further analysis.

Greenhouse Planting and Generation of Seedlings

Once an ear has been selected for planting, kernels are removed from the ear and placed into a small white envelope, upon which is written the ear's pedigree (including the unique family and plant designation of the parent ear) and the unique family designation. A family consists exclusively of kernels taken from a single ear. For winter plantings, the number of kernels used per family is always fifteen due to the business model of Friendly Isle Growing Services, whose fields and equipment are used in our winter plantings. In summer, the number can vary by our own discretion, based on what is needed. In the summer plantings these kernels are hand-planted into small peat pots in the Starcher Hall greenhouse. The peat pots are filled with soil specifically prepared by Bob Sheppard and placed into flats in groups of 32

(The number of peat pots that can fit into a single flat). The soil is made up of a ratio of 3.5 pounds of Osmocote® Classic 14-14-14 fertilizer (8.2% Ammoniacal Nitrogen, 5.8% Nitrate Nitrogen, 14% Phosphate, 14% Potash) in 1 cubic yard of Sun Gro® Professional Growing Mix (Everris; Sun Gro Horticulture 2015). The first and last peat pots planted in the greenhouse on a given day are marked with a white plastic stake with the date of planting written on it in medium black sharpie™. The first kernel of each family is marked with a colored plastic stake with the family number, and the first and last four pots of each family are marked with smaller colored wooden stakes. The colors of both the wooden and plastic stakes cycle in order to visually differentiate between the different families. If a family is split between multiple flats, the first peat pot in the additional flats is also marked with a plastic stake identical to the one marking the first pot in the family. After planting, the kernels are watered and left in the greenhouse to induce germination.

Transplanting seedlings to field

Once the seedlings have grown to about 10 inches tall, they are prepared for transplanting into the experimental field on the northern edge of Grand Forks. The field, located at 47.940°, -97.079°, is leased by the City of Grand Forks to the University of North Dakota. The plants are brought out of the greenhouse and placed outdoors near the field for at least 24 hours in order for the seedlings to acclimate to the new environment. On the morning of transplanting, or the afternoon before, the seedlings are trimmed down to around 10 inches in height and the sets of eight peat pots are separated into individual pots in order for the plants to fit into the transplanting machine. The transplanting machine is then connected to the back of the tractor and is used to plant the seedlings into the field in previously marked rows. Plants within rows are placed approximately 9 inches apart, with the rows themselves spaced 50 inches apart. The machine operates by first plowing a small trench in the soil before using a metal arm attached to a rotating disc to place each peat pot and seedling into the ground. The plants are manually loaded into the transplanter by a pair of operators. After the transplanter has completed a row, large wooden stakes are then used to mark the boundaries between families, using the original plastic stakes as a guide. Plants are straightened or manually planted

as necessary, and the trench is closed to cover the roots of the plants and finalize the transplanting. This is then repeated until all plants are planted in the field.

Pollination and Harvest

Once the plants have grown tall and begun producing tassels, the plants are visually inspected each day for the appearance of ear shoots emerging between the leaves and stem. Once this ear shoot has appeared and is of sufficient size and before silks appear, a small waxed bag is fitted over the shoot in order to prevent uncontrolled pollination. The bag is held in place by its edge wedged between the shoot and stem. The bags are of different colors, the chosen color is changed twice a week in a cycle in order to delineate the approximate time of shoot bagging. Once the ear has grown for a few more days and has started to produce silks, the shoot is cut back (trimmed). Cutting back involves removing the upper portion of the ear husk tissue and the enclosed silks to a level just above the tip of the young ear in order to allow for more even pollination the following day. At this point, the husk tissue may be collected for DNA isolation and sequencing, or is simply discarded in the field. If double pollination is desired, the husk is initially cut back at a higher point, and a small vertical cut is made down the additional tissue in order to insert a small paper divider. The waxed shoot bag is then replaced, continuing to protect the silks while they grow during the next day.

Meanwhile, Kraft paper bags are placed over the tassels in order to collect pollen. The following day, once temperatures have risen enough (normally around 80° F) to enable the tassels to begin shedding pollen, the bags are taken down from the tassels and the pollen inside is gently poured onto the trimmed silks of the desired ears. In the case that the pollen is only to be used on one plant, the bag is then placed over the ear and stapled around the stem, and is marked with the unique identification numbers of the plants involved in the cross or self-pollination. The large Kraft paper bag provides the ear with room to grow while continuing to protect the ear from any further pollination as well as predation from local wildlife. Once the ears in the field are fully grown with fully developed kernels, the enclosed ears in their bags are collected before being placed on a forced air dryer to prepare the ears for storage. Afterwards, a small tag with the ear's information is attached to each ear, and the bags are discarded.

Double Pollination

Double pollination is a method by which a single ear can be self-pollinated and also cross-pollinated by pollen from another plant (Sheridan and Clark, 1986). In order for double pollination to be successful, a few conditions must be met. First, the recipient plant must be grown from a kernel displaying a recessive aleurone (outermost layer of the endosperm) phenotype. In our lab, this is normally the colorless yellow phenotype, denoted as clY. Second, the donor plant is required to carry a dominant allele for the aforementioned phenotype (normally a purple coloration, or Cl, in our lab). The two requirements together allow the resulting two sets of kernels to be visually distinguished after harvesting.

When cutting back the husk tissue and enclosed silks of the ear to be double pollinated, a small divider is inserted into a small cut made in the top, which splits the silks into two halves as they grow over the next ~24 hours. After this period, pollen from the recipient plant is collected and used to self-pollinate half of the silks on the ear, while pollen from the donor plant is used to perform the cross-pollination onto the other half of the silks. These two pollination events are normally scheduled to occur 24 hours apart to reduce cross-contamination, but it can be done as early as only an hour later if time is limited. The resulting mature ear should be visually split into two halves of kernels with different phenotypes, the kernels on the side receiving the self pollen would all be yellow, while the kernels on the other side would be all purple if the cross-pollinating plant was homozygous for the distinguishing allele, or a mix of yellow and purple kernels if it was heterozygous. In the second case, care must be taken not to mix the two resulting set of kernels, as the two types of yellow kernels cannot be distinguished after being removed from the ear.

Scoring of Kernels

We take up to 100 kernels from each self-pollinated ear grown on a plant from a mutant lineage. An area of the ear containing flat, uniform kernels is normally selected, as these are easier to score, and there is little reason to suspect that the placement of the kernel on the ear will affect its genetics. The embryos in the removed kernels are visually examined for defects under low-level magnification. An example of a normal and a defective kernel is shown in

Figure 2. While mutant embryos can vary in appearance, they lack certain features, such as a visible scutellum and embryonic axis, that mark them as mutant. The proportion of kernels with underdeveloped embryos is then given as a percentage and recorded in the documentation for that ear. The theoretical expectation for the percentage of kernels containing mutant embryos for these ears is 25%, as the mutant embryos display the recessive phenotype resulting from the self-pollination of a heterozygote. The recorded percentage of kernels with mutant embryos for a given ear may vary by chance, but ears with scores less than 16% are considered statistically low ($p<0.05$), while scores above 33% are high. Ears carrying zero defective embryos are indicative of a plant not carrying the mutant allele.

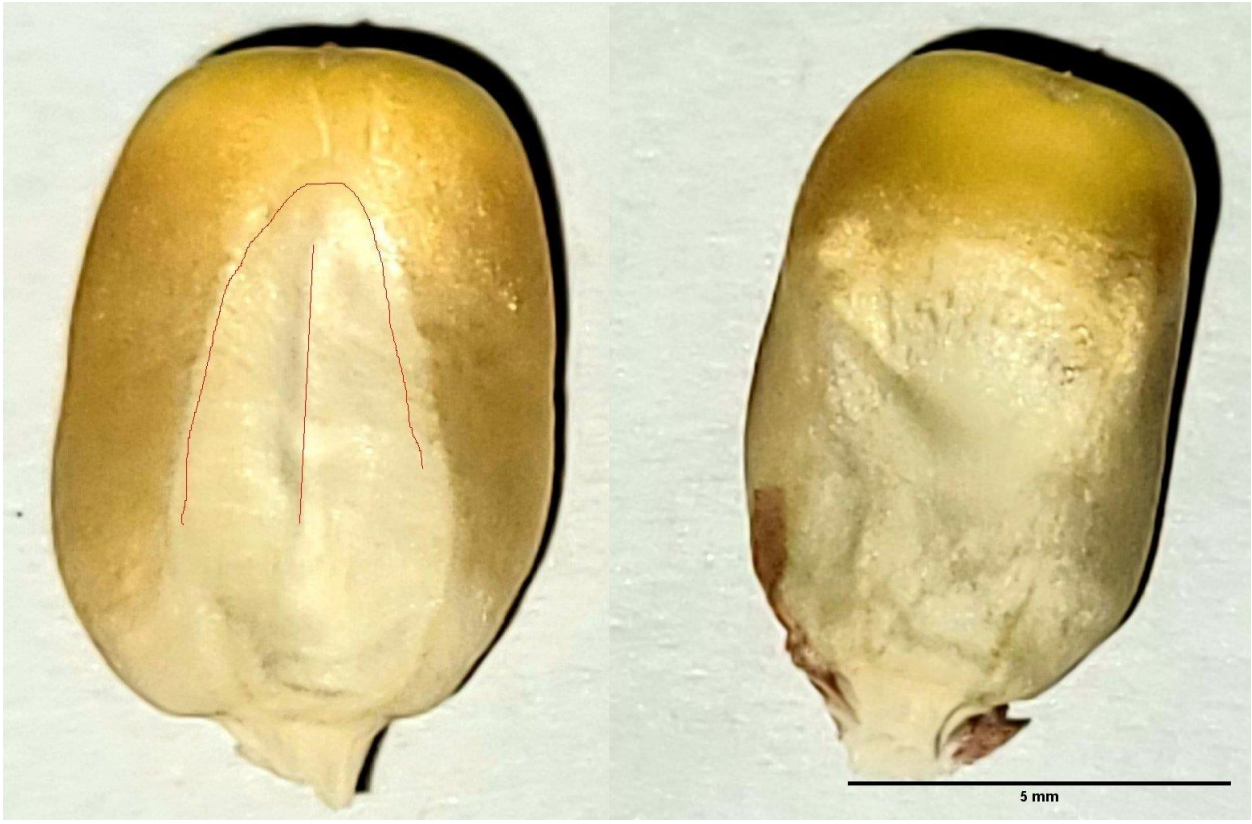

Figure 2 A comparison between a kernel with a healthy embryo (left) and a mutant embryo (right). Note the clearly defined features on the healthy embryo (Highlighted in red) that are missing on that of the right.

Results

Our methodologies produced 440 ears of corn in the final generation. After scoring each ear for segregation of normal-appearing kernels with mutant embryos (described in Methods -

Scoring), the scored ears were divided into groups based on the score's proximity and direction to the expected Mendelian score of 25%. The 'very low' group contains ears with scores ranging from greater than 0% through 13%. 'Low' contains ears with scores from greater than 13% through 16%. The 'Mendelian' group contains scores from 16% through 33%. The 'High' group contains 34% through 36%, and the 'Very high' group contains scores 37% and above. The boundaries between the groups were determined by employing a chi-square test using values of [score] and [100 - score] for the observed values and 25 and 75 for the expected values, respectively. P-values of 0.05 and 0.01 were used to differentiate between Mendelian and moderately non-Mendelian values ('low' and 'high'), and between moderate and extremely non-Mendelian values ('Very low' and 'Very high'). The p-values for scores up to 50% segregation can be found in table 1. The total data set can be found in tables 2 - 6. In total, we had 75 ears with 'very low' segregation, 21 'low', 158 'Mendelian', 7 'high', and 22 'very high' ears. Among the 440 ears, 157 did not segregate for kernels with mutant embryos. In the tables, each double column represents one of the ears in row B of Figure 1, while each of those are divided into three segments based on the three rows in row C. Each data row represents a single ear and its segregation ratio.

Analysis

After collating all the data, I proceeded with analysis in the R program using the dplyr, ggplot2 and default function packages. Techniques employed included chi-square testing for the effect of gametophyte type on the probability of the mutant allele to be passed on to the next generation, as well as the extent of segregation in the next generation. In other words, I aimed to examine whether the mutant allele is more or less likely to be passed to the next generation if is transferred via the pollen versus being transferred via the embryo sac. Similarly, I analyzed whether the gametophyte has an effect on future segregation ratios, i.e. whether after being transferred only through either the pollen or embryo sac, self-pollinating the resulting plants in the following generation results in more or less frequent occurrences of non-Mendelian ratios.

Chi–square table showing p-values of different possible scores

mutant	Normal	p-value	mutant	Normal	p-value	mutant	Normal	p-value
0	100	7.76E-09	21	79	0.355611	42	58	8.64E-05
1	99	2.98E-08	22	78	0.488422	43	57	3.23E-05
2	98	1.09E-07	23	77	0.644167	44	56	1.14E-05
3	97	3.76E-07	24	76	0.817361	45	55	3.86E-06
4	96	1.24E-06	25	75	1	46	54	1.24E-06
5	95	3.86E-06	26	74	0.817361	47	53	3.76E-07
6	94	1.14E-05	27	73	0.644167	48	52	1.09E-07
7	93	3.23E-05	28	72	0.488422	49	51	2.98E-08
8	92	8.64E-05	29	71	0.355611	50	50	7.76E-09
9	91	0.00022	30	70	0.248213			
10	90	0.000532	31	69	0.165857			
11	89	0.001224	32	68	0.105969			
12	88	0.00268	33	67	0.064672			
13	87	0.005584	34	66	0.037667			
14	86	0.011074	35	65	0.020921			
15	85	0.020921	36	64	0.011074			
16	84	0.037667	37	63	0.005584			
17	83	0.064672	38	62	0.00268			
18	82	0.105969	39	61	0.001224			
19	81	0.165857	40	60	0.000532			
20	80	0.248213	41	59	0.00022			

Table 1 Blue= Perfect Mendelian ratio (p-value = 1) Green = Mendelian scores p-value > 0.05 Yellow = Moderately non-Mendelian scores p-value > 0.01 Grey = Extremely non-Mendelian scores p-value < 0.01

Source Ear: NN365-15 dp and crossed onto B73. Selfed side Seg 6.0%		Source Ear: NN365-17 dp and crossed onto B73. Selfed side Seg 22.0%	
From selfed side		From selfed side	
NN463-11	2.00%	PP506-4	No Seg
NN463-5	18.00%	PP505-1	1.00%
		PP506-7	4.00%
		PP506-2	5.00%
		PP505-12	7.00%
		NN466-1	9.00%
		PP505-7	9.00%
		NN466-2	13.00%
		PP505-2	20.00%
		PP505-6	24.00%
From crossed side		From crossed side	
NN464-8	No Seg	NN467-6	No Seg
NN464-14	No Seg	NN467-12	No Seg
NN464-10	1.00%	NN467-14	No Seg
NN464-7	2.00%	PP507-4	No Seg
NN464-11	14.00%	PP507-9	No Seg
NN464-13	17.00%	NN467-5	1.00%
NN464-1	20.00%	PP507-7	22.00%
NN464-5	22.80%	NN467-2	23.00%
		NN467-9	24.00%
		NN467-1	25.00%

From cross out		From cross out	
NN465-2	No Seg	NN468-10	No Seg
NN465-7	No Seg	NN468-11	No Seg
NN465-8	No Seg	PP508-4	1.00%
NN465-9	No Seg	NN468-1	13.00%
NN465-10	No Seg	NN468-6	13.00%
NN465-3	1.00%	PP508-9	13.00%
NN465-6	1.00%	PP508-5	21.00%
NN465-11	12.00%	NN468-5	22.00%
NN465-4	15.00%	NN468-3	25.00%
NN465-1	22.00%	NN468-8	25.00%

Table 2 Segregation Frequencies of the Second Generation Progeny of GG447-1 (Seg. 5.0%) show that the segregation of the UND-9 mutant allele at a low, non-Mendelian ratio does not determine its segregation frequency in subsequent generations

**The NN series of plants were grown in the winter of 2017-2018 and the PP series of plants were grown in the winter of 2018-2019 from the same sources of the NN series plants listed in the same columns.*

Source Ear: NN366-3 dp and crossed onto B73. Selfed side Seg 6.0%		Source Ear: NN366-8 dp and crossed onto B73. Selfed side Seg 4.0%		Source Ear: NN366-14 dp and crossed onto B73. Selfed side Seg. 11.0%	
From selfed side		From selfed side		From selfed side	
NN469-3	No Seg	NN472-4	No Seg	NN475-4	No Seg
NN469-14	No Seg	NN472-9	No Seg	NN475-2	1.00%
NN469-13	6.80%	PP513-4	No Seg	PP517-1	1.00%
NN469-1	10.00%	NN472-1	3.00%	NN475-9	2.00%
NN469-4	15.00%	NN472-2	8.00%	PP517-2	3.00%
NN469-2	20.00%	PP513-1	8.00%	NN475-3	15.00%
NN469-7	20.00%	NN472-8	9.00%	NN475-5	19.10%
NN469-5	21.00%	NN472-6	10.00%	NN475-7	21.00%
NN469-15	22.00%	PP513-7	13.00%	NN475-10	21.00%
NN469-10	23.00%	PP513-3	26.00%	NN475-1	39.00%
From crossed side		From crossed side		From crossed side	
NN470-7	No Seg	NN473-3	No Seg	NN476-1	No Seg
NN470-15	No Seg	NN473-5	No Seg	NN476-9	No Seg

NN470-13	2.00%	NN473-10	No Seg	NN476-16	No Seg
NN470-1	3.00%	NN473-14	No Seg	NN476-3	15.00%
NN470-6	3.00%	NN473-12	1.00%	NN476-11	21.00%
NN470-3	17.00%	NN473-7	13.00%	NN476-7	23.00%
NN470-16	17.00%	NN473-8	19.00%	NN476-4	26.00%
NN470-11	18.00%	NN473-6	21.00%	NN476-15	29.00%
NN470-5	20.00%	NN473-11	26.00%	NN476-14	30.00%
NN470-2	23.00%	NN473-13	26.00%	NN476-12	32.00%
NN470-4	23.00%	NN473-9	30.00%	NN476-5	42.00%
NN470-9	24.00%	NN473-15	33.00%		
NN470-14	24.00%	NN473-2	39.00%		
NN470-10	25.00%				
NN470-12	25.00%				
NN470-8	28.00%				
From cross out		From cross out		From cross out	
NN471-2	No Seg	NN474-4	No Seg	NN477-4	No Seg
NN471-3	No Seg	NN474-13	No Seg	NN477-10	No Seg
NN471-5	No Seg	NN474-14	12.00%	NN477-11	No Seg
NN471-8	No Seg	NN474-10	19.00%	NN477-1	15.00%
NN471-9	No Seg	NN474-1	21.00%	NN477-2	21.00%
NN471-15	No Seg	NN474-3	26.00%	NN477-13	28.00%
NN471-7	14.00%	NN474-12	27.00%	NN477-14	29.00%
NN471-11	16.00%	NN474-2	28.00%	PP520-14	29.00%
NN471-14	18.00%	NN474-16	35.00%	NN477-7	33.00%
NN471-1	22.00%	NN474-9	42.00%	NN477-5	35.00%
NN471-6	22.00%				
NN471-12	22.00%				
NN471-4	23.00%				

Table 3 Segregation Frequencies of the Second Generation Progeny of GG448-3 (Seg. 8.0%) show that the segregation of the UND-9 mutant allele at a low, non-Mendelian ratio does not determine its segregation frequency in subsequent generations

**The NN series of plants were grown in the winter of 2017-2018 and the PP series of plants were grown in the winter of 2018-2019 from the same sources of the NN series plants listed in the same columns.*

Source Ear: NN368-6 dp and crossed onto B73. Selfed side Seg 5.0%		Source Ear: NN368-22 dp and crossed onto B73. Selfed side Seg17.0%		Source Ear: NN368-26 dp and crossed onto B73. Selfed side Seg 3.0%	
From selfed side		From selfed side		From selfed side	
NN478-4	No Seg	PP526-4	No Seg	NN484-1	No Seg
PP521-4	No Seg	PP526-10	No Seg	PP528-1	No Seg
PP521-9	No Seg	NN481-2	19.00%	PP528-2	No Seg
PP521-11	No Seg	NN481-1	20.00%	PP528-4	No Seg
PP522-3	No Seg	PP526-3	22.00%	PP528-7	No Seg
PP522-4	No Seg	PP526-6	23.00%	PP528-16	No Seg
NN478-1	3.00%	NN481-11	25.00%	PP529-12	No Seg
NN478-2	5.00%	PP525-2	25.00%	NN484-19	2.00%
NN478-5	5.00%	NN481-4	26.00%	NN484-3	3.00%
NN478-3	6.70%	NN481-14	27.00%	PP529-3	16.00%
From crossed side		From crossed side		From crossed side	
NN479-1	No Seg	NN482-3	No Seg	NN485-3	No Seg
NN479-2	No Seg	NN482-9	No Seg	NN485-9	No Seg
NN479-3	No Seg	NN482-10	No Seg	NN485-10	No Seg
NN479-4	No Seg	NN482-13	No Seg	PP530-1	No Seg
NN479-5	No Seg	NN482-14	No Seg	PP530-2	No Seg
NN479-9	No Seg	NN482-2	1.00%	PP530-4	No Seg
NN479-11	No Seg	NN482-13	1.00%	PP530-5	No Seg
NN479-12	No Seg	NN482-1	5.00%	NN485-2	1.00%
NN479-15	1.00%	NN482-10	25.00%	NN485-13	1.00%
NN479-14	2.00%	NN482-9	31.00%	NN485-1	5.00%
From cross out		From cross out		From cross out	
NN480-1	No Seg	NN483-1	No Seg	NN486-10	No Seg
NN480-3	No Seg	NN483-2	No Seg	NN486-13	No Seg
NN480-5	No Seg	NN483-11	No Seg	NN486-14	No Seg
NN480-11	No Seg	PP527-4	No Seg	NN486-15	No Seg
PP524-1	No Seg	PP527-6	No Seg	NN486-16	No Seg
PP524-2	No Seg	NN483-3	1.00%	NN486-1	1.00%
PP524-3	No Seg	PP527-2	25.00%	NN486-5	1.00%
PP524-4	No Seg	PP527-8	25.00%	NN486-7	1.00%
NN480-7	17.00%	NN483-9	27.00%	NN486-8	1.00%
		PP527-12	27.00%	NN486-11	1.00%

Table 4 Segregation Frequencies of the Second Generation Progeny of GG447-5 (Seg. 13.0%) show that the segregation of the UND-9 mutant allele at a low, non-Mendelian ratio does not determine its segregation frequency in subsequent generations

**The NN series of plants were grown in the winter of 2017-2018 and the PP series of plants were grown in the winter of 2018-2019 from the same sources of the NN series plants listed in the same columns.*

Source Ear: NN370-3 dp and crossed onto B73. Selfed side Seg 42.0%		Source Ear: NN370-5 dp and crossed onto B73. Selfed side Seg 39.0%		Source Ear: NN370-7 dp and crossed onto B73. Selfed side Seg 34.0%		Source Ear: NN370-14 dp and crossed onto B73. Selfed side Seg 39.0%		Source Ear: NN370-16 dp and crossed onto B73. Selfed side Seg 21.0%	
From selfed side		From selfed side		From selfed side		From selfed side		From selfed side	
NN487-8	No Seg	NN490-1	No Seg	PP540-8	No Seg	NN496-5	No Seg	NN499-13	No Seg
PP532-4	No Seg	NN490-4	No Seg	PP541-9	No Seg	PP544-4	No Seg	NN499-7	19.00%
PP532-6	No Seg	NN490-7	No Seg	PP540-4	11.00%	PP544-5	No Seg	NN499-1	21.00%
PP533-11	No Seg	NN490-11	No Seg	PP540-3	18.00%	PP544-13	No Seg	NN499-5	27.00%
PP533-3	16.00%	NN490-12	No Seg	PP541-12	25.00%	PP545-3	No Seg		
NN487-7	18.80%	PP537-12	No Seg	PP541-7	26.00%	PP545-11	No Seg		
PP532-9	20.00%	PP537-13	25.00%	NN493-1	28.00%	NN496-1	14.00%		
PP533-14	34.00%	NN490-2	33.00%	PP540-11	33.00%	PP545-4	19.00%		
NN487-2	41.00%	PP536-5	37.00%	PP541-3	35.00%	PP544-15	27.00%		
NN487-6	43.60%	PP536-9	38.00%			PP544-14	38.00%		
From crossed side		From crossed side		From crossed side		From crossed side		From crossed side	
NN488-4	No Seg	NN491-2	No Seg	NN494-6	No Seg	NN497-2	No Seg	NN500-1	No Seg
NN488-12	No Seg	NN491-4	No Seg	NN494-9	No Seg	NN497-7	No Seg	NN500-2	No Seg
NN488-13	No Seg	PP538-14	No Seg	NN494-10	No Seg	NN497-10	8.00%	NN500-13	No Seg
NN488-2	13.00%	PP538-4	1.00%	PP543-12	No Seg	NN497-1	19.00%	NN500-10	15.00%
PP534-11	15.00%	NN491-8	18.00%	NN494-14	13.00%	NN497-11	21.00%	NN500-11	15.00%
NN488-16	17.00%	NN491-3	19.00%	NN494-8	20.00%	NN497-8	25.00%	NN500-7	18.00%
NN488-15	20.00%	PP538-9	28.00%	NN494-13	21.00%	NN497-13	25.00%	NN500-8	19.00%
NN488-1	21.00%	PP538-7	29.00%	NN494-5	23.00%	NN497-4	31.00%	NN500-14	23.00%

PP534-4	26.00%	PP538-8	35.00%	NN494-11	23.00%	NN497-9	38.00%	NN500-3	26.00%
PP534-9	51.00%	NN491-5	41.00%	PP543-7	28.00%	NN497-12	38.00%	NN500-12	26.00%
				NN494-3	30.00%				
				NN494-7	30.00%				
				PP543-9	44.00%				
From cross out		From cross out		From cross out		From cross out		From cross out	
NN489-6	No Seg	NN492-2	No Seg	NN495-15	12.00%	NN498-6	No Seg	NN501-1	No Seg
PP535-1	No Seg	NN492-3	No Seg	NN495-8	14.00%	NN498-1	12.00%	NN501-3	No Seg
PP535-2	No Seg	PP539-5	No Seg	NN495-5	21.00%	NN498-2	13.00%	NN501-11	No Seg
PP535-12	No Seg	NN492-11	1.00%	NN495-4	26.00%	NN498-4	17.00%	NN501-14	No Seg
PP535-16	No Seg	NN492-12	17.00%	NN495-16	31.00%	NN498-10	18.00%	NN501-13	2.00%
NN489-13	1.00%	NN492-9	21.00%	NN495-12	33.00%	NN498-3	28.00%	NN501-8	11.00%
PP535-11	15.00%	PP539-6	22.00%	NN495-13	41.00%	NN498-14	32.00%	NN501-7	14.00%
PP535-15	15.00%	NN492-10	35.00%			NN498-11	40.00%	NN501-12	20.00%
PP535-14	18.00%	PP539-1	41.00%			NN498-16	42.00%	NN501-10	23.00%
PP535-6	19.00%	PP539-3	42.00%			NN498-15	43.00%	NN501-2	25.00%
						NN498-7	45.00%		
						NN498-7	45.00%		
						NN498-8	49.00%		

Table 5 Segregation Frequencies of the Second Generation Progeny of GG447-13 (Seg. 26.9%) show that the segregation of the UND-9 mutant allele at a Mendelian ratio does not determine its segregation frequency in subsequent generations

**The NN series of plants were grown in the winter of 2017-2018 and the PP series of plants were grown in the winter of 2018-2019 from the same sources of the NN series plants listed in the same columns.*

Source Ear: NN371-2 dp and crossed onto B73. Selfed side Seg 9.0%		Source Ear: NN371-16 dp and crossed onto B73. Selfed side Seg 35.7%	
From selfed side		From selfed side	
PP548-1	No Seg	PP552-4	No Seg
PP548-5	No Seg	PP553-1	No Seg
PP548-7	No Seg	NN505-12	5.00%
PP549-10	No Seg	PP552-5	12.00%
NN502-3	25.00%	PP553-8	12.00%
PP548-6	25.00%	PP552-6	20.00%
PP548-9	25.00%	PP552-1	21.00%
PP549-6	26.00%	PP553-7	23.00%
PP549-4	27.00%	PP552-2	26.00%
		NN505-8	32.50%
From crossed side		From crossed side	
NN503-10	No Seg	NN506-2	No Seg
NN503-11	No Seg	NN506-8	No Seg
NN503-2	2.00%	NN506-1	12.00%
NN503-3	2.00%	NN506-12	14.00%
NN503-12	2.00%	NN506-7	15.00%
NN503-14	18.00%	NN506-13	16.00%
NN503-8	21.00%	NN506-5	25.00%
NN503-4	26.00%	NN506-4	32.00%
NN503-7	28.00%	NN506-9	34.00%
NN503-9	28.00%		
NN503-1	33.00%		
From cross out		From cross out	
NN504-4	No Seg	NN507-5	No Seg
NN504-15	No Seg	NN507-4	13.00%
PP551-3	No Seg	NN507-14	19.00%
PP551-6	No Seg	NN507-7	21.00%
PP551-8	No Seg	NN507-3	24.50%
PP551-1	21.00%	NN507-17	28.00%
NN504-11	28.00%	NN507-11	30.00%
NN504-13	28.00%		
PP551-2	28.00%		
NN504-14	29.00%		

Table 6 Segregation Frequencies of the Second Generation Progeny of GG449-6 (Seg. 44.5%) show that the segregation of the UND-9 mutant allele at a high, non-Mendelian ratio does not determine its segregation frequency in subsequent generations

**The NN series of plants were grown in the winter of 2017-2018 and the PP series of plants were grown in the winter of 2018-2019 from the same sources of the NN series plants listed in the same columns.*

Finally, I used a linear regression in the default R packages to determine whether segregation values in one generation are related to segregation values in the next, either as a whole or via one or the other of the two gametophytes.

P-value uniformity test

One of the first tests was designed to elucidate whether or not the numbers of 'very low' or 'low' were actually significantly outside the realm of normal expectations. For this, we assumed a null hypothesis that each kernel on each segregating ear (ears with zero segregating kernels were left out of this analysis) would have a 25% chance of containing a mutant embryo.

Therefore, a random selection of 100 kernels taken from the ear of a heterozygous plant would most likely have 25 kernels with mutant embryos, however any number between 1 and 100 would be theoretically possible (0 kernels would also be possible in theory, but in practice such ears are considered to be non-segregating). The relevant possible segregation frequencies (1-50% kernels with mutant embryos) has already been assigned a p-value by table 1. A p-value is the probability of that particular p-value or a lower p-value being produced from an analysis, assuming that the null-hypothesis is true. This effect is similar to the idea that rolling a 3 or less on a fair, 6-sided die has a probability of 3 in 6. Therefore, in a situation in which null hypothesis is the correct one, multiple statistical tests taken on independent sets of data should produce a uniform distribution of p-values. Examining the p-values showed that p-values in the 0 to 0.2 bracket were vastly overrepresented, as shown in figure 3. Unfortunately, the amount of data we had was not sufficient to obtain a higher resolution, and we opted to leave this branch of analysis here.

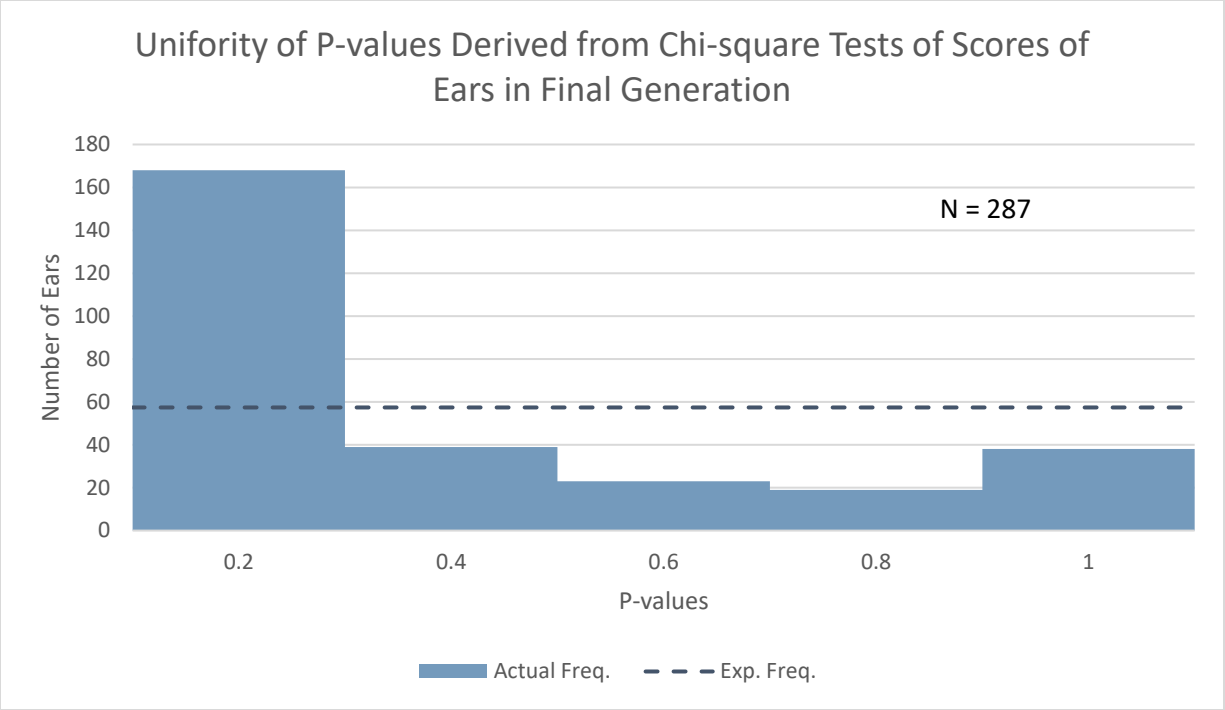

Figure 3 This graph shows the p-values of each segregating ear, after it's score was subjected to the chi-square test described in "data". The solid bars represent the observed values of the ears, whereas the dashed line represents the expected values if the scores were distributed in accordance with the null hypothesis. It is evident that scores fall much more frequently into the 0-0.2 bin than would be expected from a uniform distribution.

Segregation Distribution

After determining that there was indeed some sort of statistical anomaly in the distribution of segregation ratios we were seeing in UND-9, we wanted to see precisely how the distribution differed from that which we would otherwise expect. For this, we generated a binominal probability distribution based on 100 trails with a 25% chance of success. By multiplying each probability by the total number of ears (440), we could approximate the number of ears we would expect for each segregation ratio. Using this method, we were able to see that ears in the 'very low' section appeared at a much greater frequency than expected, at the expense of ears with a Mendelian ratio (Figure 4). We also saw that there was some overrepresentation in the 'very high' section of scores as well.

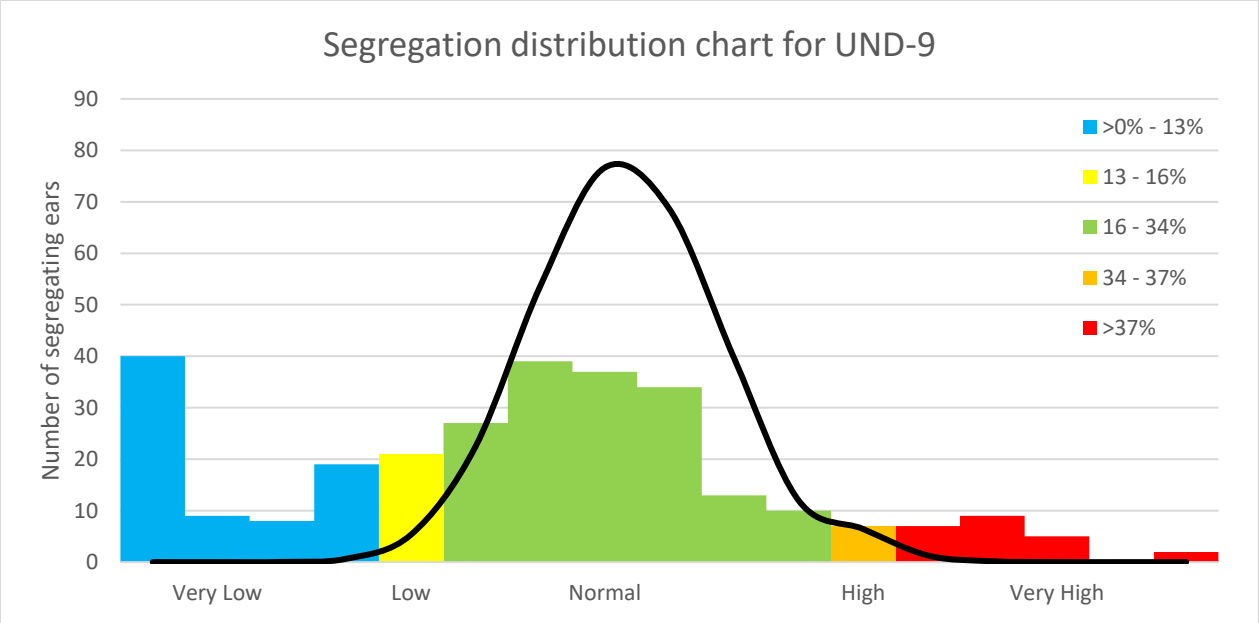

Figure 4 A graph showing the segregation ratios of all 283 ears of corn, dived by their scoring segments. The black line represents a binominal probability distribution that is based on 100 trials with a 25% chance of success.

Correlation of Parent and Offspring Segregation Ratios

Next, we performed analyses examining the heritability of the segregation ratios. The first of these analyses involved comparing the segregation ratios of the final generation to the segregation ratios of their mutant allele-carrying parent (Rows C and B in Figure 1, respectively). The resulting correlation produced a slope of 0.294 with an R-squared

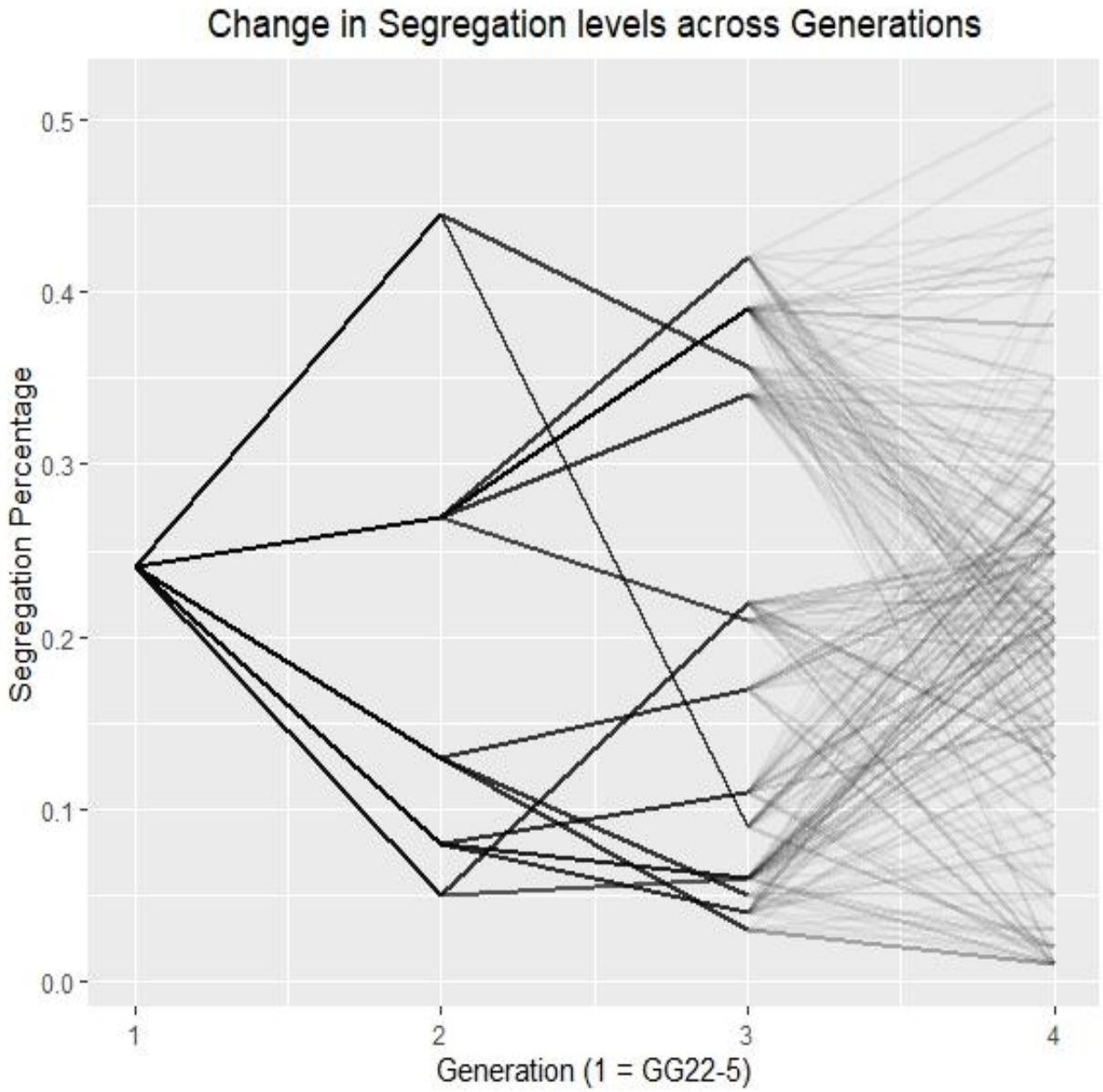

Figure 5 A graph showing the path each lineage took through each generation in terms of their segregation ratios. Generation 1 on the x-axis refers to GG22-5, and generations 2 - 4 represent line A - C on Figure 1, respectively. Values on the y-axis indicate segregation values in decimal form (e.g. 0.10 = 10%).

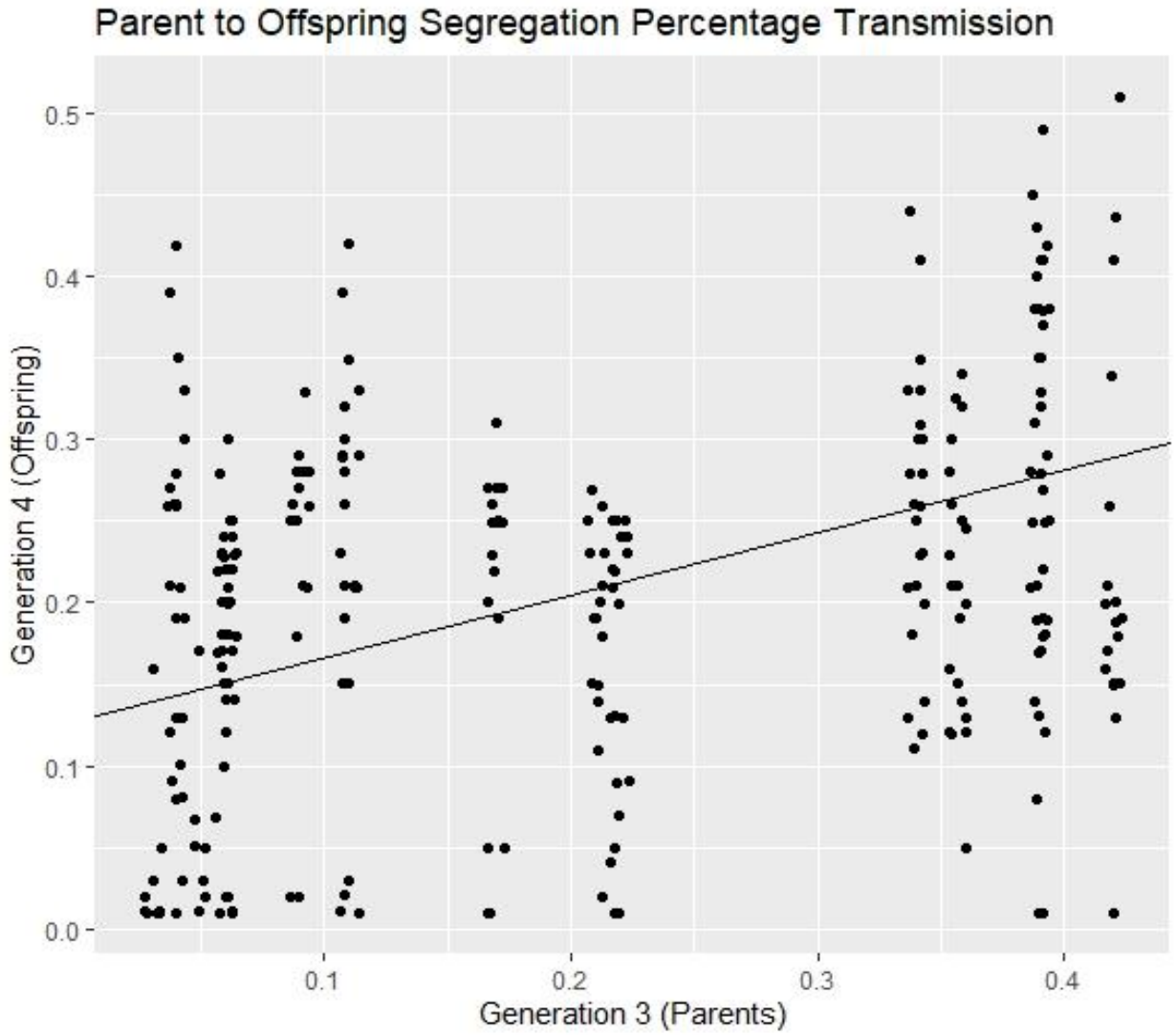

Figure 6 A graph showing the correlation between the segregation in the parent ears in the third generation (Line B in Figure 1) and the segregation of the offspring ears in the next generation (Line C in Figure 1). Data points are jittered to show overlapping data points. The trend line indicates a linear regression (R squared = 0.04). Values on axes indicate segregation values in decimal form (e.g. 0.10 = 10%).

value of 0.04, suggesting an extremely weak but positive correlation, i.e. that high segregating plants were more likely to have offspring with high segregation ratios and vice versa for low segregations (Figure 6). Furthermore, we attempted to trace segregation values throughout all four generations (Figure 5) and were able to draw a few conclusions. First, high segregating ears seemed for the most part to return to Mendelian levels of segregation in the next generation, with only a few remaining in the higher sections. Second, parents with low or normal levels of segregation were able to produce offspring with either low or normal scores, with no apparent resistance to doing so. Third, parent ears in all scoring sections were able to produce offspring ears with high segregation ratios, though the offspring with the highest scores came from parents that already had high scores. All of these, but particularly the third point, likely contributed to the slight positive correlation found in the earlier analysis.

Transmission via Differing Gametes and the Resulting Effect on Segregation Values

Finally, we examined whether transmission of the mutant allele or the segregation of succeeding generation was affected by whether said mutant allele was transmitted to the next generation via either the male or female gametophyte (Tables 7 and 8), using methodologies described in an earlier section. Using a chi-square test, we determined that there was little evidence to suggest that either of these characteristics were affected by differences in transmission via different gametophytes. The expected values were calculated by multiplying the row and column totals for each square and dividing by the table total. For example, in table 7, the expected value for “Cross-out (Male) x Seg” was (283 x 148)/440. We also examined whether there was an effect on the inheritance of segregation ratios, and again found no evidence of such (Figure 7). Ultimately, we have concluded that this mutation’s activity is either wholly unaffected by transmission by the two sex gametes, or that any such effect is identical.

Observed	Cross-out (Male)	Cross on (Female)	Self (Both)	Total
Seg	93	103	87	283
No seg	55	55	47	157
Total	148	158	134	440

Expected	Cross-out (Male)	Cross on (Female)	Self (Both)	Total
Seg	95.19091	101.6227	86.18636	283
No seg	52.80909	56.37727	47.81364	157
Total	148	158	134	440

P = 0.898005

Table 7 Tables showing the chi-square test for whether transmission of the UND-9 allele is affected by its passage through the male or female gametophytes

Observed Frequency	Cross-out (Male)	Cross on (Female)	Self (Both)	Total
Low Seg	33	35	35	103
Norm Seg	48	60	45	153
High Seg	12	8	7	27
Total	93	103	87	283

Expected Frequency	Cross-out (Male)	Cross on (Female)	Self (Both)	Total
Low Seg	33.84806	37.48763	31.66431	103
Norm Seg	50.27915	55.68551	47.03534	153
High Seg	8.872792	9.826855	8.300353	27
Total	93	103	87	283

P = 0.607656

Table 8 Tables showing the chi-square test for whether segregation of the UND-9 phenotype is altered by passage through either the male or female gametophyte.

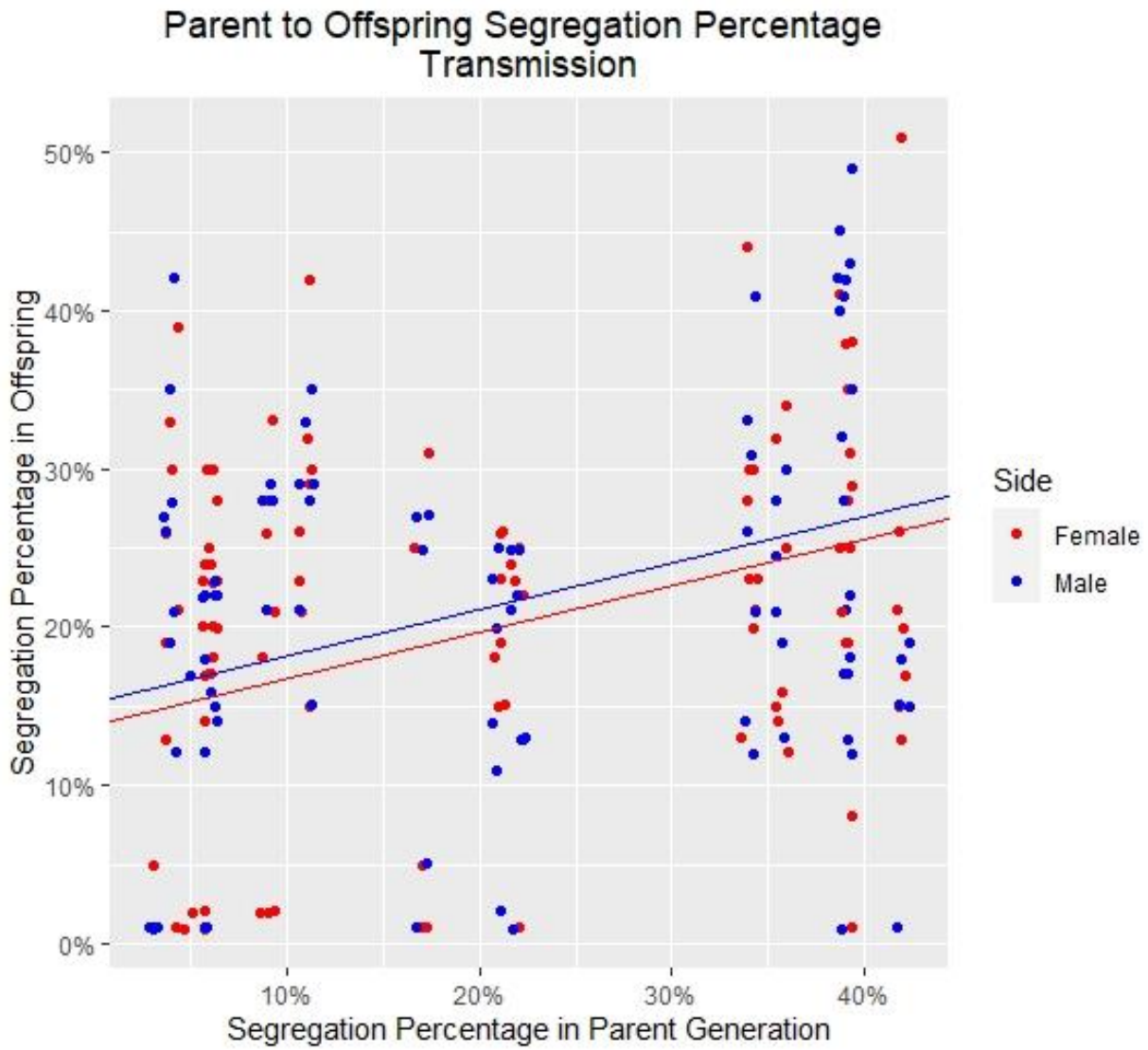

Figure 7 A graph showing the correlation between the segregation in the parent ears in the third generation (Line B in Figure 1) and the segregation of the offspring ears in the next generation (Line C in Figure 1), colored based on whether the UND-9 allele in the offspring generation had passed through the male or female gametophytes. Data points are jittered to show overlapping data points. The trend lines indicate linear regressions for each data set (R squared = Male: 0.047, Female: 0.04),

Discussion

Our research and analysis has not yet resulted in a satisfactory explanation for the behavior of the UND-9 allele, though we have already eliminated a few hypotheses and determined that the abnormal behavior in question does in fact exist. We have also determined that abnormal segregation ratios may be heritable to a degree.

However, despite the apparent lack of progress, we have since begun developing a new model to explain this behavior based on the gene *striate2* that undergoes a similar pattern of having its phenotype suppressed when transferred to a new genetic background (Park et. al., 2000). The *striate2* mutant allele, in its homozygous form, causes white stripes to appear on the leaves of the maize plant where chloroplasts do not develop properly. However, two different alleles of the *inhibitor of striate* gene, or *isr*, are able to reduce this striping effect to varying degrees by preventing the proliferation of cells with defective chloroplasts and produce leaves with normal coloration but narrower width.

In our model, UND-9 is suppressed by an as of yet unknown number of genes via an unknown mechanism in order to allow the embryo to develop as normal. This may happen because the protein encoded by the gene affected by UND-9 is not directly necessary for the development of the embryo, but is instead a signaling protein that can be bypassed, and in fact is bypassed in some inbred lines of maize, but not in others. This could also allow kernels homozygous for the UND-9 allele to survive and grow into a plant that could then produce very high segregation ratios, dependent on the precise dosages required for suppression to take place. Alternatively, the suppressive genes could simply have a similar function to the UND-9 gene, but with reduced functionality or expression that prevents a single allele from rescuing the phenotype single-handedly.

In our model, we assume that each suppressor allele grants a ‘suppression score’ to the embryo, and over a certain threshold, the deleterious effects of the UND-9 are suppressed, resulting in a normal-appearing embryo. This means that each kernel can have one of three outcomes. In the first, the UND-9 locus contains at least one wild-type allele, and embryo development proceeds as normal, regardless of the suppression score. We expect this to

happen in 75% of kernels from self-pollinated plants that are heterozygous for the mutation. In the second case, the UND-9 allele is homozygous, and the suppression score is below the necessary threshold, allowing the mutation to halt embryo development. Finally, the third outcome is that the UND-9 allele is homozygous as in the second possibility, but the suppression score is above the threshold, and the kernel embryo develops sufficiently to appear normal during scoring. It is also possible that such an embryo could go on to develop into a fully grown plant and self-pollinate, which would also have the effect of producing 100% homozygous mutant embryos in succeeding generations, were it not for the effect

Now to consider a few different scenarios. In all scenarios, we will assume that we are self-pollinating a plant that is heterozygous for the UND-9 mutant allele and produces an ear with 100 kernels in the standard 1:2:1 ratio for the expected genotypes. If there are no suppressive genes, this ear will always produce a perfect 25% segregation ratio. We will also assume that any of the genes in question are unlinked and will assort independently in successive generations. I will also be referring to the UND-9 allele as *m*, and its wild-type variant as *+* in order to improve clarity moving forwards. Further, any plant that carries the mutation but is unable to show the phenotype (because it is fully suppressed in all possible genotypes e.g. the parent plant is homozygous for all suppressor alleles) will be considered by our methodology to be non-segregating and will be left out of any further breeding attempts.

In the first scenario, we'll assume there is one gene (we will call it *A*), and only one allele is needed for suppression of the mutant phenotype, i.e., it acts dominantly. If the parent plant is heterozygous for the allele, then there is a 25% chance for an *m/m* kernel to escape suppression (*m/m aa*), resulting in a 25% x 25% = 6.25% chance for a given kernel to contain an embryo expressing the mutant phenotype, as shown in Figure 8. As mentioned earlier, our lab's goal has been to transfer the mutation into B73 by crossing. The B73 plants have no prior relation to the mutagenesis experiment, and being inbreds gives them a high level of homozygosity, so it can therefore be reasonably extrapolated that they have a genotype of *+/+ AA*. The *A* allele had to have come from somewhere, and if it had been in W22, then the UND-9 mutation would not have been discovered at all. Therefore, any seeds from this cross onto B73 would have been either *AA* or *Aa*, and *+/+* or *+/m*, of which only *Aa +/m* would be able to

produce any kernels with mutant embryos after being grown and self-pollinated. This is identical to our original ear, meaning that segregation is now permanently locked at 6.25% and cannot recover, which does not match the data.

	+ A	+ a	m A	m a
+ A	+/+ AA	+/+ Aa	+/m AA	+/m Aa
+ a	+/+ Aa	+/+ aa	+/m Aa	+/m aa
m A	+/m AA	+/m Aa	**m/m AA**	**m/m Aa**
m a	+/m Aa	+/m aa	**m/m Aa**	**m/m aa**

Figure 8 A Punnett square showing the resulting ear from the self in our first simulation (One gene, needs only one suppressive allele). The bolded genotypes are homozygous for the UND-9 mutant allele, while underlined genotypes are suppressing the mutant phenotype, leaving only 1 of the 16 possible genotypes able to display the mutant phenotype.

With this possibility eliminated, we can move on to the next one. This time, there is still one repressive gene, but it must be homozygous for the suppressive allele in order for the embryo to be developed. For continuity, we will continue to call the active allele *A*, even though it is now recessive in its action. Once again, we will use a *+/m Aa* plant to carry the mutant, and a *+/+ AA* plant to receive the cross. This time, we see that the self-pollinated kernels (+/m Aa x +/m Aa) have a 25% chance to carry the mutant genotype, and a 75% chance that the phenotype is not suppressed, for a total segregation ratio of 18.75%. This on its own is higher than our data suggests, and further we can see that the attempt to cross it onto B73 (+/+ AA x +/m Aa) again suffers from the same locking in of the genotype that our first set of plants had. It's clear from this that one gene alone is unable to account for all of the patterns we're seeing in the data.

With one suppressant gene off the table, our next option is to look at two genes. Here we have several options opened up to us. We could make it so that each gene requires only one or both of the suppressive alleles, or even that they only need to share a certain number of such alleles between them. For example, requiring 2 alleles would allow for suppression to happen if one gene is homozygous for the suppressive allele, or if both are heterozygous.

In our next simulation, we'll examine the idea that both genes need to be at least heterozygous for suppression to happen. Therefore, if either is homozygous for the non-

suppressive allele, the kernel will be able to express the UND-9 phenotype. Therefore our segregation for a self-pollinated ear from a plant heterozygous in all three genes (+/m Aa Bb) is 25% x (1 - 75% x 75%), or 10.9%. Crossing the plant onto B73 with the genotype *+/+ AA BB* will produce kernels with a genotype identical to our first plant in this particular situation, but also plants with the genotype *+/m AA Bb* (or *Aa BB*, which is functionally identical), which produces an ear with 6.25% segregation and returns us back to our first, one heterozygous gene situation. Over time, we'd expect the population to start with 10.9% ears which would eventually be replaced with 6.25% ears.

Next we'll assume that one homozygous gene (*AA*) and one heterozygous gene (*Bb*) is necessary for suppression of the UND-9 phenotype to occur. Our first self-pollinated ear has a segregation ratio of 25% x (1 - 75% x 25%) = 20.3%. Crossing onto our standard B73 again allows for the creation of identical plants. It also has the possibility of removing one of the genes from consideration by making it homozygous for the suppressing allele and returning us to one of the one-gene situations. Therefore, the next generation would have a mix of 20.3%, 18.75%, and 6.25% segregation ratios. This spread fits the distribution of our last generation well, but still does not allow for recovery of the phenotype to higher ratios.

Our next situation will require that the suppression of the mutant phenotype requires both genes to be homozygous. Again, we see the previous pattern continue. Our first ear will have a segregation ratio of 25% x (1 - 25% x 25%) = 23.4%, while the next generation will have ratios of 23.4% and 18.75%.

Our final simulations will examine the possibility of a threshold situation, where it is only necessary to have a certain number of suppressive alleles, without regards to which gene is involved. For a threshold of 4 alleles, we can see that this is identical to the double homozygous situation that we have already examined, and a threshold of 3 is identical to the one homozygous and one heterozygous example. Therefore, we are left with only needing to look at thresholds for one and two alleles.

For a one allele threshold, our heterozygous self-pollinating plant will produce an ear with a ratio of 25% x 25% x 25% = 1.56%. If either gene becomes homozygous for the repressing

allele in the cross, then the threshold is automatically passed for all kernels grown in all future generations, so any cross will have to produce more triple heterozygotes in order to be useful to continuing the experiment.

Finally, we have a two-allele threshold situation. Evaluating the self is more complex than in previous situations, but comes out to 25% * (1/16 + 4/16) = 7.81% (Figure 9), while the crosses land in the same situation as the previous consideration. It seems clear that these 'threshold' models are insufficient to explain our data, at least with only two genes.

	+ A B	+ A b	+ a B	+ a b	m A B	m A b	m a B	m a b
+ A B	+/+AABB	+/+AABb	+/+AaBB	+/+AaBb	+/mAABB	+/mAABb	+/mAaBB	+/mAaBb
+ A b	+/+AABb	+/+AAbb	+/+AaBb	+/+Aabb	+/mAABb	+/mAAbb	+/mAaBb	+/mAabb
+ a B	+/+AaBB	+/+AaBb	+/+aaBB	+/+aaBb	+/mAaBB	+/mAaBb	+/maaBB	+/maaBb
+ a b	+/+AaBb	+/+Aabb	+/+aaBb	+/+aabb	+/mAaBb	+/mAabb	+/maaBb	+/+aabb
m A B	+/mAABB	+/mAABb	+/mAaBB	+/mAaBb	**m/mAABB**	**m/mAABb**	**m/mAaBB**	**m/mAaBb**
m A b	+/mAABb	+/mAAbb	+/mAaBb	+/mAabb	**m/mAABb**	**m/mAAbb**	**m/mAaBb**	**m/mAabb**
m a B	+/mAaBB	+/mAaBb	+/maaBB	+/maaBb	**m/mAaBB**	**m/mAaBb**	**m/maaBB**	**m/maaBb**
m a b	+/mAaBb	+/mAabb	+/maaBb	+/maabb	**m/mAaBb**	**m/mAabb**	**m/maaBb**	**m/maabb**

Figure 9 A Punnett square showing the results of the self in our two gene, two-allele threshold test. The bolded genotypes are homozygous for the UND-9 mutant allele, while underlined genotypes are suppressing the mutant phenotype, leaving only 1 of the 16 possible genotypes able to display the mutant phenotype.

We could continue to expand the suppressive gene model with more and more genes, adding further levels of complexity, but it unlikely to improve its accuracy. We have already examined a situation that produces a spread of data similar to what we are observing, in the two-gene, heterozygous-homozygous model. The problem with it, and all the other models, lies not within the number of genes or their dominance patterns, but within an assumption made earlier. We had made the assumption that the B73 that was crossed into the experiment with every generation would be homozygous for the suppressive alleles on each gene. If this is true, then it is clearly impossible for the experimental lineage to lose these alleles and thereby recover its segregation ratio, which is seen regularly within the data. This assumption, if dropped, will vastly open up the model to more complexity as it will allow us to examine multiple branching paths at each generation. However, this will potentially allow us to get past this obstacle. Alternatively, we can examine the effect of repeated self-pollinations on the likely

segregation ratios in order to prevent the wild-type B73 from causing the genetics to stagnate. This would also create extensive branching probabilities that would allow us to further explore the possibilities of this multi-gene model. There are also many other oddities in the data that need to be answered, such as the existence of above average segregating ears, and we can hopefully shine a light on them with a deeper examination.

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