5-2021

PRODUCTION, EVALUATION, AND SELECTION OF ELITE QUALITY PROTEIN POPCORN (QPP) HYBRIDS

Leandra Parsons
University of Nebraska-Lincoln

PRODUCTION, EVALUATION, AND SELECTION OF ELITE QUALITY PROTEIN POPCORN (QPP) HYBRIDS

by

Leandra Marshall Parsons

A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Agronomy and Horticulture

(Plant Breeding and Genetics)

Under the Supervision of Professor David R. Holding

Lincoln, Nebraska

May, 2021

PRODUCTION, EVALUATION, AND SELECTION OF ELITE QUALITY PROTEIN POPCORN (QPP) HYBRIDS

Leandra M. Parsons, Ph.D.
University of Nebraska, 2021
Advisor: David R. Holding

In 2017, twelve Quality Protein Popcorn (QPP) inbred lines were developed and selected as premier dent by popcorn crosses fit for hybridization and testing. These QPP inbred lines were derived from specific Quality Protein dent Maize (QPM) by ConAgra Brands® popcorn line crosses to produce high lysine, vitreous popcorn lines capable of near-equal popping characteristics compared to the original popcorn parents. The QPP hybridization project commenced in the summer of 2018 utilizing these 12 inbred QPP lines and crossing them in a full diallel. Since then, the production of QPP hybrids has employed a diverse set of selection factors evaluating agronomic, popping quality, protein quality, and sensory traits. In 2021, six QPP hybrids were selected for continued evaluation based on agronomic, protein, and popping characteristics, and two QPP hybrids were ultimately selected based on the results from a sensory study.

Dedication

I am thankful to know the grace of God made available through Jesus Christ's atonement on the cross and entrust this work to Him.

"For when we were yet without strength, in due time Christ died for the ungodly. For scarcely for a righteous man will one die: yet peradventure for a good man some would even dare to die. But God commendeth his love toward us, in that, while we were yet sinners, Christ died for us. Much more then, being now justified by his blood, we shall be saved from wrath through him." ~Romans 5:6-9

"That if thou shalt confess with thy mouth the Lord Jesus, and shalt believe in thine heart that God hath raised him from the dead, thou shalt be saved." ~Romans 10:9

"Therefore being justified by faith, we have peace with God through our Lord Jesus Christ:" ~Romans 5:1

"And whatsoever ye do in word or deed, do all in the name of the Lord Jesus, giving thanks to God and the Father by him." ~Colossians 3:17

Acknowledgements

Dr. David Holding has continually supported this breeding project from its inception. He guided the previous breeding endeavor of producing inbred lines and set me up well for hybrid production. Without his guidance, hands-on field work, innovative practices, and honest support of my endeavors these hybrids would not have been produced. His kindness and graciousness in both personal and academic spheres encouraged me to continue pursing my Ph.D. throughout the 'highs' and 'lows' of the program, and I can say with confidence that I could not have been placed within a better program or under the leadership of a better advisor.

My committee members Dr. Edgar Cahoon, Dr. James Schnable, Dr. Steve Baenziger, and Dr. Oscar Rodriguez have all influenced the research and writing of this project. Dr. Cahoon's kindness and knowledge in plant biochemistry made his class one of my most enjoyable experiences at UNL. Dr. Schnable's advice in keeping diversity in selection allowed for our program to expand beyond just a few hybrids. Dr. Baenziger's applied knowledge in crop breeding and selection indices, and his continual willingness to introduce me to a variety of people who helped along the way, allowed for my confidence in our BC_3 breeding scheme and 2020 Selection Index (I am also very thankful for Blaine Johnson's wisdom in the field of selection indices). Finally, Dr. Rodriguez continually kept the door open between ConAgra® Brands and our project. His willingness to perform hands-on experimentation (volume expansion tests, popability, flake morphology, etc.), ship essential seed to our seed room, advise concerning popcorn parent heterotic pools and hybrid potential, and be a continual resource for any popcorn

related question (population density, etc.) did not go unnoticed, and I am very thankful for his consistent accessibility and membership on my committee.

Acknowledgement is due to ConAgra Brands® for its funding and popcorn parental lines and Dr. Ying Ren for inbred line production. Additionally, collaborations with Dr. Ruthie Angelovici and Dr. Abou Yobi from University of Missouri-Columbia, field-work by Payton Knutzen-young and Preston Hurst, and general help from Abigail Osterholt and Elsa Rasmussen are all appreciated.

Funding Acknowledgement

This research was financially supported by ConAgra Brands® and the University of Nebraska-Lincoln.

Preface

The results presented in Chapter 2 were accepted for publication in *Frontiers in Plant Science – Plant Breeding* (Parsons, L., Ren, Y., Yobi, A., Hurst, P., Angelovici, R., Rodriguez, O., & Holding, D. R. (2020). Production and Selection of Quality Protein Popcorn Hybrids Using a Novel Ranking System and Combining Ability Estimates. *Frontiers in Plant Sci.* 11:698. doi: 10.3389/fpls.2020.00698).

Results presented in Chapter 3 were accepted for publication in *Frontiers in Plant Science*. (Parsons, L., Ren, Y., Yobi, A., Angelovici, R., Rodriguez, O., and Holding, D.R. (2021). Final Selection of Quality Protein Popcorn Hybrids. *Frontiers in Plant Sci.* 12:658456. doi: 10.3389/fpls.2021.658456).

Results presented in Chapter 4 have been submitted for publication in the *Journal of Sensory Studies* (L. Parsons, O. R. Rodriguez, D. R. Holding, *"Original Sensory Evaluation of Quality Protein Popcorn reveals Improved Diversity in Taste and Texture compared to Conventional Varieties,"* (submitted for publication in Jour. Sens. Studies, January 2021).)

Table of Contents

CHAPTER 1: LITERATURE REVIEW

1. Maize

To date, genetic and archeological data suggests *Zea mays* ssp. *mays* (hereafter referred to as 'maize') was domesticated from its wild progenitor teosinte, *Zea mays* ssp. *parviglumis,* in one event in the Balsas river valley of Mexico around 7000 B.C. (Lorant et al., 2018) (Figure 1). Since this time, maize has diversified into two main domesticated subspecies and a myriad of varieties adapted to a wide range of environments and grown predominantly for food, fuel, and feed (Fang et al, 2019; Tenaillon and Charcosset, 2011). Classification of maize varieties is traditionally based on endosperm composition, appearance (vitreousness vs. opacity), and kernel morphology, and commonly fall into dent (*Zea mays* ssp. *indentata*) or flint (*Zea mays* ssp. *indurata*) subspecies, or specifically 'field' (dent), sweet, or popcorn (*Zea mays* ssp. *everta*) classifications (Brown and Darrah, 1985; Sandhu et al., 2004).

Dent corn, as the name insinuates, characteristically forms a dent in the crown of the kernel at harvest (~15.5% kernel moisture) due to unequal drying of the hard outer and softer inner white starch (Ensminger et al., 1993). Due to less soft, white, starchy kernel endosperm, popcorn varieties do not form a dent after drying and commonly have a spherical and vitreous morphology. Though these different subspecies have intrinsic characteristics that define their classifications, such as the morphology of dent maize and popability of popcorn, peripheral positive and negative attributes of each subspecies warrant additional description.

1.1 Dent Maize

The earliest recorded experiments in dent maize breeding began more than two centuries ago in the United States with the experimental hybridization of varieties separately traced to Mexico and the eastern U.S. seaboard (Anderson and Brown, 1952). These experimental, single cross hybrids (traced to memoirs written in 1813) were providentially successful in producing distinctively long-eared, strong stalked, and high yielding progeny (Bailey, 1814; Anderson and Brown, 1952). These results gave way to multiple experimental maize crosses during the United States' migration through the Great Plains during the 19th century. In 1840, sold maize seed bags consisted of a conglomerate population of more than 250 open-pollinated varieties (a number that would quadruple by the end of the century; Mikel, 2008). These Corn Belt dent lines would become progenitor lines to the first experimental dent double and single-cross hybrid varieties produced in the United States between the 1930s and 1960s (Brown and Darrah, 1985; Mikel, 2008).

Due to its widespread cultivation and amassed materials, it is of no surprise that dent maize was one of the first model systems for genomic research. Genetic studies in dent maize have been traced back to the 1900s, including two notable discoveries by Barbara McClintock in 1931 (the realization of a physical exchange of genetic material during genetic recombination) and 1950 (the discovery of transposable elements) and facilitated one of the earliest mutant linkage studies published in 1935 (Emerson et al., 1935; Hake and Ross-Ibarra, 2015; Creighton and McClintock, 1931; McClintock, 1950). This particular linkage study was the first to specifically highlight maize mutant allele *opaque-2* (originally found in Connecticut in the 1920s) and its effect on kernel endosperm vitreousness (Singleton and Jones, unpublished; Emerson et al., 1935; Vietmeyer, 2000).

Though the genetic action of *opaque-2* wasn't fully realized until 1990, studies from 1964 onward realized this mutant's capacity in rebalancing kernel endosperm proteins to confer elevated lysine and tryptophan levels (Mertz et al., 1964, 1965, Schmidt et al, 1990). These findings served as the foundation for the Center of International Maize and Wheat Improvement (CIMMYT) to embark on a multi-decade, humanitarian dent maize breeding program aimed to better satisfy dietary requirements for communities in developing countries (Vivek et al., 2008).

1.1.1 Quality Protein Maize

The *opaque-2* allele is not isolated in its ability to foster elevated lysine and tryptophan levels in the maize kernel. In fact, more than 10 maize alleles have been identified that have various effects on kernel storage protein formation and confer an opaque endosperm phenotype and/or elevated essential amino acids in the kernel (Wang et al., 2019). However, *opaque-2* was determined to be most suitable for genetic manipulation by CIMMYT and other programs aimed at breeding high quality protein maize varieties because of its simple, predictable recessive inheritance and comparatively low yield reduction (Vivek et al., 2008; Wang et al., 2019).

During the inception of CIMMYT's breeding scheme, it was understood that *Opaque-2* was an α-zein (*Zea mays* proteins) prolamin regulatory gene. The mutant counterpart, *opaque-2,* was hypothesized to manifest an opaque mutant phenotype due to the down-regulation of zeins, disruption of storage protein production and/or protein body formation, and subsequent increase in lysine and tryptophan (Bjarnason and Vasal, 1992). Other breeding attempts found *opaque-2*'s pleotropic effects of low yields, higher pest/rot susceptibility, slow dry-down, and soft, chalky endosperm (impractical for machine

harvesting) unmanageable for realistic commercialization (Salamini et al, 1970; Dudley and Moll, 1969; Bjarnason and Vasal, 1992). However, studies from the University of Missouri in 1969 suggested variability in endosperm vitreousness/opacity in *opaque-2* backgrounds, and such a discovery allowed space for plausible restoration of endosperm hardiness without sacrificing amino acid biofortification (Paez et al., 1969; Prasanna et al., 2001). Though funding and efforts in breeding *opaque-2* biofortified dent maize genotypes prematurely diminished after realizing the substantiality of its negative agronomic impact, larger institutions such as CIMMYT continued to look for avenues to dissuade these effects. Alongside the study published in 1969, CIMMYT researchers identified variation in *opaque-2* endosperm hardness in temperate and tropical maize lines in the 1970s (Bjarnason and Vasal, 1992). With locations unknown, genes involving the restoration of kernel endosperm vitreousness were termed 'endosperm modifiers' while genes involving lysine and tryptophan levels (other than *opaque-2*) were termed 'amino acid modifiers' and both were phenotypically selected (Vivek et al., 2008). During its two-decade backcross-recurrent selection breeding program (further details to follow), CIMMYT successfully produced numerous 'Quality Protein Maize' (QPM) inbred lines conferring high lysine and high tryptophan restored-vitreousness endosperm, and hybrids were robustly tested and selected (600-1000 hybrids per year) across the globe until the 2000s (Prasanna et al., 2001). Since this time, QPM cultivation has become a staple in countries worldwide and serves as the primary food crop for many maize-based communities (Yasabu, 2019) (Figure 2).

Though CIMMYT's work was principally aimed at achieving QPM inbred line production and bettering QPM hybrid quality, QPM inbred lines have been deposited at

various germplasm and U.S. plant introduction stations for independent, original research and QPM inbred registration (Worral et al., 2015). Research utilizing QPM cultivars has ranged from evaluating degree of drought stress to antioxidant fermentation and porridge acceptability testing (Oladeji et al., 2017; Chiuta and Mutengwa, 2018; Maseta et al., 2016), and breeding programs aimed at conversion of normal maize lines into QPM varieties have expanded outside of CIMMYT's realm. However, these conversions notably remained in the flint and dent subspecies pools apart from sweet or popcorn classifications. In fact, to date there is no sweet corn conversion into QPM in literature, and the first cross between QPM dent maize and popcorn was described in 2018 (Ren et al., 2018). These breeding programs aimed at integrating the *opaque-2* allele into different subspecies and classifications of maize may well develop the next frontier for QPM research.

1.2 Popcorn

Like dent maize, the history of popcorn begins in the New World as either a Native American crop or consequential mutation from parched maize (prepared kernels for storage) after early European colonization (Eldredge and Thomas, 1959). Unlike dent maize, popcorn kernels do not change shape while drying, and popcorn kernels are distinct from other subspecies due to this sphericity, as well as a nearly negligible soft, opaque center and overall small kernel size. Cultivation of popcorn in the United States was rare until the 1900s, and though the number of acres planted slowly rose throughout the century, dent maize production and research continued to surpass the niche market of popcorn. To date, 99% of U.S. acres planted to maize are dent maize varieties, while all other specialty crops, including both popcorn and sweet corn, are planted on less than 1%

(Nebraska Corn Board, 2019). Due to its relatively limited production, use, and revenue, popcorn research and breeding has lagged behind field corn and is resultantly an agronomically inferior crop. Moreover, due to an observable negative correlation between agronomic traits that directly affect yield and popcorn quality traits such as popping expansion volume and popability, bettering popcorn agronomics has proven to be a difficult task.

1.2.1 Popcorn Breeding

In 1984, Robbins and Ashman undertook the first dent by popcorn breeding experiments aimed at bettering popcorn agronomics, and since this time numerous other groups have tried this method of popcorn improvement with limited success (Robbins and Ashman, 1984; Crumbaker et al., 1949; Johnson and Eldredge, 1953). A proof-of-concept study in 2018 successfully crossed popcorn varieties with QPM dent varieties, cultivars with their own past set of agronomic challenges, in a backcross breeding program and fostered high lysine, vitreous, pop-able Quality Protein Popcorn, or QPP, inbred lines (Ren et al., 2018). These QPP inbred lines exhibited a biofortified endosperm akin to QPM, but the kernels were phenotypically indistinct from the original popcorn parental lines. This study was the first to demonstrate the restoration of popcorn quality traits after hybridization to dent maize and offered an opportunity for hybrid generation within its unique germplasm pool due to the production of twelve inbred lines.

1.2.2 Backcross-Recurrent Selection

Alike to QPM breeding, the successful crossing between dent and popcorn subspecies required a detailed breeding program and strategy referred to as ‘backcross-recurrent’ selection. For introduction, almost all traditional breeding programs (or breeding

programs that do not utilize genetic engineering) start with a female by male cross, the progeny of which are called F_1 (filial) seeds. Particularly in a backcross program, the purpose of this cross is to integrate genetic material from the 'donor' parent (either male or female) to the 'recurrent' parent. A simple example of this is the breeding of QPM, in which the donor parent was *opaque-2* germplasm and the recurrent parent was a more agronomically robust cultivar. The purpose of these crosses was solely to integrate the desired genetic material from the donor parent (the *opaque-2* gene and possible endosperm/amino acid modifiers). In any general backcross breeding program, at this point these F_1 seeds are grown and crossed back to the recurrent parent again. Considering simple Mendelian genetics, the F_1 seed and plant carry half of the maternal and half of the paternal genetic material, and if it is self-pollinated, the F_2 seed would carry the same proportions. However, since the F_1 is crossed back to the recurrent parent for a 'backcross', or the production of a BC_1 generation, the proportion of genetic material becomes a ratio of 75:25 recurrent parent to donor parent. It is key to note theoretically (in infinitely large population not under selection), if self-pollinated generations occur between backcrosses, the proportion of genetic material from the donor or recurrent parent will not change (though the percentage of homozygosity *does* significantly change). However, if genetically selected, chosen lines are continued to self-pollinate and selection occurs for multiple rounds, this random proportion of genetic material will likely skew toward the recurrent parent if the amount of genetic material desired from the donor parent is solely and specifically selected (i.e. forward, marker-assisted selection) and the rest is purposefully eliminated (background selection). Since selection procedures for different breeding programs are diverse, for this purpose we will

assume a normal distribution of genetic material in the population and random selection of the generations (albeit a theoretical and unrealistic assumption). Taking a BC_1 as 75:25 recurrent to donor contribution of genetic material, a BC_2 follows as 87.5:12.5, and a BC_3 as 93.75:6.25. For another perspective, theoretically, if *randomly selected* from the beginning, roughly 6.25% of the BC_3 generation would house the desired genetic material from the donor parent and no more than 6.25% of the donor parent's genetic material would be available for further selection rendering a very inefficient system with limited success.

In CIMMYT's breeding program, backcrosses were made only after selection of successful modification of the kernel endosperm, a selection requirement that would sometimes take more than three self-pollinated generations (Vasal, 2002). For a brief description of terminology, progeny from F_1 seeds (and plants) that were self-pollinated would be considered the F_2 generation. Similarly, the harvested seed from a self-pollinated BC_1 would be considered a BC_1F_2. If a BC_1F_2 was again self-pollinated, the progeny would be called a BC_1F_3. If this BC_1F_3 population was crossed back to the recurrent parent, it would be considered a BC_2. The terminology for the progeny after a backcross only depends on the level of backcrossing and not on the number of previous self-pollinations. Though a backcross' progeny's title does not reflect the number of 'self-ed' generations, these generations are very important in changing the genetic composition of the progeny if any selection measures are involved. Self-pollinating and selecting superior progeny allowed for a skew of the contributed genetic material and rapid and efficient breeding of QPM (Vasal, 2002). By integrating all components, self-pollination, selection, and backcrossing, Quality Protein Maize genotypes were

efficiently produced that had a selected mixture of donor stock and *opaque-2* germplasm rendering agronomically sound, high quality protein, fully modified cultivars (Vasal, 2002). This backcross-recurrent selection method proved very useful in its ability to select for holistically competitive QPM cultivars, and therefore a modified but similar breeding scheme was used for the production of Quality Protein Popcorn inbred lines (Figure 3). Final BC_2F_5 and BC_3F_4 QPP inbred lines were both produced and hybridized to test in the summers of 2018, 2019, and 2020 (Ren et al., 2018; Parsons et al., 2020; Parsons et al., 2021).

2. Methods of Selection

Though the primary aim of the QPP program was to increase lysine and tryptophan in the QPP endosperm to biofortify popcorn while restoring and/or maintaining all necessary popcorn quality traits, an important secondary goal was to improve agronomic traits of the original parental popcorn lines and subsequent hybrids. After successful inbred line production using an adapted backcross-recurrent selection method, QPP hybrids were compared to original popcorn hybrids of the same underlying popcorn pedigree without QPM germplasm introgression and multiple traits were evaluated to best gauge agronomics, popcorn quality, and protein quality traits.

To select the best hybrids fit for competitive comparison to original popcorn hybrids, QPP crosses underwent a four year breeding and selection program initially comprised of 132 hybrids and culminating in the selection of two hybrids fit for potential commercialized production. Multiple methods were used to efficiently rank hybrids, including a selection index, combining ability estimates, an observation of increasing agronomic improvement with increasing parental genetic diversity, taste-testing, flake

morphology assessment, and protein profiling (Parsons et al., 2020; Parsons et al., 2021). These assessment methods will be further described in the following sections.

2.1 Heterosis

Maize hybrid experimentation was first introduced by Charles Darwin in 1858 after observing a 25% increase in plant height in maize hybrids compared to open-pollinated varieties (Darwin, 1858). This particular study drew interest from William Beal, a professor from Michigan State College, who proceeded to experiment with the hybridization of open-pollinated varieties and observed yield heterosis (Crabb, 1993). However, maize heterosis through open-pollinated varieties was unpredictably influenced by inbreeding and the varieties were difficult to market and mass produce. Inbreeding depression, or the reduction of progeny fitness associated with reduced genetic diversity by inbreeding (i.e. self-pollination), occurred too frequently in open-pollination. Realizing this variability, George Harrison Shull and Edward Murray East independently discovered the utility and predictability of the 'inbred-hybrid method' in 1908 by producing homozygous maize lines before intentional crossing for observed hybrid vigor (Duvick, 2001). Though both scientists arguably deserve shared credit with the discovery, a dramatic sideline involving undulated dynamics between Shull and East made for a recognition exchange during and after the discovery. Throughout the early 1900s, East campaigned and gained a majority of credit for the discovery of 'heterosis', though it was Shull who coined the term. However, the spotlight quickly turned after East's early passing in 1938 before maize hybrids hit production and Shull gained a majority of recognition after national maize hybrid success (Crabb, 1993).

At its onset, the 'inbred-hybrid' method had its complications. Inbred lines produced by both East and Shull were severely inbred depressed and unprofitable for F_1 seed production. However, a graduate student under East, Donald Jones, creatively identified the utility of the double-cross method in 1918 to both profitably yield F_1 seed for planting and high F_2 yields for producers (Jones, 1918) (Figure 4). Though the progeny from F_1 parents did not produce as optimally as progeny from F_8 inbred lines, the cost of double-cross hybrid seed was manageable for farmers and the yield high enough to make this system the running model of the time (especially considering the political and economic climate of 1918). This 'double-cross method' seemed to sustain farmers while researchers in the public and private sector pushed inbred breeding experimentation forward in a very similar manner to modern inbred generation and selection.

The first mention of a diallel system in maize, or the crossing of multiple inbreds together in a reciprocal fashion, is found in 1942 by Sprague and Tatum (Sprague and Tatum, 1942). This pivotal paper described two main concepts that have been since utilized in maize breeding: the diallel system and genetic combining abilities. Since the genetic explanation for hybrid vigor still remains elusive, the experimental diallel to test best hybrid combinations remains the most informative methodology for testing heterosis (Duvik, 2001; Birchler et al., 2010; White et al., 2020). Akin to the methodology, statistical analysis of the diallel structure is predominantly conducted using Griffing's diallel models described in 1956 (Griffing, 1956). Though many different theories since the 1950s have emerged postulating the underlying genetic explanation of hybrid vigor and determination of heterotic pools, the tangible principles and methodology of maize

inbred generation and hybrid production and testing have arguably remained static for almost a century (Birchler et al., 2010).

Indeed, contributions from East, Shull, and Jones from 1908-1911 effectively describe the running explanation of heterosis: *"This [heterotic] stimulation has been shown to be correlated more or less closely with the degree of heterozygosity,"* (Jones, 1917). Along this vein breeders soon formed heterotic groups with differing levels of heterozygosity, leading the way for the delineation of maize heterotic groups (or pools) by genetic diversity for best predicted heterotic capacity (Adams and Shank, 1959; Moll et al., 1965; Reif et al., 2005; Springer and Stupar, 2007). This overarching idea that progeny heterosis is generally negatively correlative to the degree of parental genetic relatedness was both an opportunity for observation and a useful method during Quality Protein Popcorn hybrid assessment and selection.

2.2 Genetic Combining Abilities

Sprague and Tatum first defined the terms 'general' and 'specific combining abilities' in 1942 to describe the average and specific performances of a line in general or specific crosses, respectively (Sprague and Tatum, 1942). When crossing in a full diallel system, the general combining ability (GCA) reflected relative trait performance values of a line in hybrid combination with all other lines within the diallel, while the specific combining ability of a particular cross represented the unexplainable increase or decrease in trait value after measuring GCAs of the parents.

An illustration of these concepts for further explanation may be profitable. For example, in a diallel system of seven maize inbreds labeled 'A' through 'G', maize parental inbred line 'A' measured an average 19 cm ear length across all of its hybrid combinations and

parental inbred line ‘B’ averaged 17 cm ear length across all hybrid combinations. If an average ear length of 18 cm was measured across all possibly hybrid combinations between inbreds ‘A’ through ‘G’ (i.e. the overall mean of the population), inbred ‘A’ would have a +1 cm GCA for ear length and inbred ‘B’ would have a -1 cm GCA. Given these GCAs, the cross between inbreds ‘A’ and ‘B’ would theoretically foster progeny with an average 18 cm ear length. If so, the specific combining ability for the ‘A’ x ‘B’ cross would be 0 cm. However, if that cross sustainably rendered 20 cm long ears, the SCA for the ‘A’ x ‘B’ cross would equal +2 cm.

The potential utilization for these trait combining abilities was quickly realized by the maize breeding community after Sprague and Tatum published their piece, and a pipeline for efficient statistical analysis for measurement and significance determination of these combining abilities was published by Griffing in 1956 specific to full diallel systems, the design used by Sprague and Tatum (Sprague and Tatum, 1942; Griffing, 1956). Between 1956 and the 1980s, a majority of published maize diallel systems followed the field design required for Griffing’s analysis. However, one major limitation to this model was the inability to test an unequal number of designated male and female inbred lines, or in more statistical terms, ‘non-orthogonal data’. The advanced statistical proficiency required to manipulate these types of datasets began emerging in the 1980s with the development of the ‘REML’ (restricted maximum likelihood) program. The REML software was capable of manipulating non-orthogonal datasets to ultimately calculate unbiased GCA and SCA values (Robinson et al., 1982). This initial software served as the foundation for statisticians to build, alter, and advance theoretical and computational proficiency for estimating genetic effects. Out of these emerging statisticians was a man

named Arthur Gilmore, a brilliant Australian scholar, who would soon produce perhaps the most well-known program utilized to estimate genetic variances called 'ASREML'. (Gilmore, 1996). This particular program, made available in 1996, utilized similar manipulation processes as REML but in a more straightforward approach, and the program has arguably become the current standard for computing genetic effects in both plant and animal breeding (AAABG, 2013).

GCA and SCA estimates were utilized for the selection of elite Quality Protein Popcorn hybrids in and after the summer of 2019. Genetic estimates were calculated for more than ten traits and 44 hybrids from 12 paternal and four maternal inbred lines. The ASREML program allowed for the non-orthologous dataset of this partial diallel and general and specific combining ability estimates, standard errors, heritabilities, and genetic repeatability values for multiple traits were calculated through this program and served as descriptive tools for ultimate selection (Parsons et al., 2020).

2.3 Popcorn Quality Trait Assessment

Popcorn quality trait evaluation and testing began in the United States more than a century ago. The earliest available record of popcorn quality trait investigation may be found in Sturtevant's 1894 bulletin piece, '*Notes on Maize'* in which he describes a hypothetical process and explanation of popping (Sturtevant, 1894). In the decades that followed, multiple scientists wrote pieces further postulating the explanation for the starch-moisture interaction in the popcorn endosperm with applied heat (Kraemer, 1903; Wilbert, 1903; Lyerly, 1942; Carr and Ripley, 1920).

2.3.1 Expansion Volume

This introductory exploration of popcorn quality traits understandably transitioned into the analysis of expansion volume and popping characteristics by F.C. Stewart in 1923, in which he wrote the first record of an articulated methodology for popcorn expansion volume, stabilizing heat, and determining the moisture content in the popcorn immediately prior to popping (Stewart, 1923) (Figure 5). F. Constance Stewart would go on to publish another piece in 1936 to describe the viability of popping in relation to age and other parameters utilizing his previously published methods (Stewart, 1936). Though expansion volume as typically measured today in a volume per weight unit differed slightly from Stewart, his notion of quantitatively describing quality popcorn traits laid the foundation for further studies that developed popability, flake morphology, and expansion volume measurements. Stewart's method of a volume per volume expansion measurement was prevalently used until breeders shifted to a volume per gram unit measurement somewhere between 1985 and 1990. Though Stephen Dofing's pair of expansion volume papers describing this new way of measurement in 1990 and 1991 is predominantly cited in current literature, the equation can actually be found in a lesser known paper a few years prior (Lin and Anantheswaran, 1988; Dofing et al., 1990 and 1991). It is very plausible that Dofing utilized a measurement first conceptualized by his colleagues Lin and Ananthwaran in 1988, as all three scientists were from the University of Nebraska-Lincoln a year prior. However, immediately before the 1988 paper, both Lin and Ananthwaran moved to Pennsylvania State University. Intentional or not, Dofing's lack of reference to his former colleagues' methodology landed him with the current reference standard for popcorn expansion volume methodology (Sweley et al., 2012).

It is understandable that expansion volume became one of the first quality traits of popcorn to be studied due to its defining immediacy to 'popcorn' varieties, but other traits such as popability, hull dispersion, flake morphology, kernel size, color and morphology, and flake texture and flavor were explored throughout 1943-1993 (Eldredge and Lyerly, 1943; Grogan et al., 1958; Eldredge and Thomas, 1959; Hoseney et al., 1983; Matz, 1984; Lin and Anantheswaran, 1988; Mohamed et al., 1993; Ziegler, 2001; Quinn et al., 2005; Sweley et al., 2013). These trait measurements will be described in turn beginning with popability and hull dispersion, traits closely linked to expansion volume in regard to methodology.

2.3.2 Popability

Popability measurements are evaluated as a percentage of fully popped kernels after an attempt to pop a defined number of kernels (Song et al., 1991). Alike to expansion volume, popability is influenced by multiple factors including genetics, environmental conditions, moisture content endosperm vitreousness, and a disproportionate lack of heat during popping (Eldredge and Lyerly, 1943; Hoseney et al., 1983). Interestingly, a study in 2012 found no correlation between unpopped kernel physiochemical parameters and unpopped kernels - results instead suggesting that unpopped kernels may be an artifact of a disproportionate lack of heat and/or inadvertent shielding during the popping process (Sweley et al., 2012). Nevertheless, a measure of unpopped kernels has become a standard and important trait to evaluate for overall popcorn quality, though ironically this type of measurement was considered useless and arbitrary during the first fruits of popcorn experimentation (Stewart, 1923; Ozturk et al., 2020).

2.3.3 Hull Dispersion

Hull dispersion is a majorly qualitative trait assessed after popping. More dispersion of the outer pericarp (used interchangeably with 'hull'), or unattachment from popped flakes, has been noted as desirable to consumers since 1943 (Eldredge and Lyerly, 1943). This trait is best assessed after popping by evaluating the brown 'shell' left remaining on the popped flake. Relatively, the more connective hull marks a lower hull dispersion score (Zeigler, 2001; Sweley et al., 2013). Flake morphology has been identified as an interactor with hull dispersion; butterfly flakes tend to have more effective hull dispersion while mushroom morphologies tend to retain hulls after popping (Eldredge and Thomas, 1959; Watson, 1988; Sweley et al., 2011). Though consumer satisfaction has been clearly correlative to more effective hull dispersion, popped, coated popcorn products require predominantly mushroom morphology due to better hardiness and resistance to breakage during coating and packaging (Eldredge and Thomas, 1959; Matz, 1984, Sweley et al., 2011 and 2013).

2.3.4 Flake Morphology

Popcorn flakes were first categorized into 'butterfly', 'intermediate', and 'mushroom' in 1959 by Eldredge and Thomas. 'Butterfly' flakes were considered irregular protrusions that were branched or pronged, 'mushroom' popcorns simply popped into a symmetrical, spherical shape, and intermediate flakes were termed what is currently considered in literature 'unilateral' (Eldredge and Thomas, 1959; Sweley et al., 2011 and 2012). Sweley et al. from the University of Nebraska-Lincoln proposed a more specific, quantitative and categorical system for terming flake morphology in 2011, parsing out the 'butterfly' morphology into 'unilateral' (previously termed 'intermediate'), 'bilateral' (previously 'butterfly'), and 'multilateral', while maintaining the 'mushroom'

morphology described in 1959 (Sweley et al., 2011) (Figure 6). Recent publications suggest that these four categories are emerging as the new popcorn classifications for flake morphologies (Sweley et al., 2014; Ranathunga et al., 2016; García-Pinilla et al., 2019; Parsons et al., 2020).

2.3.5 Kernel Size

Popcorn kernel size was attributed as a significant influence to expansion volume as early as 1927 and was considered a continuous trait until 1943 when size was given three basic classifications (Willier and Brunson, 1927; Eldredge and Lyerly, 1943). Eldredge and Lyerly described multiple varieties of popcorn in their 1943 '*Popcorn in Iowa*' bulletin as producing 'small', 'medium', and 'large' kernels and relating, though not correlating, these sizes to other popcorn quality traits such as hull dispersion, expansion volume, and flake texture (Eldredge and Lyerly, 1943). Though these categories were relative, popcorn seed producers soon followed a grading labeling system detailing the length of the kernels sold. Grades 11-17 corresponded sequentially to 11/64 to 17/64 of an inch in kernel length, and popcorn or sorghum planter plates were recommended for accurate planting populations (Eldredge and Thomas, 1959). This grading system reasonably produced the current sieving method of determining kernel size, with kernels passing between 4.36-4.76 mm (categorized as 'small'), 4.76-5.16 mm, 5.16-5.56 mm, 5.56-5.95 mm (categorized as 'medium'), and greater than 5.95 mm round hole sieves (categorized as 'large') as to correlate to 11/64 through 15/64 of an inch (Lin and Anantheswaran, 1988; Song et al., 1991; Ceylan and Karababa, 2001). Willier and Brunson's 1927 study also introduced a secondary means of counting kernels by weight and allotting kernels into these three main categories (Willier and Brunson, 1927). The current measure used

is a 10 gram sample composed of 52-67 ‘large’ kernels, 68-75 ‘medium’ kernels, or 76-105 ‘small’ kernels (Ziegler et al., 1984; Matz, 1984; Sweley et al., 2013). Both means of considering kernel size are found in literature, though counting kernels per 10 grams is perhaps more frequent in recent publications.

2.3.6 Kernel Color

Though there are many popcorn varieties that hold vivid coloration in the aleurone or pericarp, there are only two main types of popped kernel color (or flake color): white and yellow (Eldredge and Thomas, 1959; Park and Maga, 2000). Red, purple, blue, and black colorations are found in the pericarp or aleurone, and though possibly appealing in kernel appearance, these darker shells were found to give the popped white flakes an unattractive, shady appearance and are uncommonly marketed (Eldredge and Lyerly, 1943) (Figure 7). A study in 2000 statistically validated a suggestion in 1959 that yellow popcorn ranked higher than white popcorn in both color and aroma appeal to a tasting panel despite the white endosperm’s association with tenderness and more effective hull dispersion (Eldredge and Thomas, 1959; Park and Maga, 2000). Despite skepticism for vivid coloration in the kernel, some specialty breeding programs can be sometimes found in literature. For example, a 2012 breeding program crossed commercial yellow popcorn with a vivid purple line overexpressing anthocyanin in the pericarp to produce a high anthocyanin, purple-kernel product (Lago et al., 2012). Likely due to the use of a yellow recurrent parent, anthocyanin levels in the purple popcorn popped flakes were significantly higher than the yellow control (thanks to traditionally less hull/pericarp dispersion where the anthocyanin was housed) while maintaining equal consumer preference to the original yellow (Lago et al., 2012). Overall, though multiple small-

scale breeding programs market colored seed, major popcorn producing companies currently rely on white and yellow endosperms, pericarps, and aleurones for commercialization (Sweley et al., 2013).

2.3.7 Kernel Morphology

Early popcorn studies linking kernel size to expansion volume started considering different variables composed of 'kernel size', such as breadth, length, sphericity and thickness (Willier and Brunson, 1927; Lyerly, 1942; Haugh et al., 1976; Lin and Anantheswaran, 1988; Pordesimo et al., 1990; Mohamed et al., 1993). These studies concluded that small, 'horny', vitreous kernels produced the largest flakes, and three classes of popcorn morphologies emerged around the 1940s: rice-shape, pearl-shape, and Japanese Hulless (Eldredge and Lyerly, 1943). The Japanese Hulless subcategory was slowly phased out throughout the 1950s, possibly due to its irregularly short and thick cob, indiscriminate kernel rows, low yields, and intermixing morphology pools, and the two predominant kernel morphologies of rice-shape and pearl-shape have since been produced (Lyerly, 1942; Grogan et al., 1958; Eldredge and Thomas, 1959; Ziegler et al., 1985; Carter et al., 1989; Ceylan and Karababa, 2001) (Figure 8). The rice types of kernels are rounded, long and slender, and have a sharp point at the kernel tip, while the pearl morphologies are characterized by short and thick kernels rounded at the top of the kernel (Eldredge and Thomas, 1959). Pearl types were known to have characteristically higher yields and larger expansion volume and are the most abundant types of commercialized popcorn products to date, though varieties with blended morphologies are also possible (Grogan et al., 1958; Eldredge and Thomas, 1959; Ziegler, 2001; Sweley et al., 2013).

2.3.8 Flake Texture

Popcorn has been enjoyed as a pastime snack in the United States for well over a century. The 1912 'The Book of Parties and Pastimes' devoted an entire chapter to 'popcorn parties', describing games in which men and women would race to shell, pop, and eat an ear of popcorn, while the cookbook 'Foods that Will Win the War: And How to Cook Them' timely published in 1918 describes multiple recipes utilizing popcorn as a flour or popped complement to apples (Dawson and Telford, 1912; Goudiss and Goudiss, 1918). Popcorn breeding and utilization were ramping up in the United States through the early 1900s, yet the qualitative and/or quantitative evaluations of flake texture or flavor were not deeply considered until the 1940s. Only one reference before this time can be found in a short 1921 paper observing the texture of the popcorn kernel endosperm in relation to popcorn expansion volume (Weatherwax, 1921). Alike to many other popcorn quality traits, the first publicized mention of popcorn flake texture is found in Lyerly and Eldredge's pieces in 1942 and 1943 in which they note certain popcorn varieties having a course-texture in the popped flakes (Lyerly, 1942; Eldredge and Lyerly, 1943). Flake texture started to become more frequently cited in literature around the 1980s and became one of the top four (after price, flavor, and appearance) important consumer quality traits and one of the top two that derived from the popcorn variety itself. Understandably, popcorn texture and hull dispersion are closely related as less hull dispersion lends itself to a less desirous course texture, and therefore flake morphology (butterfly vs. mushroom in this case) also is a significant variable in flake texture (Matz, 1984). Flake texture has slowly become a more specific and quantitative measurement over the past decades. 'Tenderness' and 'crispness' were first descriptors of flake consistency, then later a high

quality ‘texture’ was defined further as crisp then soft as opposed to chewy and adhesive (Song et al, 1991; Zeigler, 2001; Sweley et al., 2013). Park and Maga described the first study attempting to quantify popcorn texture with sensory and instrumental methods and relating it to panel rankings of ‘crispiness’ and ‘tenderness’ but found insignificant differences in texture between flake morphologies and kernel color, results in disagreement with previous notions (Eldredge and Thomas, 1959; Park and Maga, 2000). However, Sweley et al. delved further into the evaluation of texture in relation to flake type and found that unilateral flake morphologies were significantly higher in taste panel satisfaction in regard to overall flavor, butter flavor, saltiness, texture density, crispness, and crunchiness compared to both bilateral and multilateral flake morphologies (Sweley et al., 2011).

Though popcorn flake texture and flavor are arguably subjective and/or qualitative, breeders and food scientists have determined to identify ways in which to scale these important factors. Moreover, although conflicting results are found in literature, the common conception for ideal popcorn texture involves the lack of hull attachment after popping, a butterfly flake morphology (ideally unilateral), and a crisp flake that turns soft rather than adhesive.

2.3.9 Flake Flavor

Like popcorn flake texture, flake flavor analysis has become more defined over the century. Until the 1970s, popcorn ‘flavor’ was considered alongside expansion volume as the two most important consumer-driven traits (Willier, 1927). This concise definition for favored popcorn varieties was expanded in 1943 to include other quality and agronomic traits, but popcorn ‘flavor’ was considered with premier importance despite its

unclear description of ‘good’ and ‘distinctive’ (Eldredge and Lyerly, 1943; Brunson and Smith, 1944). Moreover, these ‘important’ sensory traits were also ill-defined in breeding programs, and without clear selection techniques popcorn taste and flavor seemed to take a backseat to other selectable, albeit less consumer-driven, quality traits in popcorn breeding efforts for the better half of the 20th century. However, a group in 1970 utilized innovative analytical chemistry technology, gas chromatography coupled to mass spectrometry (GC-MS), to identify the volatiles characteristic of popcorn (Walradt et al., 1970). Overall the group characterized 58 different compounds, 23 of which could be attributed to popcorn’s characteristic smell and flavor. Pyrazines, furans, pyrroles, carbonyls, and decorated phenols were listed as the primary aromatic compounds comprising popcorn (Walradt et al., 1970). Buttery et al. identified more distinctive compounds in 1997 through different sample preparations for GC-MS, and these results were combined in holistic studies analyzing consumer preferences and compound relative concentration by Sweley et al. and flake morphology and kernel color by Park and Maga and Ceylan and Karababa (Schieberle, 1991; Buttery et al., 1997; Park and Maga, 2000; Ceylan and Karababa, 2001; Sweley et al., 2011). The overall results from these studies seem to concur that enhanced popcorn flavor is majorly attributed to pyrazine compounds which may be correlated to a unilateral flake morphology and/or yellow rice and pearl shaped kernels – traits amiable toward selection.

A description of popcorn flavor would be incomplete without preliminarily realizing that most popcorn flavors commercially sold stem from artificial coatings, butter, oil, and salt mixed in microwave bags and ready-to-eat popcorn (Matz, 1984; Ziegler et al., 1985; Carter et al., 1989). In fact, Matz’s book ‘Snack Food Technology’ described popcorn

flavor as relatively unimportant since freshly popped popcorn aroma and taste soon dissipate leaving the producer a bland baseline to improve with additives (Matz, 1984). Nevertheless, breeders and researchers have found continued interest in identifying the aromatic compounds associated with popcorn's unique aroma and flavor for competitive sales of uncoated and coated popcorn kernels alike, and experimentation has taken successful strides in identifying these molecules.

2.3.10 Popcorn Quality Traits Evaluated for QPP Hybrid Selection

The final twelve BC_2F_5 Quality Protein Popcorn inbred lines were selected in 2017 and hybrids were produced in a full diallel with separate reciprocals in 2018. Qualitative observations on maternal capabilities such as standability, ear length and overall seed set were considered and 44 hybrids out of 132 were selected for further analysis. During the 2019 field season, expansion volume, popability, color, and flake morphology were quantitatively/qualitatively analyzed and five QPP hybrids were selected for continued analysis. During the 2020 field season, popcorn quality traits expansion volume, popability, color, flake morphology, hull dispersion, taste, texture, smell, appearance, and overall likability were considered alongside agronomic traits for final selection of best QPP hybrids.

2.4 Protein Profiling

2.4.1 Brief history of the *opaque-2* mutant and the production of Quality Protein Maize hybrids

As previously mentioned, the *opaque endosperm-2* mutant was first discovered in the 1920s and publicly characterized in 1935 as a mutant on Chromosome 7 conferring a chalky, soft starch endosperm (Singleton and Jones, unpublished; Emerson et al., 1935).

This mutant was of little interest until the 1960s when Purdue University chemist Edwin Mertz and corn geneticist Oliver Nelson observed higher levels of essential amino acids lysine and tryptophan in *opaque-2* mutant kernels (Mertz et al., 1964). Oliver Nelson's background knowledge in zeins and high-protein, high vitreous maize breeding and Edwin Mertz's biochemical understanding of zein and non-zein amino acid constituents cumulatively led the team's decision to pursue opaque phenotypes for high-lysine protein. Mertz hypothesized that less zein formation, a notion considered by Nelson as interchangeable with less endosperm vitreousness, would foster elevated levels of non-zein proteins, and therefore higher lysine. Together these researchers mined through multiple opaque phenotypes, and two mutants, *floury-2* and *opaque-2*, showed this predicted effect (Mertz et al., 1964; Nelson et al., 1965; Crow and Kermicle, 2002; Larkins, 2019).

Predominantly due to *opaque-2's* superior lysine increase compared to *floury-2* (though publishing order no doubt aided *o2* research momentum), *o2* became the premier biofortifying maize mutant for food quality researchers and maize breeders. Rats, swine, and other monogastric animals showed significant average daily gain increases when fed *o2* vs conventional maize diets (as high as 5-fold improvements; Figure 9), and human trials showed promising effects both in child stature and response to kwashiorkor, a protein deficiency disease found in children in developing countries (Mertz et al., 1965; Cromwell et al., 1967; Gipp and Cline, 1972; Crow and Kermicle, 2002).

The utilization of this mutant for larger-scale experiments and breeding became difficult, however, due to the same phenotype that initially drew researcher interest. *Opaque-2* mutant maize lines were unmanageably susceptible to pest and diseases, soft, and low

yielding. Moreover, combine harvesting and milling was difficult as the kernels broke easily. Papers published between the 1970s and 80s were mixed; some researchers wrote with optimism noting phenotypic variability and a future genetic respite from the chalky kernel with improved breeding, while others deemed the *opaque-2* cause fruitless (Lambert et al., 1969; Salamini et al., 1970; Denić, 1983; Crow and Kermicle, 2002; Tandzi et al., 2017). Despite scant optimism, out of all major companies in the United States only Crow's Hybrid Corn Company in Illinois continued breeding after the 70s for the *o2* mutation (Crow and Kermicle, 2002). Other than Crow's company, only two other breeding programs at the University of Natal in South Africa and The International Maize and Wheat Improvement Center (CIMMYT) in Mexico continued breeding the *opaque-2* mutation with the hope of fostering effective high-lysine maize lines (Prasanna et al., 2001).

Ultimately, the breeding program at CIMMYT had premier success for multiple reasons. CIMMYT's robust Quality Protein Maize (QPM) breeding team included breeders, pathologists, entomologists, and biochemists concurrently selecting for vitreous, agronomically sound, high lysine lines, involved laboratory support for rapid biochemical and genetic marker services, and was composed of a single, dynamic, multi-faceted breeding strategy that adjusted as researchers discovered better breeding options (Vasal, 1999; Prasanna et al., 2001). Multiple *opaque-2* conversion programs (or the introgression of *opaque-2* into wild-type backgrounds) noted kernel vitreousness variation in *opaque-2* germplasm; however, these kernels were predominantly shrugged off and discarded as somehow wild-type or anomalous (Vasal, 1999). The notion of *opaque-2* 'modifier genes' able to partially restore endosperm vitreousness was first

introduced in a 1969 paper from the University of Missouri, and the segregation of kernels via vitreousness commenced as a premier selection technique for restoring kernel hardness (Paez et al., 1969; Vasal, 1999; Figure 10). In 1974, CIMMYT revised their breeding program strategy to begin targeting the introgression of unknown genetic modifiers through phenotypic selection of vitreousness (Vasal et al., 1982).

The program's overarching strategy for the production of QPM cultivars was two-fold: first, developing QPM versions through conversion of 'normal', or wild-type endosperm, maize lines into *opaque-2* carrying lines with vitreous endosperm and second, developing hard endosperm QPM gene pools. The first aim of producing QPM entailed a backcross-recurrent breeding program involving an *o2* donor to a 'normal' parent with recurrent selection of the BC_1 generation (as previously described). In his summary of CIMMYT's QPM breeding program, S.K. Vasal (a seasoned QPM breeder for CIMMYT) denoted that sometimes four or more self-pollinated recurrent selection generations were required to foster suitable vitreousness before an additional backcross to the recurrent parent could be made. This type of breeding facilitated the use of improved, or more vitreous, recurrent parents in each backcross and noticeable progress after each crossing. Modification and protein quality were tested after each backcross to ensure maintenance. This novel selection scheme became known as 'modified backcross cum recurrent selection' and was very successful in producing some of the first CIMMYT QPM breeding lines (Vasal et al., 1980; Vasal, 1999; Babu and Prasanna, 2013).

Sixteen years after Paez. et al. suggested the concept *o2* modifier genes and more than two decades after CIMMYT began breeding with *o2* germplasm, the QPM hybrid initiative at CIMMYT finally commenced in 1985 (Vasal, 1999). Logically for any

breeding program, CIMMYT prioritized the crossing of their QPM stocks to test and select optimal gene pools for hybrids with quality protein and agronomics, and testing was robust. More than 20 gene pool hybrid combinations were tested in 2-15 locations by 1993, and by 2003, more than 18 developing countries were beginning realistic trials for cultivating QPM hybrids (Vasal, 1986; Beck et al., 1990; Beck et al., 1991; Vasal et al., 1991; Vasal et al., 1992a and 1992b; Bjarnason and Vasal, 1992; Pixley and Bjarnason, 1993; Vasal, 1999; CIMMYT, 2000; Babu and Prasanna, 2013).

2.4.2 Genetic Action and Protein Profile of *Opaque-2*

The *opaque-2* mutation was rigorously introgressed into breeding programs after its potential for maize endosperm biofortification was realized in 1964. Though Mertz, Bates, and Nelson identified *opaque-2* as the sole cause for zein reduction when the mutant was first described, its ability to cause the resultant proteomic rebalancing remained undetermined until 1990 (Mertz et al., 1964). No doubt in part due to advances in genetic technologies, such as cDNA cloning and sequencing, southern blots, and fusion protein production, Schmidt et al. first hypothesized *opaque-2*'s function as a regulatory protein directly interacting with zein transcription in 1990, and he would later prove himself correct in 1992 (Schmidt et al., 1990; Schmidt et al., 1992). The 1992 paper clearly identified *opaque-2* as a leucine zipper transcriptional activator for, specifically, the 22-kD α-zein genes.

Concurrent with Schmidt's studies, researchers at Purdue University and the University of Arizona were actively pursuing a different approach for observing *o2*'s proteomic consequences. Specifically, Wallace and Larkins were interested in the differences between the *opaque-2* unmodified and QPM modified endosperm proteome, but

differentiating between these similar protein compositions proved difficult with previously published zein extraction methods. Not that these procedures were in low abundance; the name of the 'zein' protein and description as an alcohol-soluble protein originated in 1821, and a patent for a specific zein extraction protocol was allotted before the 20th century (Gorham, 1821; Lawton, 2002). In fact, before inquiring into the *opaque-2* mutant, Edwin Mertz himself published an updated procedure on the extraction of protein classes (Mertz and Bressani, 1957). Despite numerous options, Wallace and Larkins were unsatisfied with the proteins' resolution on a sodium dodecyl sulfate polyacrylamide-based gel (SDS-PAG-Electrophoresis) (Wallace et al., 1990). Procedures inspecting protein purity and abundance were evolving concurrently with extraction protocols as well as zein nomenclature and classes. SDS-PAGE was first introduced in 1970 as a possible means to differentiate proteins post-extraction by molecular weight, after which the processes to both extract and run zeins on a gel were further scrutinized (Reynolds and Tanford, 1970; Fling and Gregerson, 1986; Wallace et al., 1990). Some may consider Wallace's 1990 paper as the final benchmark for zein nomenclature, extraction, and SDS-PAGE running procedures. In the paper, zeins were described (according to a 1986 piece) based on their structural relationships within protein bodies and molecular weight (Esen, 1986; Wallace et al., 1990). The novel extraction procedure involved a complete solubilization of proteins using sodium borate and 2-mercaptoethanol, and a 70% ethanol extraction of soluble zeins from insoluble non-zeins (Wallace et al., 1990). The SDS-PAGE resolution procedure used by Wallace was according to a previously published piece with trivial differences (Fling and Gregerson, 1986; Wallace et al., 1990).

Unintentionally, the extraction, description, and resolution of zein proteins by Wallace in 1990 complemented and further validated Schmidt's work at UC-San Diego in determining *opaque-2's* function as primarily affecting the 22-kD α-zein (though the paper was not referenced in Schmidt's 1992 piece). Comparing QPM, *opaque-2* unmodified, and normal maize endosperm zein protein profiles, Wallace identified significant reductions in the 22-kD α-zein in QPM and *o2* unmodified profiles compared to normal maize. Moreover, the group observed an overproduction of the 27-kD γ-zein and foreshadowed the eminence of 27-kD γ-zein abundance in endosperm restoration in QPM germplasm (Wallace, 1990) (Figure 11).

Though it is the gene's highlighted role, it would be an overgeneralization to attribute sole action of *Opaque-2* as a 22kD α-zein activator. Like many other transcription factors, *Opaque-2* activates multiple genes involved in processes ranging from protein structuring to central metabolism; diverse effects that are synergistically coordinated to promote protected endosperm production during maize's critical time of seed development. Concurrent to Schmidt's work in California identifying *O2* as a transcription factor for 22kD α-zein, researchers in Italy were conducting almost identical work with *O2* and its effect on the 'b-32' gene, a 32-kD albumin (a water-soluble, globular type protein) (Lohmer, 1991). Lohmer et al. postulated that *O2* was the transcriptional activator for the b-32 gene and this theory was widely accepted despite Schmidt's skepticism detailed in his 1992 paper (Lohmer et al., 1991; Schmidt et al., 1992). Taken together, these two papers trailblazed the way for other research groups to further investigate *O2* and its transcriptional targets. Since 1991, Lohmer and other research groups added to the better characterization of *Opaque-2* and its genetic action

(Figure 12). In 1994, the Italian cohort identified *O2*'s activation of cytosolic pyruvate orthophosphate dikinase-1 (or cyPPDK), promoting the last step of glycolysis (Maddaloni et al., 1996). In 2003, research in Brazil identified *Opaque-2*'s action in lysine degradation and aspartate conversion pathway (Azevedo et al., 2003). Though the running model for *O2*'s regulatory network has been proposed and generally accepted, there is still much to discover concerning *O2's* influence on the maize endosperm. Transcriptomic profiling of *opaque-2* in 2011 using microarray identified 113 upregulated and 649 downregulated expressed sequence tags (ESTs; short cDNA sequence) with respect to the wildtype (Hartings et al., 2011).

Though complete elucidation of *Opaque-2*'s function is yet to come, the primary genes regulated by *O2* involve promoting *b-32* and zein synthesis, downregulating starch synthesis, and activating lysine and aspartate catabolism (Prioul et al., 2008) (Figure 12). When in its wild-type state, all of these factors aid in promoting endosperm formation and protein body production under the protection of *b-32* albumin's role of biotic resistance (Damerval and Guilloux, 1998; Prioul et al., 2008; Lanzanova et al., 2010; Hartings et al., 2011).

2.4.3 *opaque-2* Amino Acid and Endosperm Modifier Genes

Reflection on *O2's* immense regulatory role in endosperm formation rationalizes the overarchingly debilitated *o2* mutant phenotype of low yielding, soft and starchy, pest and rot susceptible lines. However, it also adds emphasis to the accomplishment of CIMMYT's breeding program in successfully alleviating most of these negative pleiotropic effects by breeding in unknown modifiers primarily based on phenotypic response. During recurrent selection, CIMMYT breeders prioritized light box screening

and tryptophan amino acid analysis for introgression of the *opaque*-2 gene and amino acid and endosperm modifiers (Figure 10). The most cost-effective and successful selection involved individually sorting kernels of multiple successive generations (F_1 to F_4) into Type 1 through Type 5 levels of vitreousness. These levels were determined based on the fraction of vitreousness observed in the kernel endosperm over a light box. Near complete vitreousness was termed 'Level 1', while complete opacity was labeled 'Level 5'. During preliminary stages of self-pollination and risk of unintentionally keeping the *Opaque-2* dominant allele from recurrent stock was at its highest, CIMMYT's protocol required the selection and continuation of 'Type 3' kernels. After a couple of self-pollinated generations, breeders would continue with 'Type 2' kernels. In CIMMYT's 'Breeding Quality Protein Maize' protocol booklet printed in 2008, the selection of 'Type 1' kernels is prohibited unless accompanied by tryptophan analysis due to the risk of selecting the dominant allele (Vivek et al., 2008).

Additionally, selecting for desired proteomic rebalancing of future QPM stock was just as important as selecting for endosperm modification. CIMMYT realized early in their QPM breeding that the homozygous introgression of the *opaque-2* allele did not necessitate an increase in lysine and tryptophan levels in the endosperm. Though the average lysine level in normal maize is approximately 2.0% of total protein in whole grain flour and 4.0% in *opaque-2* stock, these levels range from 1.5-2.8% in wild-type backgrounds and 2.6-5.0% after *o2* introgression (Moro et al., 1996). The small overlap of confidence intervals is made manifest in breeding programs that do not select for amino acid modifiers in every generation. As a result, lysine and tryptophan levels are in some degree lower than the original *opaque-2* line, though higher than the recurrent,

wild-type line (Vivek et al., 2008). To produce the most optimal lines with cost-efficiency in mind, CIMMYT analyzed the tryptophan relative content in every breeding generation as an indicator for lysine and tryptophan levels, since these two amino acids correlate at approximately a 4:1 ratio, respectively (Vivek et al., 2008).

The utilization of these two tools, tryptophan analysis and light box screening, was very successful in producing high quality protein, vitreous QPM stock. Conversely, genotypic selection for *opaque-2*, or specifically the use of marker-assisted selection (MAS), was not reported in literature until 2002 when CIMMYT breeders published a cost-benefit analysis concerning the utilization of in-gene markers for selecting the recessive allele (Dreher et al., 2003). Though the team cautiously described CIMMYT's benefit in selecting for *opaque-2* using MAS in preliminary stages of their breeding program, they warned that its cost may not outweigh the variable effectivity in other programs. Only three in-gene short sequence repeat (SSR) polymorphic markers have been discovered to help identify the *O/opaque-2* allele(s), and polymerase chain reaction products were found to be diverse depending on the populations tested. Up to 10 different *opaque-2* alleles were proposed in CIMMYT's protocols introducing MAS. These protocols cautioned programs to first identify consistent, differentiable bands marking the inbred parents and test the marker's co-dominance (relatively equal amplification of both alleles) before any large-scale implementation. CIMMYT further contended that for MAS to be truly effective, markers for *opaque-2,* amino acid modifiers, and endosperm restoration modifiers needed to be identified (Krivanek and Vivek, 2006; Vivek et al., 2008).

CIMMYT's overarching caution didn't convince some researchers completely out of genetic selection for QPM breeding. Researchers in India described a rapid breeding program converting normal maize into QPM using foreground and background selection of *opaque-2* flanking and in-gene markers (Babu et al., 2005) (Figure 13). This study utilized in-gene marker umc1066, a co-dominant SSR marker, for its selection. In addition to MAS, this breeding program tested three normal maize lines and two QPM donors in all combinations and employed agronomic trait and amino acid analyses to ultimately convert one maize line into a BC_2F_4 QPM (Babu et al., 2005). Though this study may be considered another model for the assimilation of genotypic and phenotypic selection for *opaque-2* introgression, light box screening and amino acid analyses were continually performed throughout the breeding program to ensure success (Babu et al., 2005).

As the 2005 QPM conversion study insinuates, phenotypic techniques for selecting both vitreousness and high lysine and tryptophan endosperm content are still predominantly utilized for current QPM conversions, though a few modifier genes have been identified and/or suggested. In the early 1990s researchers conceptualized that the endosperm modifiers were somehow involved with γ-zein storage protein synthesis (Lopes and Larkins, 1991). A study in 1995 utilizing segregating F_2 populations and restriction fragment length polymorphisms (RFLPs) identified two endosperm modifier loci on Chromosome 7. One modifier locus was mapped near the end of the long arm, while the second was mapped to the γ-zein storage protein (Lopes et al., 1995). This discovery, in which the authors suggested that the 27-kD γ-zein gene and relative protein content were doubled, would be verified by next generation sequencing almost two decades later

(Lopes et al., 1995; Liu et al., 2016). Studies after 1995 utilized different QPMs from diverse gene pools to identify *Opm(s)*, or *o2* modifiers, on Chromosomes 1, 7, and 9 and further supported the probability of a 27-kD γ-zein gene duplication (Holding et al., 2008). This study also originally suggested endosperm modification (or lack of) was related to programmed cell death (Holding et al., 2008) (Figure 14). Other studies indicated that quantitative trait loci (QTLs) for endosperm vitreousness/texture were observed on Chromosomes 3, 5, 6, and 8, while amino acid modifiers were on Chromosomes 7 and 8 (Gutiérrez-Rojas et al., 2010). However, further concurrent studies showed that γ-zeins were essential in providing vitreous structure to the maize endosperm and suggested validation for the former QTL study involving endosperm vitreousness (Holding et al., 2008; Wu et al., 2010). Additionally, a 2015 analysis revealed a more complex, minor QTL-involving structure for QPM's amino acid modifiers in comparison to QPM's endosperm modifiers, agreeing with previous work by Holding et al. identifying three QTLs associated with *o2* endosperm modification (Holding et al., 2011; Pandey et al., 2015). These endosperm QTLs were found to be associated with the ethylene response pathway and promotion of the glycolytic pathway – a particularly interesting find as previous researchers identified *Opaque-2*'s role in stimulating glycolysis (Maddaloni et al., 1996; Holding et al., 2011). Work surrounding the identification of amino acid modifiers compared to endosperm modifiers is limited; though a 2014 article suggested five significant QTLs for tryptophan content on Chromosomes 5, 7, and 9 though utilization of these QTLs in breeding programs is difficult to find (Babu et al., 2014).

Predominantly, the culmination of work surrounding the identification of endosperm and amino acid modifiers features the validation of the 27-kD γ-zein genetic duplication in direct correlation with QPM endosperm modification (Liu et al, 2016) (Figures 15 and 16). Moreover, this same lab identified a triplication of this gene in a 2019 study on high frequency DNA rearrangements (Liu et al., 2019). Though some studies may be found that utilize a plethora of genetic markers available for QPM conversion, current breeding strategies arguably trend toward utilizing phenotypic measurements of amino acid profiles and endosperm vitreousness while integrating zein analysis (whether by genotyping the 27-kD γ-zein duplication or through SDS-PAGE) and MAS for the *opaque-2* allele utilizing in-gene and/or flanking markers (Babu et al., 2005; Dev et al., 2018; Hossain et al., 2018; Ren et al., 2018; Parsons et al., 2020).

2.5 Utilization of Selection Indices in Breeding

The 2020 Selection Index described in Parsons et al. was produced as a novel selection equation best fit for ranking a large number of popcorn hybrids by multiple, variable, quantitative traits (Parsons et al., 2020). The concept of a selection index is not new; in fact, researchers at the University of Maine introduced the utilization of an index by analyzing sweet corn in 1909 (Pearl and Surface, 1909). These researchers outlined four 'requirements' that they determined needed to be upheld in a proper selection index: first, the index should be simple and easily calculated. Second, the index value should increase as the desirability of the 'individual' as determined by the breeder increases. Third, the variables (or traits) in the index should be weighted, and forth, the index values should decrease as the desirability of the individual decreases (Pearl and Surface, 1909). With current selection indices ranging from mixed models including REsidual or

REstricted Maximum Likelihood (REML) and/or Best Linear Unbiased Prediction (BLUP) models to calculate genetic effects while adjusting for mating design, genotype by environmental interactions and genetic effects, to inversions of the numerator relationship matrix and/or transformation of the BLUP equations by pre- and post-multiplication of the relationship matrix, it sometimes seems as if the first qualification stated by Pearl and Surface was either ignored or read by a modern-day Archimedes (Henderson, 1976; de Resende, 2016). Indeed, by 1936 breeders considering index selection introduced concerns involving the selection of non-heritable variations such as environmental factors and variances associated with both genetic and environmental parts, thus invoking the need for matrix algebra (Smith, 1936). In his same critique, Smith concurrently introduced the first index for simultaneous selection of several traits (Smith, 1936). Not long after, Lanoy Hazel, an animal science graduate student at Iowa State College, completed his dissertation describing the efficiency, principles, and requirements of a selection index (Hazel, 1941). He formed specific indices for pig and cattle breeding involving specific traits and appropriate weighting values. Hazel very clearly described the essentiality in identifying factors that affect rate of genetic change such as trait correlations and dependencies, aggregate genotypic variability of the population to be bred, difficulty in measurement accuracy, and unconscious prejudice (Hazel, 1941). He further developed his index practices and published a refined protocol for constructing selection indices in 1943 (Hazel, 1943). Throughout this time, selection indices produced were only applicable to the population of interest. Hazel and Terrill constructed a selection index for sheep selection at weaning age and different indices for poultry were constructed in 1946 and 1947 (Hazel and Terrill, 1946; Panse, 1946; Lerner

et al., 1947). These two poultry indices described different traits and different economic weightings, but both groups emphasized the need for prior knowledge of economically important traits and utilization of economic weighting within the selection index. James Legates, another animal breeding Ph.D. candidate from Iowa State College, described his crafted dairy cow selection index in 1949 and emphasized the need for repeatability estimates of data, correlations between half-sisters both maternally and paternally, and the heritability of the trait of interest (Legates, 1949).

Plant breeders realized the efficiency of such selective breeding during Smith's introduction to the concept in 1936 in which he provided an example with maize (Smith, 1936; Hutchinson, 1939). In 1951, Robinson and Comstock discussed the production of a selection index by apportioning genetic variance into additive, non-additive, and environmental effects, and identifying phenotypic trait correlations to better select premium lines (Robinson and Comstock, 1951). The calculated coefficients implemented in the Robinson and Comstock selection index due to trait covariances were related to heritability, an estimate that Smith argued for the use of throughout his writing (Smith, 1936). The next few decades after Robinson and Comstock would render multiple personalized yet generally similar versions of selection indices for animal and plant breeding. Williams argued in his 1962 critique that breeders maintained an overemphasis on genetic values and underemphasized economic values, after which multiple indices primarily and solely utilizing economic value were proposed (Williams, 1962; Elston, 1963; Henderson, 1963).

In all, a complete historical description of the formulation of selection indices and the subsequent adaptations would fill tedious pages with stories already written (Baker,

2020). In his book 'Selection Indices in Plant Breeding', Baker summarizes *"the optimum selection index for improving a specified linear function of genotypic values is a linear function of phenotypic values in which the weights attached to each phenotypic value are chosen to maximize the correlation between genotypic worth and the selection index… other modifications to the selection index methodology include the use of a base index, where relative economic values are used for index coefficients, and a weight-free index based solely on observed phenotypic values,"* (Baker, 2020, pg. 7). In short, selection indices have been tailored to include or exclude genetic parameters and economic weighting of evaluated traits based on the tested population and discretion of the breeder. Alike to previous breeders' judgements, the 2020 Selection Index is a tailor-made equation specifically designed for the ranking and assessment of a Quality Protein Popcorn hybrid population utilizing genetic repeatability and economic weighting estimates. However, unlike previous indices, this equation is readily transferable to other crop and animal breeding systems and fulfills the first of Pearl and Surface's four requirements of a Selection Index – simplicity.

2.5.1 The 2020 Selection Index

Selection indices are more commonly used for recurrent selection of inbred lines than final hybrid selection (Hallauer and Eberhart, 1970; Johnson et al., 1988; Tardin et al., 2007; Marinho et al., 2014). Thus, the utilization of a heritability estimate in most reported selection equations is more appropriate and common than genetic repeatability. Though these two terms are often interchanged, for this purpose the definition of heritability may be described as the proportion of genetic variation transferable to the next generation rather than the amount of genetic variation in a population's phenotypic

trait, the proportion known as ‘additive variance’ (Robinson et al., 1907). Genetic repeatability may be self-defined as the proportion of total variance calculated through multiple measurements of a trait due to differences among the tested individuals. This measure therefore becomes more useful when evaluating for consistency rather than heritability (Dohm, 2002). Separating these terms enables the distinction between selection indices utilized for either recurrent selection programs or hybrid selection in that heritability estimates are more helpful for inbreeding programs and genetic repeatability estimates are better utilized in hybrid selection indices. Since quantitative trait QPP analysis began at the hybrid stage, the selection index formulated needed to have a genetic repeatability measure. Additionally, as many selection indices previously prescribed, an economic weighting value appended to each evaluated trait would be necessary to select the most optimal hybrids fit for commercialization (Williams, 1962; Mulamba and Mock, 1978). Dynamic consumer and producer preferences dictate trait weighting; thus, these coefficients are essentially subjectively chosen by experts in the field and may fluctuate. Though the most optimal value may not necessarily be utilized, some weighting (albeit imperfect) still provides a reflection of trait ranking in economic importance toward industry and consumer choice. Previous studies comparing multiple selection indices in various crops have indicated that the Mulamba and Mock index, first described in 1978, is most efficient and successful in anticipated selection (Neves et al., 2011; Rosado et al., 2012; Vivas et al., 2012; Almeida et al., 2014; Entringer et al., 2016; Azeredo et al., 2017; Crevelavi et al., 2017; Vieira et al., 2017; Crevelavi et al., 2018; Leite et al., 2018; Crevelavi et al., 2019).

The Mulamba and Mock index is relatively simple in its computation. Considered the 'sum of ranks', each individual (or groups to be tested, i.e. hybrid) is given an integer ranking per trait starting with '1' and ending at the total number of genotypes tested. The final value for the specific genotype or individual is the sum of the rankings per trait (Vieira et al., 2017). Economic weights and/or heritability estimates may accompany these trait values before summing all traits for one final value, as shown by the equation (Mulamba and Mock, 1978; Vieira et al., 2017):

$$I = \sum_{j=1}^{n} p_j r_j \tag{1}$$

In the equation above, I is the final ranking value of an individual, p is the economic weight given by the breeder on the j^{th} trait, and r is the rank of the individual in relation to the j^{th} trait. Other derivations of the Mulamba and Mock have included heritability estimates within the products to be summed for the final individual value (Azeredo et al., 2017). The 2020 Selection Index adapted this equation to allow for a continuous ranking of individuals per trait with a genetic repeatability estimate, as shown below:

$$X_h = \sqrt{\sum_{i=1}^{m} \left(\frac{y_{i,h}}{y_{i,max}} - 1\right)^2 I_i \, (\sigma_{i,h}/\sigma_{i,max})} \tag{2}$$

The product of $\left(\frac{y_{i,h}}{y_{i,max}} - 1\right)^2$ serves as the replacement to the integer ranking identified in the Mulamba and Mock equation if $y_{i,h}$ is the i^{th} trait value of hybrid 'h' and $y_{i,max}$ is the maximum average trait value across the tested population. This comparison in itself serves as a relative ranking of trait value. Furthermore, after this quotient is subtracted from 1, the remainder is squared to represent an exponential distance away from the

maximum trait value, thus amplifying the distance between the best individual and the rest of the population while under the square root. This term is then multiplied with the economic weighting of trait *'i'* (I_i) and the relative value of the individuals' trait variation, as calculated by the term: $\sigma_{i,h}/\sigma_{i,max}$ where $\sigma_{i,h}$ is the standard deviation of hybrid '*h'* trait '*i',* and $\sigma_{i,max}$ is the maximum standard deviation value of trait '*i'*. Calculating all values relatively allows for equal influence from inherently different traits. For example, 100-grain weight data may vary by an average 10 gram standard deviation, while ear grain weight may vary by as much as 50 grams. Without standardizing standard deviation through dividing the maximum deviation of that particular trait, the final continuous rank of the hybrid (X_h) without unintentionally offer more weight to traits with larger variances. The final ranking is then the square root of the product to expand the range of rankings (without changing rank order) for interpretation and evaluation.

The 2020 Ranking Index was a novel method to select best QPP hybrids from a larger population, and it proved useful in this context. However, this model can be applied to any testable population, plant or animal, with multiple quantitative traits. Standard deviation best reflects genetic repeatability if testing final cultivars or progeny, while heritability values could replace standard deviation in the Ranking Model for inbred line ranking (or sires/dams). The trait of 'cost' can also be implemented into the ranking model (i.e. semen straws) with a decided selection intensity. This model may be adapted into a useable format for researchers and on-the-ground producers alike to independently rank and select a diverse array of products, from maize genotypes to clean-up bulls.

3. Quality Protein Popcorn Hybrid Evaluation and Selection

The first introgression of Quality Protein Maize *opaque-2* alleles and essential amino acid and endosperm modifier genes into multiple popcorn lines while maintain popcorn quality traits was a significant success (Ren et al., 2018). Very rarely have dent by popcorn crosses been reported for agronomic and/or quality improvement though popcorn is known to be agronomically inferior to dent maize. The introgression of QPM into popcorn lines for inbred line production had a two-fold aim in bettering endosperm protein quality and overall popcorn agronomics. A four year backcross-recurrent selection breeding program fostered twelve QPP BC_2F_5 inbred lines with superior protein quality and scope for agronomic improvement (Figure 17). In the summer of 2018 these QPP inbred lines were crossed in a full diallel and 44 QPP hybrids were chosen for analysis (Parsons et al., 2020). Out of these 44 hybrids, the most elite five BC_2F_5 derived hybrids were selected. In the spring of 2020, relative crosses between BC_3F_4 QPP inbred lines were made for a total of ten QPP hybrids fit for final selection in the 2020 summer season. These ten QPP hybrids were compared with five ConAgra® Brands popcorn varieties for major agronomic traits such as yield and test weight, key protein quality improvement (i.e. lysine), popcorn quality traits such as expansion volume and flake type, and sensory traits such as appearance, aroma, taste, and overall likability. The holistic evaluation and selection of these QPP hybrids provided the most robust and thorough comparative analysis feasible and ultimately rendered two top Quality Protein Popcorn hybrids fit for future commercialization.

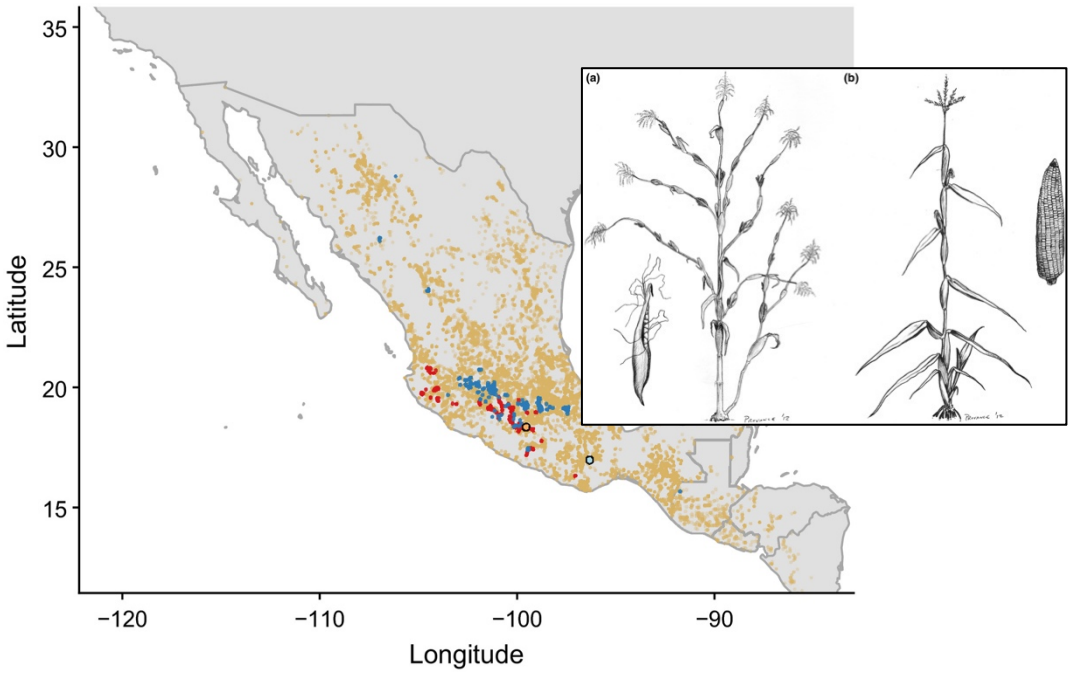


Figure 1. Locations of progenitor species and/or wild relatives of maize. Balsas river valley shown in the orange circle, oldest cobs to date found in the light blue circular area. (a) teosinte, (b) maize (Stitzer and Ross-Ibarra, 2018).

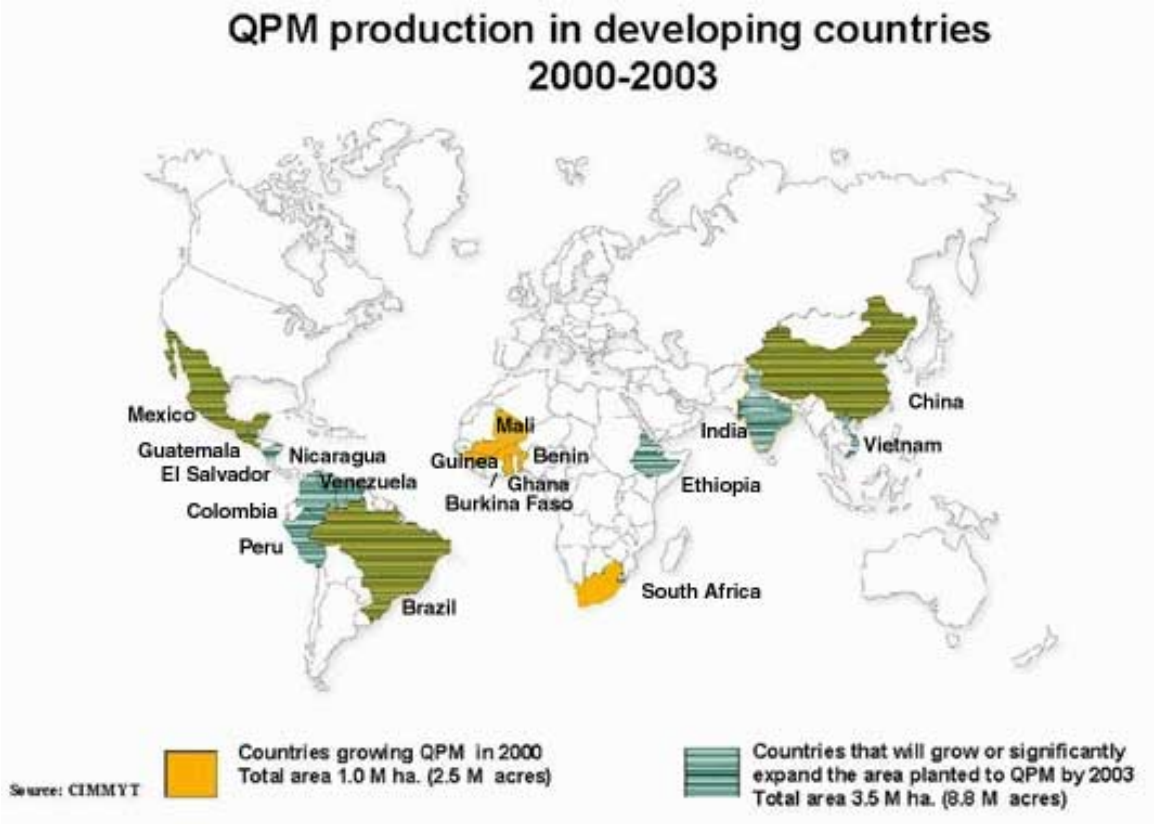


Figure 2. Map of Quality Protein Maize cultivation across the world in 2000 (CIMMYT, 2000).

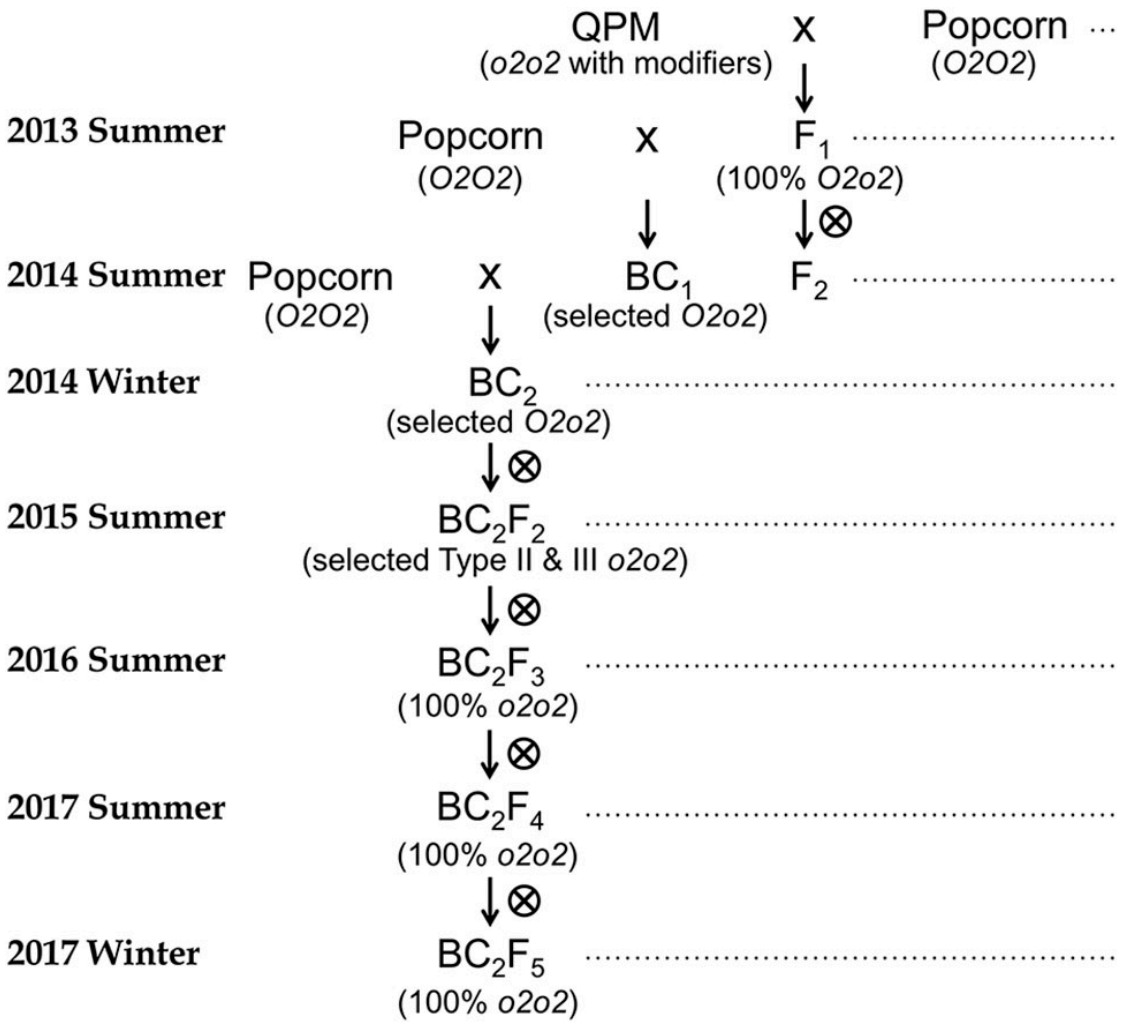


Figure 3. Backcross-recurrent selection breeding scheme for the inbred production of Quality Protein Popcorn (Ren et al., 2018).

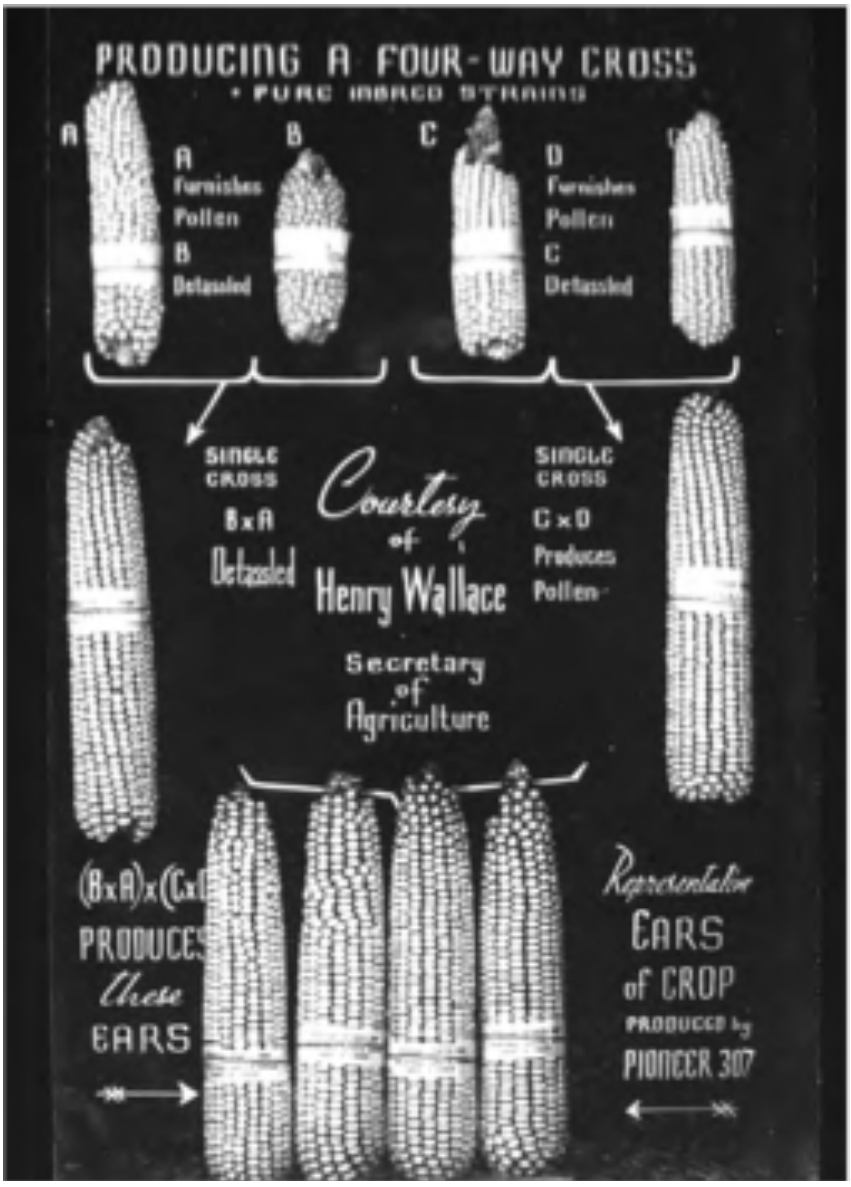


Figure 4. Double-cross hybrid method (Pioneer, 2018).

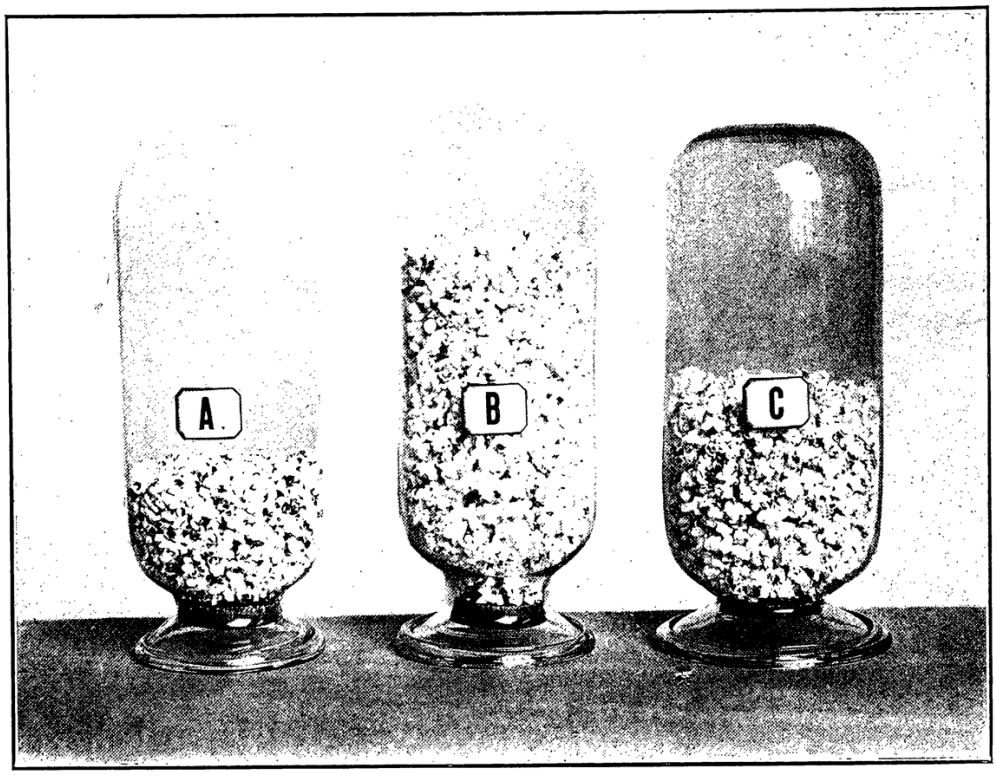


PLATE IV.—EFFECT OF ADDING WATER TO VERY DRY POPCORN.
A. 1,007 cc., from 80 grams of corn; no water added.
B. 3,027 cc., from 80 grams of corn plus 7 cc. water.
C. 2,030 cc., from 80 grams of corn plus 11 cc. water.

Figure 5. The first analysis of popping expansion volume (Stewart, 1923).

Figure 6. Display of popcorn morphologies: upper left, mushroom. Upper right, unilateral. Lower left, multilateral, lower right, bilateral (Sweley et al., 2013).

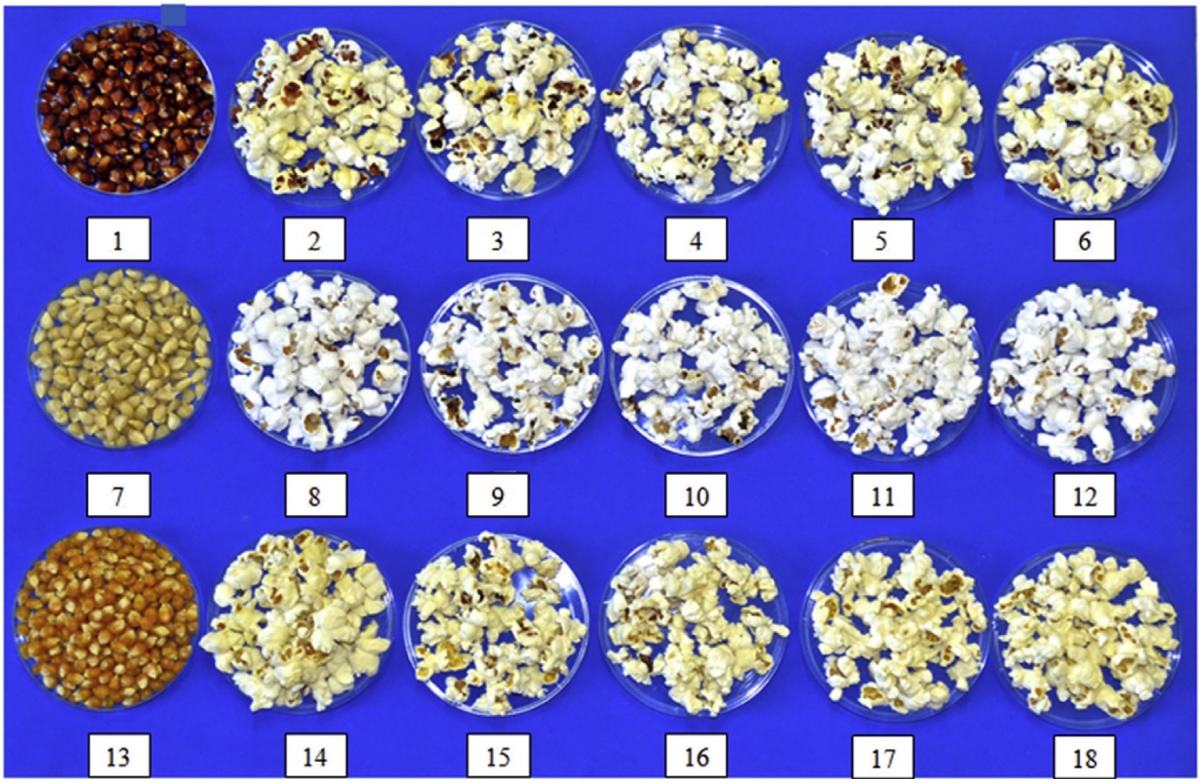


Figure 7. Red, white, and yellow popcorn types. Labels 2-6, 8-12, and 14-18 represent different popping methods on red, white, and yellow popcorn respectively (Paraginski et al., 2016).

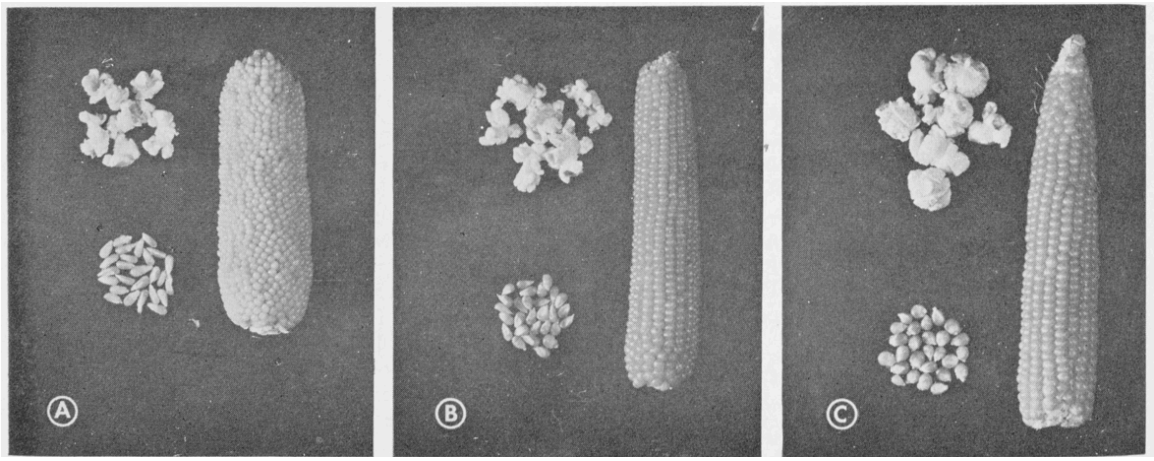


Figure 8. Different types of popcorn varieties. (a) Japanese Hulless (rice kernel morphology), (b) Yellow Pearl, (c) South American Hybrid (pearl kernel morphology) (Eldredge and Thomas, 1959).

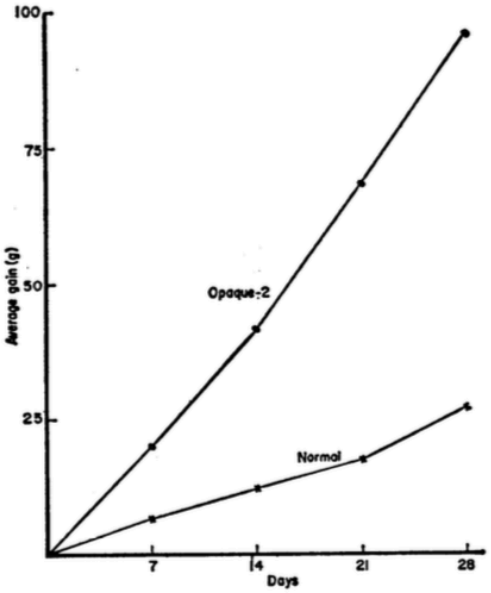


Table 1. Amino acids in *opaque-2* and normal defatted corn (expressed as grams per 100 g of protein). Values for tryptophan were not available.

Amino acid	*Opaque-2*	Normal corn
Lysine	4.7	2.8
Histidine	3.0	3.0
Ammonia	2.7	3.4
Arginine	6.5	4.8
Aspartic acid	10.6	6.7
Glutamic acid	17.9	20.8
Threonine	3.9	3.6
Serine	4.9	4.8
Proline	8.1	10.0
Glycine	4.8	3.8
Alanine	6.9	7.9
Valine	5.5	5.0
Cystine	1.4	1.2
Methionine	1.9	2.0
Isoleucine	3.8	4.0
Leucine	9.8	13.9
Tyrosine	3.6	4.0
Phenylalanine	4.8	5.2

Figure 9. Growth of rats on an *opaque-2* or normal maize-based diet (Mertz et al., 1965)

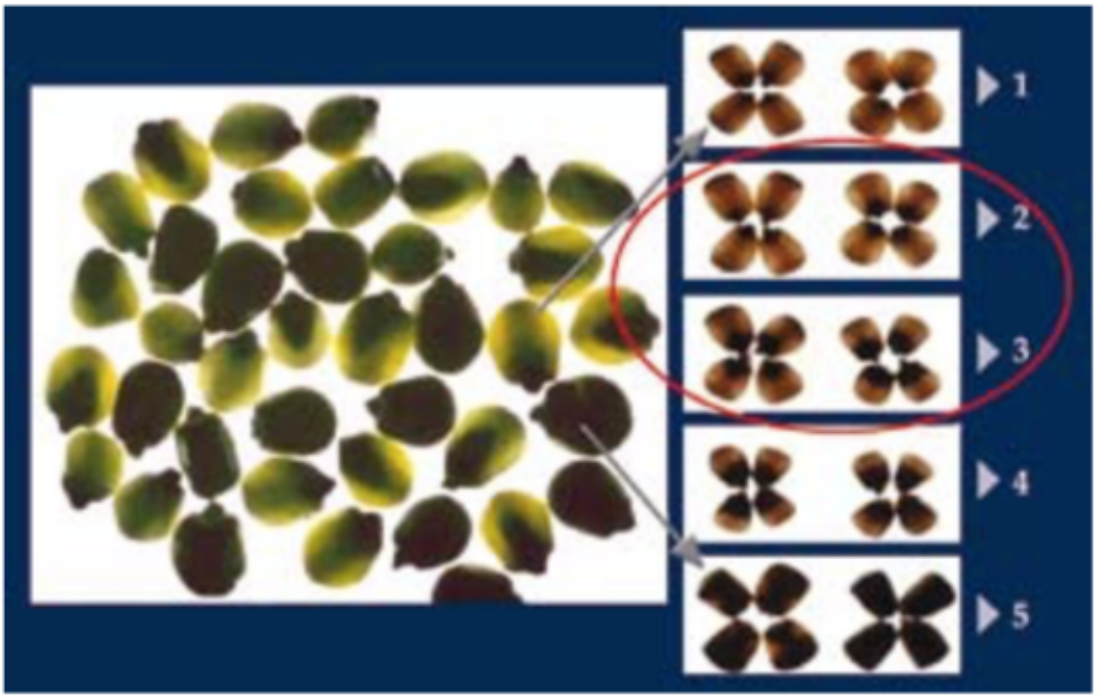


Figure 10. Selective scale of endosperm vitreousness in CIMMYT's Quality Protein Maize breeding booklet (Vivek et al., 2008).

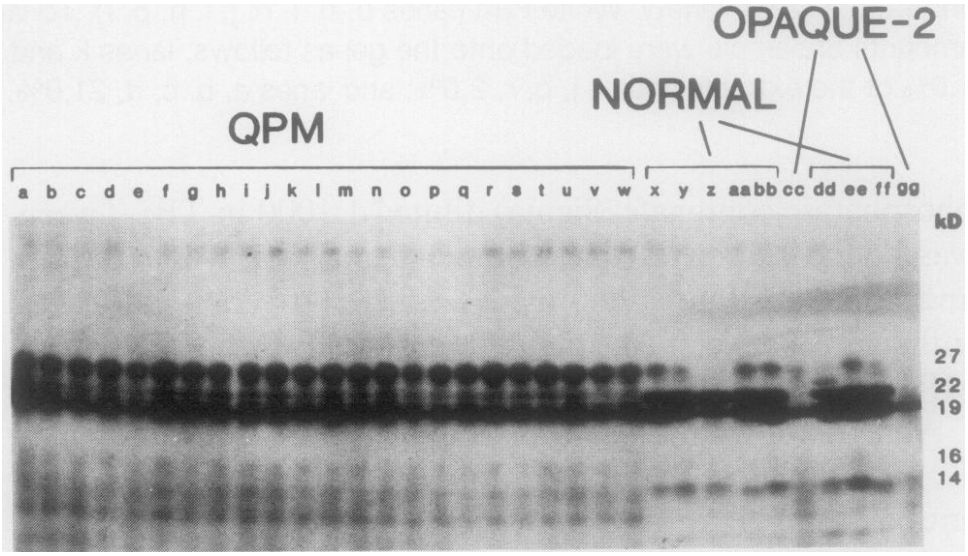


Figure 11. SDS-PAGE of QPM, wild-type, and *opaque-2* cultivar zeins using novel Wallace protocol (Wallace et al., 1990).

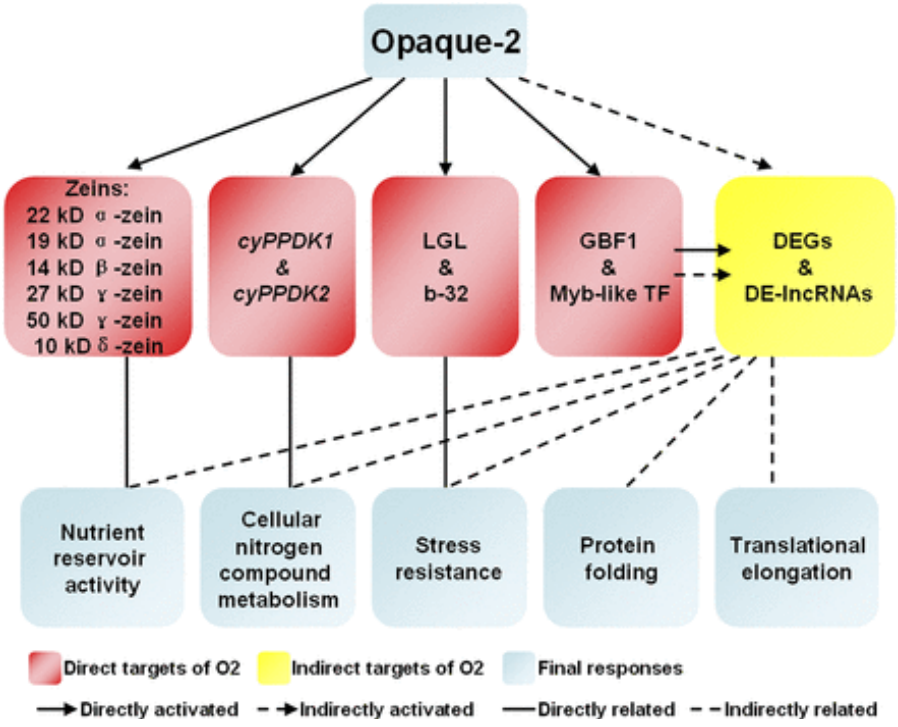


Figure 12. Transcriptional regulation of *Opaque-2* (Mach, 2015).

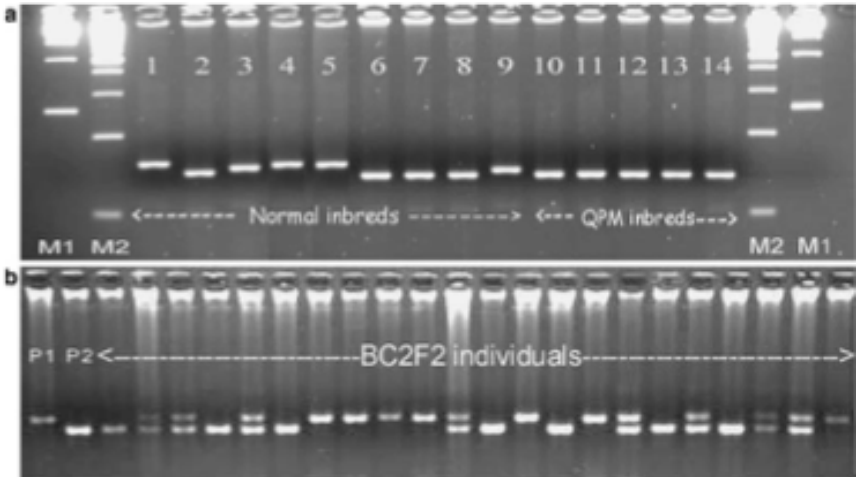


Figure 13. Utilization of *umc1066* as a foreground selectable marker for QPM conversion (a) analysis of *umc1066* amplicons in 14 parental lines (b) utilization of *umc1066* in BC_2F_2 to discriminate between wild-type and mutant alleles (Babu et al., 2005).

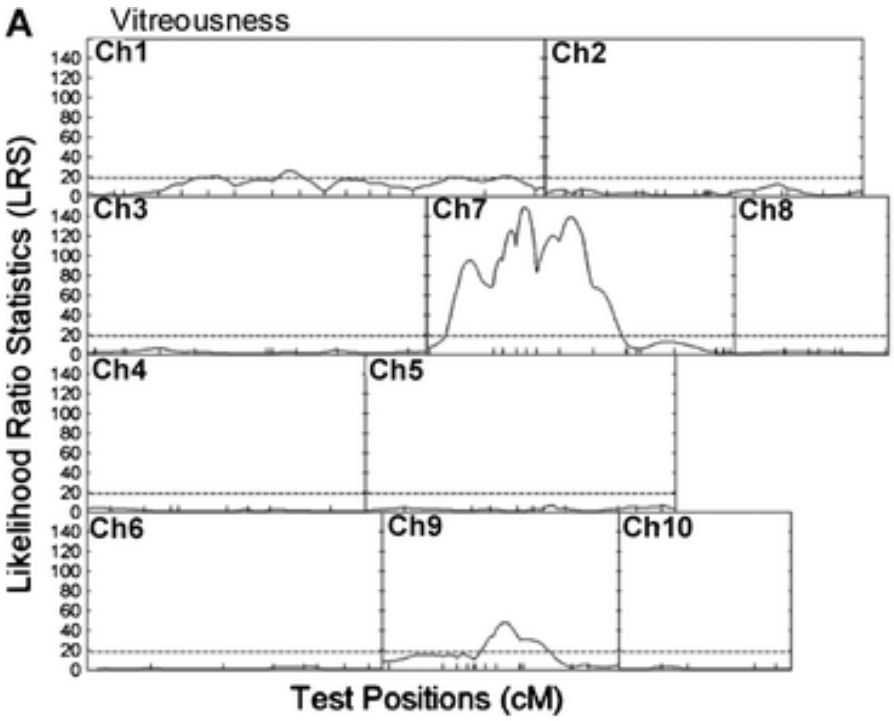


Figure 14. Quantitative Trait Loci for Vitreousness using a QPM by *o2* cross population (Holding et al., 2008).

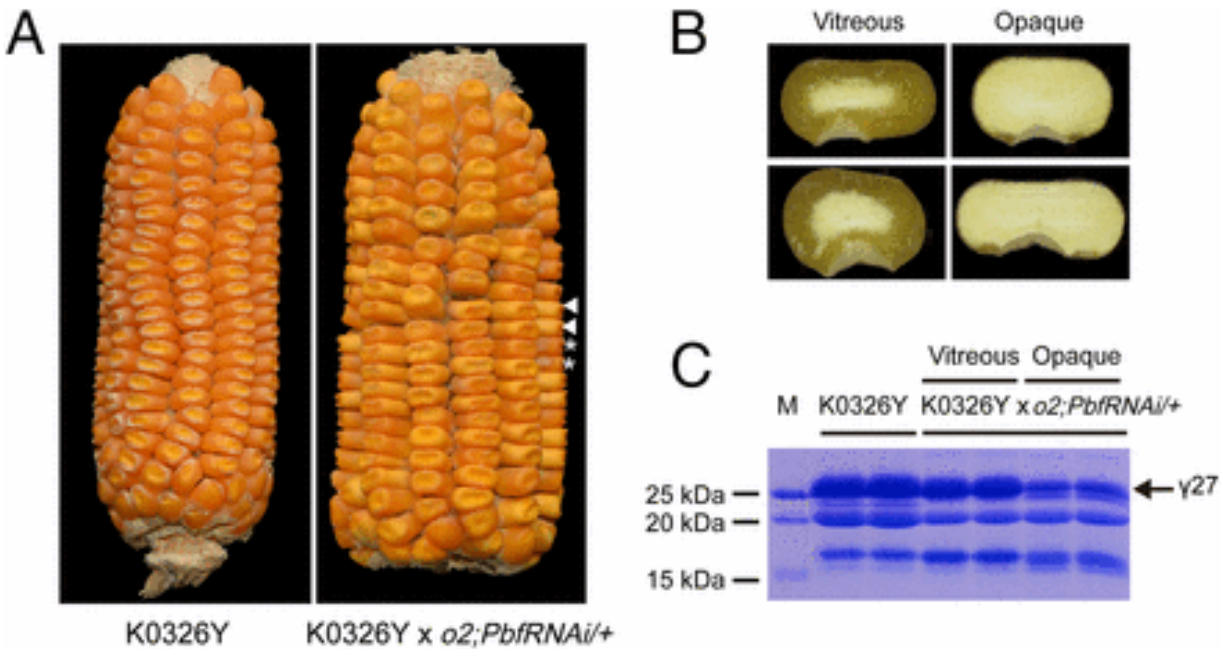


Figure 15. K0326Y (QPM) by *o2* (reduced 27 kD γ-zein through RNAi) progeny show correlation between reduced 27 kD γ-zein and opacity (a) Parental QPM (K0326Y) ear and F_1 progeny when crossed to RNAi *o2* line reducing 27 kD γ-zein (b) cross-section of segregating F_1 opaque and vitreous kernels (c) SDS-PAGE zein gel showing reduction of 27 kD γ-zein in opaque kernels and more prolific 27kD γ-zein production in vitreous kernels (Liu et al., 2016).

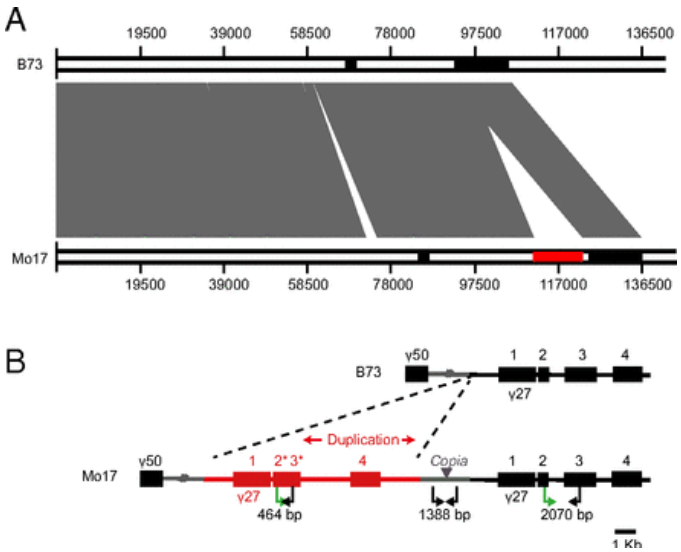


Figure 16. Sequence alignment of B73 (wild-type) and Mo17 (QPM). (a) Orthologous regions on Chromosome 7 mapped between cultivars (b) Red region in (a) expanded; sequenced duplication of the 27kD γ-zein genes 1, 2, 3, and 4 (Liu et al., 2016).

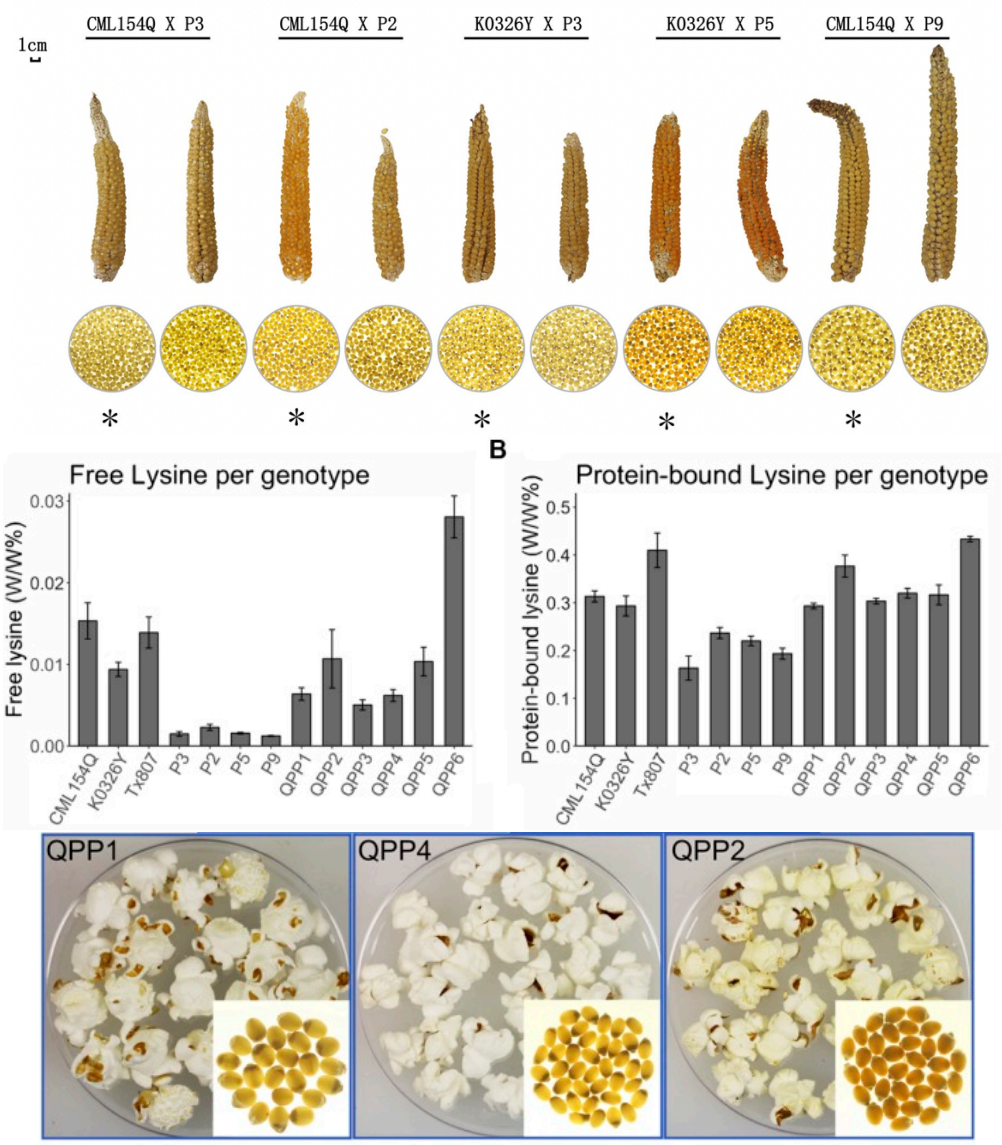


Figure 17. Ten out of twelve Quality Protein Popcorn inbred lines produced in 2017. Inbreds with an asterisk represent protein profiled QPP inbreds. Inbreds showed adequate popping characteristics (Ren et al., 2018).

References

Adams, M.W., and Shank, D.B. (1959). The Relationship of Heterozygosity to Homeostasis in Maize Hybrids. *South Dakota Agricultural Experiment Station.* 418: 777-786.

Almeida, L.M., Viana, A.P., Júnior, A.T.A., Júnior, J.B.C. (2014). Breeding full-sib families of sugar cane using selection index. *Cienc. Rural.* 44:4.

Amann, R.I., Ludwig, W., Schleifer, K. (1995). Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation. *Microbiological Reviews,* 59:1, 143-169.

Anderson, E., Brown, W.L. (1952). Origin of corn belt maize and its genetic significance. In: Gowen J.W. (ed). Heterosis. Ames, Iowa: Iowa State University Press, pg 124-128.

Association for the Advancement of Animal Breeding and Genetics. (2013). A.R. Gilmore. Available at: http://www.aaabg.org/aaabghome/specialmems.php

De Azeredo, A.A.C., Bhering, L.L., Brasileiro, B.P., Cruz, C.D., Silveira, L.C.I., Oliveira, R.A., Bespalhok Filho, J.C., and Daros, E. (2017). Comparison Between Different Selection Indices In Energy Cane Breeding. Genetics And Molecular Research 16:1. doi:10.4238/gmr16019535

Azevedo, R.A., Damerval, C., Landry, J., Lea, P.J., Bellato, C.M., Meinhardt, L.W., Guilloux, M.L., Delhaye, S., Toro, A.A., Gaziola, S.A., Berdego, B.D.A. (2003). Regulation of maize lysine metabolism and endosperm protein synthesis by opaque and floury mutations. *Eur. J. Biochem.* 270, 4898-4908.

Babu, R., Nair S. K., Kumar A., Venkatesh S., Sekhar J. C., Singh N. N. (2005). Two-generation marker-aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). Theor. Appl. Genet. 111, 888–897. doi: 10.1007/s00122-005-0011-6

Babu, R., Prasanna, B.M. (2013). Molecular Breeding for Quality Protein Maize (QPM) *In: Tuberosa R., Graner A., Frison E. (eds) Genomics of Plant Genetic Resources. Springer, Dordrecht.*

Babu, B.K., Agrawal, P.K., Saha, S., Gupta, H.S. (2015). Mapping QTLs for opaque2 modifiers influencing the tryptophan content in quality protein maize using genomic and candidate gene-based SSRs of lysine and tryptophan metabolic pathway. *Plant Cell Rep.* 34, 37-45.

Bailey, L.B. (1814). Memoirs of the Philadelphia Society for Promoting Agriculture. Philadelphia, PA: Johnson and Warner, 147:3, pg. 303-325.

Baker, R.J. (2020). Selection Indices in Plant Breeding. CRC Press: New York, NY.

Beck, D.L., Vasal., S.K., Crossa, J. (1991). Heterosis and Combining Ability among Subtropical and Temperate Intermediate-Maturity Maize Germplasm. *Crop Sci.* 31:1, 68-73.

Beck, D.L., Vasal., S.K., Crossa, J. (1990). Heterosis and Combining Ability of CIMMYT's Tropical Early and Intermediate Maturity Maize (*Zea Mays* L.*)* Germplasm. *Maydica.* 35:1, 279-285.

Bik, E. (2016). The Hoops, Hopes, and Hypes of Human Microbiome Research. *Yale J. Biol Med.* 89:3, 363-373.

Birchler, J.A., Yao, H., Chudalayandi, S., Vaiman, D., and Veitia, R. (2010). Heterosis. *Plant Cell.* 22:7, 2105-2112.

Bjarnason M. and Vasal, S.K (1992). Breeding of Quality Protein Maize (QPM). In: Janik, J. (ed). Plant Breeding Reviews, Lafayette, Indiana: John Wiley & Sons Inc. Chapter 7.

Boler, B.M.V., Serao, M.C.R., Bauer, L.L., Staeger, M.A., Boileau, T.W., Swanson, K.S., Fahey, G.C. (2011). Digestive physiological outcomes related to polydextrose and soluble maize fibre consumption by healthy adult men. *Brit J of Nutrit*. 106:12, 1864-1871.

Brown, W.L., Darrah, L.L. (1985). Origin, Adaptation, and Types of Corn. *National Corn Handbook.* 10.

Brunson, A.M., and Smith, G.M. (1944). Hybrid Popcorn. *Agron. Jour.* 37:3, 176-183.

Burton, J.P., Cadieux, P., Reid, G. (2003). Improved understanding of the bacterial vaginal microbiota of women before and after probiotic instillation. *Appl. Environ. Microbiol.* 69, 97–101.

Buttery, R.G., Ling, L.C., and Stern, D.J. (1997). Studies on Popcorn Aroma and Flavor Volatiles. *J. Agric. Food Chem.* 45, 837-843.

Carr, R.H. and Ripley, E.F. (1920). What puts 'pop' in popcorn? *Proc. Indiana Acad. Sci.* 261-279.

Carter, P.R., Hicks, D.R., Doll, J.D., Schulte, E.E., Schuler, R., Holmes, B. (1989). Popcorn. *Alt. Field Crops Man.* Available at: https://hort.purdue.edu/newcrop/afcm/popcorn.html

Carvalho-Wells., A.L., Helmolz, K., Nodet, C., Molzer, C., Leonard, C., McKevith, B., Theileck, F., Jackson, K.G., Tuohy, K.M. (2010). Determination of the in vivo prebiotic potential of a maize-based whole grain breakfast cereal: a human feeding study. *British Journ. of Nutrition.* 104, 1353-1356.

Ceylan, M. and Karaba, E. (2001). Comparison of sensory properties of popcorn from various types and sizes of kernel. *Jour. of the Sci. of Food and Ag.* 82: 127-133.

Chiuta, N.E., Mutengwa, C.S. (2018). Response of Yellow Quality Protein Maize Inbred Lines to Drought stress at Seedling Stage. *Agronomy* 8, 287.

CIMMYT (2000). N2976. SeedQuest: News Release by Mexico City, Mexico and Washington, D.C.

Costabile, A., Deavile, E.R., Morales, A.M., Gibson, G.R. (2016). Prebiotic Potential of a Maize-Based Soluble Fibre and Impact of Dose on the Human Gut Microbiota. *PLOS ONE.* 11:1.

Costabile, A., Klinder, A., Fava, F., Napolitano, A., Fogliano, V., Leonard, C., Gibson, G.R., Tuohy, K.M. (2008). Whole-grain wheat breakfast cereal has a prebiotic effect on the human gut microbiota: a double-blind, placebo-controlled, crossover study. *Br J Nutr.* 99, 110–120.

Creighton, H.B., McClintock, B. (1931). A correlation of cytological and genetical crossing-over in Zea Mays. *Proceedings of the National Academy of Sciences of USA.* 17:492–497. doi: 10.1073/pnas.17.8.492.

Crabb, R. (1993). The Hybrid Corn-Makers. West Chigaco, Illinois: West Chicago Publishing Company.

Crevelari, J.A., Durães, N.N.L., Pereira, M.G. (2017). Prediction of genetic gains and correlations in corn hybrids for silage. *Austrailian Journ. of Crop Sci.* 11:11, 1411-1417.

Crevelari, J.A., Durães, N.N.L., Bendia, L.C.R., Silva, A.J.S., Azevedo, F.H.V., Azeredo, V.C., Pereira, M.G. (2018). Assessment of agronomic performance and prediction of genetic gains through selection indices in silage corn. *Aust. Journ. of Crop. Sci.* 12:05, 800-807.

Crevelari, J.A., Pereira, M.G., Azevedo, F.H.V., Vieira, R.A.M. (2019). Genetic improvement of silage maize: predicting genetic gain using selection indexes and best linear unbiased prediction. *Rev. Ciênc. Agron.* 50:2

Crow, J.F., and Kermicle, J. (2002). Oliver Nelson and Quality Protein Maize. Genetics. 160: 819-821.

Cromwell, G.L., Pickett, R.A., and Beeson, W.M. (1967). Nutritional Value of *OPAQUE-2* Corn for Swine. Purdue University Agricultural Experiment Station, Journal Paper No. 2996. 1325-1331.

Crumbaker, D. E., Johnson, I., Eldredge, J. (1949). Inheritance of popping volume and associated characters in crosses between popcorn and dent corn. Agron. J. 41, 207–212. doi: 10.2134/agronj1949.00021962004100050009x

Damerval, C., Guilloux, M.L. (1998). Characterization of novel proteins affected by the o2 mutation and expressed during maize endosperm development. *Mol Gen Genet.* 257:354-361.

Darwin, C. The Origin of Species (Penguin Books USA Inc., New York, 1858).

David, L.A., Materna, A.C., Friedman, J., Campos-Baptista, M.I., Blackburn, M.C., Perrota, A., Erdman, S.E., Alm, E.J. (2014). Host lifestyle affects human microbiota on daily timescales. *Genome Biol.* 15:7, R89.

David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E., Ling, A.V., Devlin, A.S., Varma, Y., Fischbach, M.A., Biddinger, S.B., Dutton, R.J., Turnbaugh, P.J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature.* 505:7484, 559–563.

Dawson, M., and Telford, E.P. (1912). The Book of Parties and Pastimes. Philadelphia, PA: David McKay.

Denić, M. (1983). Genetic Basis of Storage Protein Synthesis in Maize *in Seed Proteins: Biochemistry, Genetics, Nutritive Value.* Edited by W. Gottschalk and H.P. Müller. Hingham, MA: Martinus Nijhoff / DR W. Junk Publishers.

Dev, D., Chourasia, K.N., Koujalagi, D. (2018). Quality protein maize: An overview. *Journ. of Pharm. And Phytochem.* 7:2, 3486-3492.

Dofing S.M., Thomas-Compton, M.A., Buck, .J.S. (1990). Genotype × popping method interaction for expansion volume in popcorn. Crop Sci. 30, 62–65.

Dofing, S. M., ĎCroz-Mason N., Thomas-Compton, M. A. (1991). Inheritance of Expansion Volume and Yield in Two Popcorn ⨯ Dent Corn Crosses. Crop Sci. 31,715-718. doi:10.2135/cropsci1991.0011183X003100030035x

Dohm, M.R. (2002). Repeatability estimates do not always set an upper limit to heritability. *Functional Ecology.* 16:2, 273-280.

Dominianni, C., Wu, J., Hayes, R.B., Ahn, J. (2014). Comparison of methods for fecal microbiome biospecimen collection. *BMC Microbiology.* 14:103.

Dreher, K., Khairallah, M., Ribaut, J.M., Morris, M. (2003). Money matters (I): costs of field and laboratory procedures associated with conventional and marker-assisted maize breeding at CIMMYT. *Molec Breeding.* 11, 221-234.

Dudley, J.W., and Moll, R.H. (1969). Interpretation and Use of Estimates of Heritability and Genetic Variances in Plant Breeding. *Crop Sci.* 9:3, 257-262.

Duvik, D. (2001). Biotechnology in the 1930s: the development of hybrid maize. *Nature Reviews Genetics.* Vol 2, 69-74.

Eldredge, J.C., and Lyerly, P.J. (1943). Popcorn in Iowa. *Bulletin P:* Vol 2: Bulletin P54, Article 1.

Eldredge, J.C., and Thomas, W.I. (1959). Popcorn… Its production, processing, and utilization. Bulletin P: Vol 7: Bulletin P127, Article 1.

Elston, R.C. (1963). A weight-free index for the purpose of ranking or selection with respect to several traits at a time. *Biometrics.* 19, 85.

Emerson, R. A., Beadle, G. W., & Fraser, A. C. (1935). A summary of linkage studies in maize. Ithaca, N.Y: Cornell University.

Ensminger, M.E., Ensminger, A.H., Konlande, J.E., Robson, J.R.K. (1993). Foods & Nutrition Encyclopedia, 2nd Edition, Volume 1, pg 483-484. New York, New York: CRC Press.

Entringer, G.C., Vettorazzi, J.C.F., Santos, E.A., Pereira, M.G., Viana, A.P. (2016). Genetic gain estimates and selection of S1 progenies based on selection indices and REML/BLUP in super sweet corn. *Australian Journ. of Crop Sci.* 10:3, 411-417.

Esen, A. (1986) Separation of alcohol-soluble proteins (zeins) from maize into three fractions by differential solubility. *Plant Phys.* 80: 623-627.

Fang, H., Fu, X., Wang, Y., Xu, J., Feng, H., Li, W., Xu, J., Jittham, O., Xuan, Z., Zhang, L., Yang, N., Xu, G., Wang, M, Li, X., Li, J., Yan, J., Yang, X. (2019). Genetic basis of kernel nutritional traits during maize domestication and improvement. *the plant journal* 101:2, 278-292.

Finegold, S.M., Sutter, V.L., Mathisen, G.E. (1983). Normal Indigenous Intestinal Flora *in Human Intestinal Microflora in Health and Disease.* ed. David J. Hentges. New York: New York, Academic Press.

Fling, S.P., Gregerson, D.S. (1986). Peptide and protein molecular weight determination by electrophoresis using a high-molarity tris buffer system without urea. *Analytical Biochem.* 155:1, 83-88.

Franks, A.H., Harmsen, H.J.M., Raangs, G.C., Jansen, G.J., Schut, F., Welling, G.W. (1998). Variations of Bacterial Populations in Human Feces Measured by Fluorescent In Situ

Hybridization with Group-Specific 16S rRNA-Targeted Oligonucleotide Probes. *Appl. Environ Microbiol.* 64:9, 3336-3345.

García-Pinilla, S. Gutiérrez-Lópeza, G.F, Hernández-Sáncheza, H., González-Barbosab J.J. García-Armentac, E., Alamilla-Beltrána, L., Dorantes-Álvareza, L. (2019). 2-Dimensionhot-air popcorn morphology development. *Journal of Food Engineering.* 259: 29-33.

Gibson, G.R., Roberfroid, M.B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.* 125, 1401–1412.

Gill, H.S. (2003). Probiotics to enhance anti-infective defences in the gastrointestinal tract. *Best Pract. Res. Clin. Gastroenterol.* 17, 755–773.

Gipp, W.F., and Cline, T.R. (1972). Nutritional Studies with *Opaque-2* and High Protein *Opaque-2* Corns. *Journal of Animal Science.* 34:6, 963-968.

Gorham, J. (1821). Analysis of Indian corn. *Quart. J. Sci. Lit. Art.* 2, 206-208.

Gomez, A., Petrzelkova, K.J., Burns, M.B., Yeoman, C.J., Amato, K.R., Vlckova, K., Modry, D., Todd, A., Robinson, C.A.J., Remis, M.J., Torralba, M.G., Morton, E., Umaña, J.D., Carbonero, F., Gaskin, R., Nelson, K.E., Wilson, B.A., Stumpf, R.M., White, B.A., Leigh, S.R., Blekhman, R. (2016). Gut Microbiome of Coexisting BaAka Pygmies and Bantu Reflects Gradients of Traditional Subsistence Patterns. *Cell Rep.* 14:9, 2142–2153.

Goudiss, C.H., and Goudiss, A.M. (1918). Foods that will Win the War. New York, NY: World Syndicate Company, The Forecast Publishing Co.

Griffing, B. (1956). Concept of general and specific combining ability in relation to diallel crossing system. *Australian Journal of Biological Science*, 9: 463-493.

Grogan, C.O., Singleton, O.V., and Zuber, M.S. (1958). Popcorn Culture in Missouri. *Univ. of Missouri Ag. Exp. Station.* Bulletin 718.

Gutiérrez-Rojas, A., Betrán, J., Paul Scott, M., Atta, H., Menz, M. (2010). Quantitative Trait Loci for Endosperm Modification and Amino Acid Contents in Quality Protein Maize. *Crop Sci.* 50, 870-879.

Hake, S., & Ross-Ibarra, J. (2015). Genetic, evolutionary and plant breeding insights from the domestication of maize. *eLife*, 4, e05861. https://doi.org/10.7554/eLife.05861

Hallauer, A.R., and Eberhart, S.A. (1970). Reciprocal full-sib selection. Crop Sci. 10, 315-316

Harmsen, H.J.M., Wildeboer-Veloo, A.C.M., Grijpstra, J., Knol, J., Degener, J.E., Welling, G.W. (2000). Development of 16S rRNA-Based Probes for the Coriobacterium Group and the Atopobium Cluster and Their Application for Enumeration of Coriobacteriaceae in Human Feces from Volunteers of Different Age Groups. *Appl Enviro. Microbiol.* 66:10, 4523-4527.

Harmsen, H.J.M., Raangs, G.C., He, T., Degener, J.E., Welling, G.W. (2002). Extensive Set of 16S rRNA-Based Probes for Detection of Bacteria in Human Feces. *Appl. Envioron. Microbiol.* 68:6, 2982-2990.

Hartings, H., Lauri, M., LAzzaroni, N., Pirona, R., Motto, M. (2011). The Zea mays mutants opaque-2 and opaque-7 disclose extensive changes in endosperm metabolism as revealed by protein, amino acid, and transcriptome-wide analyses. *BMC Genomics* 12:41, 1-18.

Haugh, C.G., Lien, R.M., Hanes, R.E. and Ashman, R.B. (1976). Physical properties of popcorn. *Trans. ASAE*, 19:168, 171–176.

Hazel, L.N. (1941). Principles of a Selection Index which Involves Several Characteristics and Utilizes Information Concerning Relatives. [Ph.D. Thesis: Iowa State College].

Hazel, L.N. (1943). The Genetic Basis for Constructing Selection Indexes. *Iowa Agricultural Experiment Station,* Journal Paper No. J-1121.

Hazel, L.N., Terrlll, G.E., (1946), The construction and use of a selection index for range Bambouillet lambs, (Abstract) *Jour. Anim, Scl,* 5:412.

Henderson, C.R. (1963). *Selection index and expected genetic advance, in Statistical Genetics and Plant Breeding, Publ. No 982,* Hanson, W.D. and Robinson, H.F., Eds., National Academy of Sciences, National Research Council, Washington, D.C., 1963, 141.

Henderson, C.R. (1976). A Simple Method for Computing the Inverse of a Numerator Relationship Matrix Used in Prediction of Breeding Values. *International Biometric Society.* 32:1, 69-83.

Holding, D. R., Hunter, B. G., Chung, T., Gibbon, B. C., Ford, C. F., and Bharti, A. K. (2008). Genetic analysis of opaque2 modifier loci in quality protein maize. *Theor. Appl. Genet.* 117, 157–170.

Holding, D. R., Hunter, B. G., Klingler, J. P., Wu, S., Guo, X., and Gibbon, B. C. (2011). Characterization of opaque2 modifier QTLs and candidate genes in recombinant inbred lines derived from the K0326Y quality protein maize inbred. *Theor. Appl. Genet.* 122, 783–794.

Hoseney, R.C., Zeleznak, K., and Abdelrahman, A. (1983). Mechanism of popcorn popping. *Journ. of Cereal Sci.* 1:1, 43-52.

Hossain, F., Muthusamy, V., Pandey, N., Vishwakarma, A.K., Baveja, A., Zunjare, R.U. Thirunavukkarasu, N., Saha, S., Manjaiah, K.M., Prasanna, B.M., Gupta, H.S. (2017). Marker-assisted introgression of *opaque-2* allele for rapid conversion of elite hybrids into quality protein maize. *Journ. of Gen.* 97:1, 287-298.

Hutchinson, J.B. (1939). The Application of Genetics to Plant Breeding. *Seventh International Genetic Congress, Edinburgh, August 1939.*

Johnson, I., and Eldredge, J. (1953). Performance of recovered popcorn inbred lines derived from outcrossed to dent corn. Agron. J. 45, 105-110. doi: 10.2134/agronj1953.00021962004500030005x

Johnson, B.E., Dauer, J.P., Gardner, C.O. (1988). A model for determining weights of traits in simultaneous multitrait selection. Appl. Math. Modelling. Vol 12, August.

Jones, D.J. (1917). Dominance of Linked Factors as a Means of Accounting for Heterosis. *Genetics.* 2:466-479.

Jones, D.J. (1918). The Effect of Inbreeding and Crossbreeding upon Development. *Connecticut Agri. Exp. Station Bulletin.* 246-250.

Kaufmann, P., Pfefferkorn, A., Teuber, M., Meile, L. (1997). Identification and quantification of Bifidobacterium species isolated from food with genus-specific 16S rRNA targeted probes by colony hybridization and PCR. *Appl Environ Microbiol.* 63, 1268–1273.

Kimura, K., McCartney, A.L., McConnell, M.A., Tannock, G.W. (1997). Analysis of fecal populations of bifidobacteria and lactobacilli and investigation of the immunological responses of their human hosts to the predominant strains. *Appl Environ Microbiol*. 63, 3394–3398.

Kraemer, H. (1903). The structure of the corn grain and its relation to popping. *Science.* 17, 683-684.

Kreader, C.A. (1995). Design and evaluation of Bacteroides DNA probes for the specific detection of human fecal pollution. *Appl Environ Microbiol.* 61, 1171–1179.

Krivanek, A.F., Vivek, B. (2006). Science behind breeding QPM. *Mexico,* D.F.: CIMMYT.

Lago, C., Landoni, M., Cassani, E., Doria, E., Nielsen, E., and Pilu, R. (2012). Study and characterization of a novel functional food: purple popcorn. *Molec. Breeding.* 31, 575-585.

Lambert, R.J., Alexander, D.E., Dudley, J.W. (1969). Relative Performance of Normal and Modified Protein (Opaque-2) Maize Hybrids. *Crop Science.* 9:2, 242-243.

Lanzanova, C., Torri, A., Motto, M., Balconi, C. (2010). Characterization of the maize b-32 ribosome inactivating protein and its interaction with fungal pathogen development. *Maydica.* 56:1.

Larkins, B. (2019). Proteins of the Kernel *in Corn: Chemistry and Technology*. edited by Sergio O. Serna-Saldivar. Cambridge, MA: Woodhead Publishing.

Lawton, J.W. (2002). Zein: A History of Processing and Use. *Cereal Chem.* 79:1, 1-18.

Legates, J.E. (1949). A selection index for butterfat production in Jersey cattle utilizing the fat yields of the cow and her relatives. [Ph.D. Thesis: Iowa State College].

Leite, W.S., Unêda-Trevisoli, S.H., Silva, F.M., Silva, A.J., Maurio, A.O.D. (2018). Identification of superior genotypes and soybean traits by multivariate analysis and selection index. *Rev. Ciênc. Agron.* 49:3.

Lerner, J. M., Asmundson, V.S., and Cruden, D.M, 1947*,* The improvement of New Hampshire fryers. *Poultry Science* 26:515-524,

Lin, Y.E. and Anantheswaran, R. C. (1988). Studies on Popping of Popcorn in a Microwave Oven. *Journ. of Food Science.* 53:6, 1746-1749.

Liu, H., Shi, J., Sun, C., Gong, H., Fan, X., Qui, F., Huang, X., Feng, Q., Zheng, X., Yuan, N., Li, C., Zhang, Z., Deng, Y., Wang, J., Pan, G., Han, B., Lai, J., Wu, Y. (2016). Gene

duplication confers enhanced expression of 27-kDa γ-zein for endosperm modification in quality protein maize. *PNAS.* 113:18, 4964-4969.

Liu, H., Huang, Y., Li, X., Wang, H., Ding, Y., Kang, C., Sun, M., Li, F., Wang, J., Deng, Y., Yang, X., Huang, X., Gao, X., Yuan, L., An, D., Wnag, W., Holding, D.R.., Wu, Y. (2019). High frequency DNA rearrangement at qγ27 creates a novel allele for Quality Protein Maize breeding. *Commun*. *Biol*. 2:460.

Lohmer, S., Maddaloni, M., Motto., M., Di Fonzo, N., Hartings, H., Salamini, F., and Thompson, R.D. (1991). The maize regulatory locus Opaque-2 encodes a DNA-binding protein which activates the transcription of the b-32 gene. *The EMBO Journ.* 10:3, 617-624.

Lopes, M.A., Larkins, B.A. (1991). Gamma-Zein Content is Related to Endosperm Modification in Quality Protein Maize. *Cell Biology & Molec Genetics.* 31:6, 1655-1662.

Lopes, M.A., Takasaki, K., Bostwick, D.E., Helentjaris, T., Larkins, B.A. (1995). Identification of two *opaque-2* modifier loci in Quality Protein Maize. *Molec and Gen. Genetics MMG.* 247, 603-613.

Lorant, A., Ross-Ibarra, J., M.I. T. (2018). Genomics of long- and short-term adaptation in maize and teosinte. *PeerJ Preprints* 6:e27190v1.

Lyerly, P.J. (1942). Some Genetic and Morphological Characteristics Affecting the Popping Expansion of Popcorn. *Agro. Journal.* 34:11, 986-999.

Maathuis, A., Hoffman, A., Evans, A., Sanders, L., Venema, K. (2009). The Effect of the Undigested Fraction of Maize Products on the Activity and Composition of the Microbiota Determined in a Dynamic in Vitro Model of the Human Proximal Large Intestine. *Journ. of the American College of Nutr.* 28:6.

Mach, J. (2015). Clarifying the Opaque: Identification of Direct Targets of Maize *Opaque2. The Plant Cell.* 27, 3.

Maddaloni, M., Donini, G., Balconi, C., Rizzi, E., Gallusci, P., Forlani, F., Lohmer, S., Thompson, R., Salamini, F., Motto, M. (1994). The transcriptional activator *Opaque-2* controls the expression of a cytosolic form of pyruvate orthophosphate dikinase-1 in maize endosperms. *Mol Gen Genetics.* 250: 647-654.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., Schleifer, K.H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology.* 142, 1097-1106.

Marinho, C.D., Gravina, G.A., Sebastião, L.C., Almeida, N.C., Daher, R.F., Brasileiro, B.P., Paula, T.O.M, Amaral Júnior, A.T. (2014). Indexes in the comparison of pre-commercial genotypes of common bean. Ciência Rural, 44:7, 1159-1165. doi: 10.1590/0103-8478cr20121155

Maseta, E., Mosha, T.C.E., Laswai, H., Nyaruhucha, C.N. (2016). Nutritional Quality, Mycotoxins and Antinutritional Factors in Quality Protein Maize-Based Supplementary Foods for Children in Tanzania. *Internat. J. of Sciences.* 5:7. doi: 10.18483/ijSci.1082.

Matz, S.A. (1984). 'Snack Food Technology'. Westport, Connecticut: AVI Publishing Company, Inc.

McClintock, B. (1950). The origin and behavior of mutable loci in maize. *Proceedings of the National Academy of Sciences of USA.* 36:344–355. doi: 10.1073/pnas.36.6.344

Mertz, E. T., O. A. Veron, L. S. Bates and O. E. Nelson. (1965). Growth of rats fed on opaque-2 maize. *Science*, 148: 1741-1742.

Mertz, E.T., Bates, L.S., Nelson, O.E. (1964). Mutant gene that changes protein composition and increases lysine content of maize endosperm. *Science.* 145, 279-280. doi: 10.1126/science.145.3629.279

Mertz, E.T., Bressani, R. (1957). Studies on corn proteins: 1. A new method of extraction. *Cereal Chem.* 34, 63-69.

Mikel, M. (2008). Genetic Diversity and Improvement of Contemporary Proprietary North American Dent Corn. *Crop Sci.* 48:5, 1686-1695. doi: https://doi-org.libproxy.unl.edu/10.2135/cropsci2008.01.0039.

Mohamed, A.A., Ashman, R.B., Kirleis, A.W. (1993). Pericarp Thickness and Other Kernel Physical Characteristics Relate to Microwave Popping Quality of Popcorn. *Jour. Food Sci.* 58:2, 342-346.

Moll, R.H., Lonnquist, J.H., Fortuno, J.V., Johnson, E.C. (1965). The Relationship of Heterosis and Genetic Divergence in Maize. Genetics 52, 139-144.

Moro, G.L., Habben, J.E., Hamakers, B.R., Larkins, B.A. (1996). Characterization of the variability in lysine content from normal and *opaque-2* maize endosperm. *Crop Sci.* 36, 1651-1659.

Mulamba, N.N., and Mock, J.J. (1978). Improvement of yield potential of the Eto Blanco maize (Zea mays L.) population by breeding for plant traits. Egypt. J. Gen. Cytol. 7, 40-51.

Nebraska Corn Board. (2019). Field Corn vs. Food Corn. *Nebraska Corn Board.* Available at: https://nebraskacorn.gov/issues-initiatives/your-food/field-corn-vs-food-corn/

Nelson, O.E., Mertz, E.T., and Bates, L. (1965). Second Mutant Gene Affecting the Amino Acid Pattern of Maize Endosperm Proteins. Science. 150:3702, 1469-1470.

Neves, L.G., Bruckner, C.H., Cruz, C.D., Viana, A.P., Barelli, M.A.A. (2011). Gain Prediction with Different Selection Indices for Yellow Passion Fruit Characterization. *Rev. Bras. Frutic., Jabotical – SP.* 33:4, 1322-1330.

Obregon-Tito, A.J., Tito, R.Y., Metcalf, J., Sankaranarayana, K., Clemente, J.C., Ursell, L.K., Xu, Z.Z., Treuren, W.V., Knight, R., Gaffney, P.M., Spicer, P., Lawson, P., Marin-Reyes, L., Trujillo-Villarroell., O., Foster, M., Guija-Poma, E., Troncoso-Corzo, L., Warinner, C., Ozga, A.T., Lewis, C.M. (2015). Subsistence strategies in traditional societies distinguish gut microbiomes. *Nat Commun.* 6, 6505.

Oladeji, B.S., Akanbi, C.T. & Gbadamosi, S.O. (2017). Effects of fermentation on antioxidant properties of flours of a normal endosperm and quality protein maize varrieties. *Food Measure* 11, 1148–1158 doi: https://doi.org/10.1007/s11694-017-9491-8

Olsen, G.J., Lane, D.J., Giovannoni, S.J., Pace, N.R., and Stahl, D. A. (1986). Microbial ecology and evolution: a ribosomal RNA approach. *Annu. Rev. Microbiol.* 40:337–365.

Ou, J., Huang, J., Song, Y., Yao, S., Peng, X., Wang, M., Ou, S. (2016). Feruloylated Oligosaccharides from Maize Bran Modulated the Gut Microbiota in Rats. *Plant Foods for Human Nutrition.* 71, 123-128.

Ouwehand, A.C., Kirjavainen, P.V., Shortt, C., Salminen, S. (1999). Probiotics: mechanisms and established effects. *Int. Dairy J.* 9, 43–52.

Ozturk, A., Erdal, S., Pamukeu, M., Ozata, E., and Coskuner, Y. (2020). Performances of Popcorn Hybrids in Three Geographical Regions of Turkey Based on Yield and Quality Traits. *International Journal of Life Sciences and Biotechnology*. 3:1, 27-40.

Paez, A.V., Ussary, J.P., Helm, J.L., Zuber, M.S. (1969). Survey of Maize Strains for Lysine Content. *Agronomy Journal.* 61:6, 886-889.

Pandey, N., Hossain, F., Kumar, K., Vishwakarma, A.K., Muthusamy, V., Saha, S., Agrawal, P.K., Guleria, S.K., Reddy, S.S., Thirunavukkarasu, N., Gupta, H.S. (2015). Molecular characterization of endosperm and amino acids modifications among quality protein maize inbreds. 135:1, 47-54.

Panse, V. G, 1946, An application of the discriminant function for selection in poultry. *Jour.Genetics* 47:242-248.

Paraginski, R.T., Souza, N.L., Alves, G.H., Ziegler, V., Oliveira, M., Elias, M.C. (2016). Sensory and nutritional evaluation of popcorn kernels with yellow, white and red pericarps expanded in different ways. *Journ. of Cereal Sci.* 383-391.

Park, D., and Maga, J.A. (2000). Color, Texture, and Sensory Evaluation of Selected Hybrids of Popped Popcorn. *Jour. of Food Quality.* 24: 563-574.

Parsons, L., Ren, Y., Yobi, A., Hurst, P., Angelovici, R., Rodriguez, O., & Holding, D. R. (2020). Production and Selection of Quality Protein Popcorn Hybrids Using a Novel Ranking System and Combining Ability Estimates. *Frontiers in Plant Sci*. 11, 698.

Parsons, L., Ren, Y., Yobi, A., Angelovici, R., Rodriguez, O., and Holding, D.R. (2021). Final Selection of Quality Protein Popcorn Hybrids. *Frontiers in Plant Sci.* 12:658456. doi: 10.3389/fpls.2021.658456

Pearl, R., Surface, F.M. (1909). Selection Index Numbers and Their Use in Breeding. *The American Naturalist.* 43: 511, 385-400.

Peterson, J., Garges, S., Giovanni, M., McInnes, P., Wang, L., Schloss, J.A., Bonazzi, V., McEwen, J.E., Wetterstrand, K.A., Deal, C., Baker, C.C., Francesco, V.D., Howcroft, T.K., Karp, R.W., Lunsford, R.D., Wellington, C.R., Belachew, T., Wright, W., Giblin, C., David, H., Mills, M., Salomon, R., Mullins, C., Akolkar, B., Begg, L., Davis, C.,

Grandison, L., Humble, M., Khalsa, J., Little, A.R., Peavy, H., Pontzer, C., Portnoy, M., Sayre, M.H., Starke-Reed, P., Zakhari, S., Read, J., Watson, B., Guyer, M. (2009). The NIH Human Microbiome Project. *Genome Res.* 19, 2317-2323.

Pioneer®. (2018). Agronomy Sciences Research Summary. *PHI.* Available at: https://www.pioneer.com/CMRoot/Pioneer/US/Non_Searchable/agronomy/agronomy_research_summary/2018-ARSB-Final-Optimized.pdf

Pixley, K.V., Bjarnason, M.S. (1993). Combining Ability for Yield and Protein Quality among Modified Endosperm opaque-2 Tropical Maize Inbreds. *Crop Sci.* 33:6, 1229-1234.

Pordesimo, L.O., Anatheswaran, R.C., Fleischmann, A.M., Lin, Y.E., and Hanna, M.A. (1990). Physical Properties as Indicators of Popping Characteristics of Microwave Popcorn. *Journ. of Food Sci.* 55:5, 1352-1335.

Prasanna B. M., Vasal S. K., Kassahun B., Singh N. N. (2001). Quality protein maize. Curr. Sci. 81, 1308–1319.

Prioul, J.L., Méchin, V., Damerval, C. (2008). Molecular and biochemical mechanisms in maize endosperm development: The role of pyruvate-Pi-dikinase and Opaque-2 in the control of C/N ratio. *Comptes Rendus Biologies.* 331:10, 772-779.

Proctor, L. (2019). Priorities for the next 10 years of human microbiome research. *Nature.* 569, 623-625.

Quinn, P.V., Hong, D.C., and Both, J.A. (2005). Increasing the size of a piece of popcorn. *Physica A.* 353:637-648.

Ranathunga, R.A.A., Gunasekara, G.T.N., Wijewardana, D.C.M.S.I. (2016). Quality performance, proximate composition and sensory evaluation of developed flavoured instant popcorn. *Procedia Food Science.* 6:143–146.

Rastall, R.A., Gibson, G.R., Gill, H.S., Guarner, F., Klaenhammer, T.R., Pot, B., Reid, G., Rowland, I.R., Sanders, M.E. (2005). Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: An overview of enabling science and potential applications. *FEMS Micro Eco.* 52:2, 145-152.

Reid G., Sanders M.E., Gaskins H.R., Gibson G.R., Mercenier A., Rastall R.A., Roberfroid M.B., Rowland I., Cherbut C., Klaenhammer T.R. (2003). New scientific paradigms for probiotics and prebiotics. *J. Clin. Gastroenterol.* 37, 105–118.

Reif, J.C., Hallauer, A.R., and Melchinger, A.E. (2005). Heterosis and Heterotic Patterns in Maize. Maydica 50, 215-223.

Ren, Y., Yobi, A., Marshall, L., Angelovici, R., Rodriguez, O., Holding, D. (2018). Generation and Evaluation of Modified Opaque-2 Popcorn Suggests a Route to Quality Protein Popcorn. Front. Plant Sci. 9, 1803. doi: 10.3389/fpls.2018.01803

de Resende, M.D.V. (2016). Software Selegen-REML/BLUP: a useful tool for plant breeding. *Crop Breed. Appl. Biotechnology.* 16:4, 330-339.

Reynolds, J.A., Tanford, C. (1970). The Gross Conformation of Protein-Sodium Dodecyl Sulfate Complexes. *The Journ. of Biological Chemistry.* 245:19, 5161-5165.

Robbins, W. A., and Ashman, R.B. (1984). Parent-offspring popping expansion correlations in progeny of dent corn x popcorn and flint corn x popcorn crosses. Crop Sci. 24, 119-121. doi: 10.2135/cropsci1984.0011183X002400010027x

Robinson, D.L., Thompson, R., and Digby, P.G.N. (1982). REML – A program for the analysis of Non-orthologous data by Restricted Maximum Likelihood. *Compstat 1982, part II (supplement).* Vienna, Austria: Springer.

Robinson, H.F., Comstock, R.E. (1951). Genotypic and Phenotypic Correlations in Corn and Their Implications in Selection. *Agronomy Journ.* 43:6, 282-287.

Robinson, H.F., Comstock, R.E., Harvey, P.H. (1907). Estimates of Heritability and the Degree of Dominance in Corn. 41:8, 353-359.

Rosado, L.D.S., Santos, C.E.M.., Burckner, C.H., Nunes, E.S., Cruz, C.D. (2012). Simultaneous selection in progenies of yellow passion fruit using selection indices. *Rev. Ceres.* 59:1

Salamini, F., Borghi, B., Lorenzoni, C. (1970). The effect of the opaque-2 gene on yield in maize. *Euphytica.* 19: 531-538.

Sandhu, K.S., Singh, N., Kaur, M. (2004). Characteristics of the different corn types and their grain fractions: physicochemical, thermal, morphological, and rheological properties of starches. *Journal of Food Engineering.* 64:1, 119-127.

Savage, D.C. (1977). Microbial Ecology of the Gastrointestinal Tract. *Ann. Rev. Microbiol.* 31, 107-133.

Schieberle, P. (1991). Primary odorants in popcorn. J. Agric. Food Chem., 39: 1141–1144.

Schmidt, R., Burr, F., Aukerman, M.J., Burr. B (1990). Maize regulatory gene opaque-2 encodes a protein with a "leucine-zipper" motif that binds to zein DNA. *Proc. Natl. Acad. Sci. USA.* 87: 46-50.

Schmidt, R.J., Ketudat, M., Aukerman, M.J., Hoschek, G. (1992). Opaque-2 1s a Transcriptional Activator That Recognizes a Specific Target Site in 22-kD Zein Genes. *The Plant Cell.* 4, 689-700.

Schnorr, S.L., Candela, M., Rampelli, S., Centanni, M., Consolandi, C., Basaglia, G., Turroni, S., Biagi, El., Peano, C., Severgnini, M., Fiori, J., Gotti, R., Bellis, G.D., Luiselli, D.,

Brigidi, P., Mabulla, A., Marlowe, F., Henry, A.G., Crittenden, A.N. (2014). Gut microbiome of the Hadza hunter-gatherers. *Nat Commun.* 5, 3654.

Smith, H.F. (1936). A discriminant function for Plant Selection. *Annals of eugenics,* 240-250.

Song, A., Eckhoff, S.R., Paulsen, M. and Litchfield, J.B. (1991). Effects of kernel size and genotype on popcorn popping volumes and number of unpopped kernels. *Cereal Chem.,* 68: 464–467.

Song, S. J., Amir, A., Metcalf, J.L., Amato, K.R., Xu, Z.Z., Humphrey, G., Knight, R. (2016). Preservation Methods Differ in Fecal Microbiome Stability, Affecting Suitability for Field Studies. *mSystems.* 1:3, 1-12.

Sprague, G. and Tatum, L. (1942). General vs. Specific Combining Ability in Single Crosses of Corn. *Agronomy Journal*. 34, 923.

Springer, N. M., and Stupar, R. M. (2007). Allelic Variation And Heterosis In Maize: How Do Two Halves Make More Than A Whole?. Genome Research. 17:3, 264-275. doi:10.1101/gr.5347007.

Stebbings, S., Munro, K., Simon, M.A., Tannock, G., Highton, J., Harmsen, H., Welling, G., Seksik, P., Dore, J., Grame, G., Tilsala-Timisjarvi, A. (2002). Comparison of the faecal microflora of patients with ankylosing spondylitis and controls using molecular methods of analysis. *Rheumatology*. 41, 1395–1401.

Stewart, F.C. (1923). The Relation of Moisture Content and Certain other Factors to the Popping of Popcorn. *New York St. Ag. Exp. Station.* Bulletin No. 505, 5-70.

Stitzer, M.C., Ross-Ibarra, J. (2018). Maize domestication and gene interaction. *New Phytologist.* 220:2, 395-408.

Sturtevant, E.L. (1894). Notes on Maize. *Bulletin of the Torrey Botanical Club*. 21:8, 319-343.

Sweley, J.C., Rose, D.J., and Jackson, D.S. (2011). Composition and Sensory Evaluation of Popcorn Flake Polymorphisms for a Select Butterfly-Type Hybrid. *Fac. Pub. In Food Sci. and Tech.* 109.

Sweley, J.C., Meyer, M.C. Rose, D.J., and Jackson, D.S. (2012). Effects of hybrid, environment, oil addition, and microwave wattage on popped popcorn morphology. *Journ. of Cereal Sci.* 56:2, 276-281.

Sweley, J. C., Rose, D.J., and Jackson, D.S. (2012). Hybrid and environment effects on popcorn kernel physiochemical properties and their relationship to microwave popping performance. *Faculty Pub. in Food Sci. and Tech*. 115.

Sweley, J.C., Rose, D.J., and Jackson, D.S. (2013). Quality Traits and Popping Performance Considerations for Popcorn (*Zea mays* Everta). *Food Reviews International.* 29: 157-177.

Sweley, J.C., Rose, D.J., Jia, X.,, Williams, R.A., and Jackson, D.S. (2014). Effect of flake shape on packing characteristics of popped popcorn. *Journal of Food Engineering*. 127:75-79.

Tandzi, L.N., Mutengwa, C.S., Ngonkeu, E.L.M., Woïn, N., Gracen, V. (2017). Breeding for Quality Protein Maize (QPM) Varieties: A Review. *Agronomy.* 7:80, 1-16.

Tardin, F.D., Pereira, M.G., Gabriel, A.P.C., Amaral Júnior, A.T., Souza Filho, G.A. (2007). Selection Index And Molecular Markers In Reciprocal Recurrent Selection In Maize. Crop Breeding And Applied Biotechnology. 3, 225-233. doi:10.12702/1984-7033.v07n03a01

Tenaillon, M.I., and Charcosset, A. (2011). A European perspective on maize history. *Comptes Rendus Biologies.* 334:3, 221-228.

Thakkar, R.D., Tuncil, Y.E., Hamaker, B.R., Lindemann, S.R. (2020). Maize Bran Particle Size Governs the Community Composition and Metabolic Output of Human Gut Microbiota in in vitro Fermentations. *Front. Microbiology.* 11:1009.

Vasal, S.K., Ortega, A.C., Pandey, S. (1982). CIMMYT's maize germplasm management, improvement, and utilization program. *International Maize and Wheat Improvement Center.*

Vasal, S.K., Srinivasen, G., Beck, D., Crossa, J., Pandey, S., De Leon, C. (1991). Heterosis and Combining Ability of CIMMYT's Tropical Late White Maize Germplasm. *Maydica* 37, 217-223.

Vasal, S.K., Srinivasen, G., Crossa, J., Beck, D. (1992). Heterosis and Combining Ability of CIMMYT's Subtropical and Temperate Early-Maturity Maize Germplasm. *Crop Sci.* 32:4, 884-890.

Vasal, S.K., Srinivasen, G., González, F.C., Han, G.C., Pandey, S., Beck, D.L, Crossa, J. (1992). Heterosis and Combining Ability of CIMMYT's Tropical × Subtropical Maize Germplasm. *Crop Sci.* 32:6, 1483-1489.

Vasal, S.K. (1986). Studies on the combining ability of CIMMYT maize germplasm. *International Maize and Wheat Improvement Center.*

Vasal, S.K. (1999). Improving Human Nutrition Through Agriculture: The Role of International Agricultural Research. Quality Protein Maize Story. *International Rice Research Institute.* Workshop presented: October 5-7, 1999.

Vasal, S.K. (2002). Quality Protein Maize: Overcoming the Hurdles. *Journ. of Crop Prod.* 6:1-2.

Vieira, S.D., Souza, D.C., Martins, I.A., Ribeiro, G.H.M.R., Resende, L.V., Ferraz, A.K.L., Galvão, A.G., Resende, J.T.V. (2017). Selection of experimental strawberry (Fragaria x ananassa) hybrids based on selection indices. *Gen. and Molec. Research.* 16:1, 1-11.

Vietmeyer, N.D. (2000). A drama in three long acts: the story behind the story of the development of quality-protein maize. *Diversity.* 16:3, 29-32.

Vivas, M., Silveira, S.F., Pereira, M.G. (2012). Prediction of genetic gain from selection indices for disease resistance in papaya hybrids. *Rev. Ceres.* 59:6.

Vivek, B., Krivanek, A., Palacios-Rojas, N., Twumasi-Afriyie, S., and Diallo, A.O. (2008). Breeding Quality Protein Maize (QPM): Protocols for Developing QPM Cultivars. Texcoco: CIMMYT

Wallace, J.C., Lopes, M.A., Paiva, E., Larkins, B.A. (1990). New Methods for Extraction and Quantitation of Zeins Reveal a High Content of y-Zein in Modified *opaque-2* Maize. *Plant Physiol.* 92, 191-196.

Walradt, J.P., Lindsay, R.C., Libbey, L.M. (1970). Popcorn Flavor: Identification of Volatile Compounds. *J. Agr. Food Chem.* 18:5, 926-928.

Wang, S., Zhang, B., Chen, T., Li, C., Fu, X., Huang, Q. (2019). Chemical Cross-Linking Controls in Vitro Fecal Fermentation Rate of High-Amylose Maize Starches and Regulates Gut Microbiota Composition. *J. Agric. Food Chem.* 67:49, 13728-13736.

Wang, W., Niu, S., Dai, Y., Wang, M., Li, Y., Yang, W., Zhao, D. (2019). The Zea mays mutants opaque-2 and opaque-16 disclose lysine change in waxy maize as revealed by RNA-Seq. *Sci Rep* 9, 12265 doi: https://doi.org/10.1038/s41598-019-48478-6

Watson, S. A. (1988). Com marketing, processing, and utilization. Page 885 in: Corn and Corn Improvement. G. Sprague and J. Dudley, (eds). Madison, Wisconsin: American Society Agronomy

Weatherwax, P. (1921). The popping of popcorn. *Proc. Indiana Acad Sci.* 149-153.

White, M.R, Mikel, M., Leon, N., Kaeppler, S. (2020). Diversity and heterotic patters in North American proprietary dent maize germplasm. *Crop Sci.* 60:1, 100-114.

Wilbert, M.I. (1903). Why Popcorn Pops. *Amer. Jour. Pharm.* 75, 77-29.

Willier, J.G. and Brunson, A.M. (1927). Factors Affecting the Popping Quality of Popcorn. *J. Agric. Res.* 35:7, 615.

Williams, J.S. (1962). The evaluation of a selection index. *Biometrics.* 18, 375.

Woese, C.R., Fox, G.E. (1977). Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *PNAS.* 74:11, 5088-5090.

Wold, A.E. (2001). Immune effects of probiotics. *Scand. J. Nutr.* 45, 76–85

Worral., H.M., Scott, M.P., Hallauer, A.R. (2015). Registration of Temperate Quality Protein Maize (QPM) Lines BQPM9, BQPM10, BQPM11, BQPM12, BQPM13, BQPM14, BQPM15, BQPM16, and BQPM17. *Jour. of Plant. Registrations.* 9:3, 371-375.

Wu, Y., Holding, D. R., and Messing, J. (2010). γ-zeins are essential for endosperm modification in quality protein maize. *Proc. Natl. Acad. Sci. U.S.A*. 107, 12810– 12815.

Xie, Z., Wang, S., Wang, Z., Fu, X., Huang, Q., Yuan, Y., Wang, K., Zhang, B. (2019). In vitro fecal fermentation of propionylated high-amylose maize starch and its impact on gut microbiota. *Carbohydrate Polymers.* 223:1.

Yacoub, R., Jacob, A., Wlaschin, J., McGregor, M., Quigg, R.J., Alexander, J.J. (2018). Lupus: The microbiome angle. *Immunobiology.* 223:6-7, 460-465.

Yasabu, S. (2019). Sustaining the production and demand of Quality Protein Maize in Ethiopia. *CIMMYT.* Available at: https://www.cimmyt.org/news/sustaining-the-production-and-demand-of-quality-protein-maize-in-ethiopia/

Yatsunenko, T., Rey, F.E., Manary, M.J., Trehan, I., Dominguez-Bello, M.G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R.N., Anokhin, A.P., Heath, A.C., Warner, B., Reeder, J., Kuczynski, J., Caporaso, J.G., Lozupone, C.A., Lauber, C., Clemente, J.C., Knights, D., Knight, R., Gordon, J.I. (2012). 486, 222-227.

Ziegler, K.E., Ashman, R.B., White, G.M., and Wysong, D.S. (1985). Popcorn Production and Marketing. National Corn Handbook, NCH-5, Purdue University, West Lafayette, Indiana.

Ziegler, K.E. (2001). Popcorn. In Specialty Corn. A.R. Hallauer (ed.) Boca Raton, Florida: CRC Press.

CHAPTER 2: PRODUCTION AND PRELIMINARY SELECTION OF QUALITY PROTEIN POPCORN (QPP) HYBRIDS USING THE NOVEL 2020 RANKING SYSTEM AND COMBINING ABILITY ESTIMATES

1. Introduction

Popcorn (Zea mays L. ssp everta (Sturt.) Zhuk) is a type of flint corn characterized by its ability to pop under heat and become an edible, direct-to-consumer snack product. Unlike dent maize, popcorn kernels are largely composed of vitreous endosperm that spans around the kernel's small, round, starchy center (Figure 1).

This unique kernel morphology, coupled with appropriate moisture content, allows the popcorn kernel to expand into light flakes. The market for this popped snack-food has steadily increased for more than a decade, estimated around $9.06 billion in 2016 and projected to rise to more than $15 billion by 2023 (Dawande, 2018). Despite this persistent, growing demand, popcorn variety breeding and research has been largely overshadowed by other maize species and outpaced by its market growth (Dofing et al., 1991; Ziegler and Ashman, 1994; Kantety et al., 1995; Li et al., 2008). Due to primary selection of popping traits such as expansion volume and popability, traits under repulsion linkage with yield, popcorn is less optimized than other maize types in multiple agronomic traits such as pest susceptibility, stalk strength, and grain yield, and it has a relatively narrow breeding pool to integrate and improve agronomic traits (Robbins and Ashman, 1984; Sprague and Dudley, 1988; Dofing et al., 1991; Ziegler and Ashman, 1994). Previously, breeders' attempts at introducing dent corn germplasm into popcorn to improve its agronomic fitness have met with little success because of this negative correlation between expansion volume, a key popcorn quality trait, and grain yield

(Brunson, 1937; Dofing et al., 1991; Ziegler and Ashman, 1994; Pereira and Amaral Júnior, 2001; Daros et al., 2002; Li et al., 2002; Li et al., 2007; Dhliwayo, 2008; Li et al., 2008; Li et al., 2009). However, in 2018, Ren et al. described an interpopulation breeding system between popcorn lines and dent 'Quality Protein Maize' (QPM) varieties capable of increasing essential amino acid lysine in the seed proteome to more suitable levels for human dietary needs, and restored popping at early stages in the breeding program (Ren et al., 2018).

As previously described in Chapter 1, dent QPM varieties were first produced by the International Maize and Wheat Improvement Center (CIMMYT) in the 1980s. Though it was known for decades prior to QPM production that the maize *opaque-2* mutation conveyed a natural biofortification of increased lysine and tryptophan in the kernel endosperm, the integration of the homozygous mutation into commercialized varieties proved challenging (Mertz et al., 1964). Due to its action as a seed storage-protein transcription factor, the knock-out of *opaque-2* manifested a soft, 'opaque' endosperm phenotype (Figure 1). In their unmodified form, *opaque-2* varieties proved unfit for varietal production as they generally yielded less than its comparative germplasm and were more susceptible to fungus and pests, kernel processing damage, and lacked grower acceptance (Prasanna et al., 2001). To alleviate these setbacks, CIMMYT employed a large-scale breeding program involving multiple *opaque-2* varieties and selected moderately improved vitreousness levels through back-crossed generations. Along with the *opaque-2* mutation, CIMMYT observed the necessary introgression of unknown amino acid and endosperm vitreousness restorer genes through phenotypic selection for the biofortified, vitreous QPM end product (Babu et al., 2005; Sofi et al., 2009; Panda et

al., 2010; Panda et al., 2010; Mbuya et al., 2011; Babu and Prasanna, 2014; Surender et al., 2014; Kostadinovic et al., 2016; Krishna et al., 2017). Though most amino acid and endosperm modifier genes remain unidentified, QTL studies have suggested that endosperm restorer genes are located on Chromosomes 1, 5, 7, and 9 (Holding et al., 2008; Holding et al., 2011; Babu et al., 2015). Biochemical and genetic data have suggested that increased expression and encoded protein of 27-kd γ-zein gene, in the continued presence of low α-zeins, is the most important component of modification (Geetha et al., 1991; Holding et al., 2008; Wu et al., 2010; Holding, 2014). In 2016, a 27-kd γ-zein gene duplication on Chromosome 7 was confirmed as the basis for this increase and that it is observed in all QPM varieties (Liu et al., 2016). Further investigation recently revealed this locus's high frequency of genetic rearrangement and introduced a novel triplication allele (Liu et al., 2019). To successfully integrate the required QPM genes into popcorn backgrounds, Ren et al. utilized the visible over-production of 27-kd γ-zein along with marker-assisted selection of the *opaque-2* mutation to select for restored vitreousness of the endosperm while maintaining elevated lysine (Ren et al., 2018). While selecting for a QPM-like proteome, key popcorn traits such as popability, kernel morphology, and kernel size were also selected throughout the breeding program (Ren et al., 2018). After two popcorn back-crosses and multiple rounds of self-pollination, 12 BC_2F_5 'Quality Protein Popcorn' (QPP) lines were selected for analysis of sufficient popcorn and QPM traits. These inbred lines had highly vitreous endosperm, a QPM-like proteome, high lysine, and similar popping characteristics to the original popcorn parents (Ren et al., 2018).

The quality of popcorn endosperm protein, like normal dent maize, is low because of its deficiency in lysine and tryptophan essential amino acids (Ren et al., 2018). Previous breeding attempts have successfully introgressed the *opaque-2* allele into popcorn germplasm but have not recovered popping characteristics (Zhou et al., 2016; Adunola, 2017). These QPP inbred lines described in Ren et al. demonstrated proof-of-concept that the target traits for quality protein could be successfully integrated from QPM into popcorn without sacrificing popability (Ren et al., 2018). However, as inbreds, they were not fit for commercialized production due to inbreeding depression and unoptimized agronomic capacity. Therefore, the objectives of this study were to generate all possible QPP hybrids and select elite hybrids with superior protein quality, popcorn quality, and agronomic traits. Overall, the cumulation of these analyses enabled efficient selection of five elite QPP hybrids of three flake types out of the tested QPP hybrid population fit for future, quantitative complex trait comparison to currently marketed popcorn varieties.

2. Materials and Methods

2.1 Plant Materials and Creation of Hybrids

QPP inbred lines were produced by crossing three QPM lines, CML154Q, K0326Y, and Tx807, with four ConAgra Brands® popcorn inbred lines, whose names are withheld for proprietary reasons (labeled P1-P4 to preserve identity). After F1 crossing in 2013, lines were back-crossed twice to the original popcorn parent and selfed five times over the course of four years. Phenotypically vitreous, *o2o2* homozygous BC_2F_5 QPP lines were produced in the winter of 2017. After evaluation, twelve BC_2F_5 QPP inbred lines (labeled 'QPP Inbreds 1-12') of single-seed descent from six dent x popcorn F_1 crosses were chosen for continued analysis (Ren et al., 2018). In the summer of 2018, these lines

were hand-planted and cross-pollinated in a full diallel to produce 132 QPP F_1 hybrids. Fifteen kernels were planted per row and rows were spaced 30 inches apart. Reciprocal hybrids were designed to grow in adjacent rows for efficiency in hand-pollination and kept separate at harvest. Qualitative assessment of all maternal cobs, F_1 grain fill, and F_1 grain vitreousness suggested QPP inbred lines '5', '6', '9', and '10' produced superior hybrids as maternal parents (Table 1).

At this stage, further selection of paternal parents was not conducted to maintain a diverse array of hybrids for continued analysis. Therefore, 44 hybrids of pedigrees '5' x '1-12','6' x '1-12', '9' x '1-12', '10' x '1-12' (maternal x paternal, excluding selfing) were selected for F_1 plant and F_2 grain prescreening analysis in the summer of 2019. These 44 hybrids were numerically named in order of maternal parent 'Inbred 5', 'Inbred 6', 'Inbred 9', and 'Inbred 10', and paternal parent Inbred '1-12' (Table 1). After relative ranking, five QPP hybrids were chosen for final, complex trait analysis taking place in the summer of 2020.

2.2 2019 Field Design

After QPP F_1 production in 2018, 44 hybrid crosses were selected for relative intermediate analysis of F_1 agronomic plant performance including ear size and F_2 seed traits in the summer of 2019. Hybrids were grown in Lincoln, Nebraska and Oakley, Kansas in a Generalized Complete Block Design (GCBD) with six experimental 10-foot row units randomized per location. Original dent QPM parents, K0326Y and CML154Q, QPP Inbred 9, QPP Inbred 10, Popcorn Parent 1, and Popcorn Parent 2 were also sown and analyzed for relative comparison to hybrid progeny. Fifteen kernels were planted per row and rows were spaced 30 inches apart. Plants developed under rain fed conditions in

both locations and were self-pollinated and harvested by hand. All original ConAgra popcorn inbred lines were provided by ConAgra Brands®. K0326Y QPM was a lab stock originally sourced from Hans Gevers (Gevers and Lake, 1992), and CML154Q and Tx807 QPMs were originally obtained from the North Central Regional Plant Introduction Station as previously described (Ren et al., 2018).

2.3 Protein Extraction and Profiling

Zein and non-zein proteins were extracted by procedures previously described (Wallace et al. 1990; Ren. et al 2018). Zein-profiles of two randomly selected F1 kernels from two 2018 field ears were analyzed for all 44 hybrids. Zein and non-zein profiles were analyzed on a random selection of 28 kernels from the 2019 F2 hybrid harvest. After selection of the five elite QPP hybrids for continued testing (Hybrids 20, 25, 28, 38, and 43), the zein profile of eight random kernels from each hybrid were analyzed to verify that the proteome was that of QPM (low α-zeins and high 27-kD γ-zein). Specifically, kernels were ground with a Wig-L-Bug® dental amalgam grinder and 50 mg (± 0.1mg) of powder were used for protein extraction with a borate, β-mercaptoethanol, SDS extraction buffer. Tubes were shaken for ~3 hours at room temperature and centrifuged at full speed (13.3 g) for 10 minutes. Protein supernatant was further separated into zein and non-zein fractions by introducing 70% ethanol and incubating at 4 ºC overnight. 150 µL of both zein and non-zein fractions were placed in a vacuum desiccator centrifuge and protein precipitated. The precipate was resuspended in 35 µL of 1X SDS-PAGE loading buffer and 5 µL samples were separated using 12% acrylamide SDS-PAGE to observe differentiable levels of staining due to particular protein abundance (termed ‘semi-quantitative’) for both zein and non-zein fractions.

2.4 DNA Extraction

Leaf tissue from QPP inbreds and QPP F_1 hybrids was collected from two-week old seedlings and DNA was extracted according to a previously published urea-based procedure (Holding et al., 2008). DNA samples were diluted to a final concentration of ~50 ng/µL utilizing Nanodrop® and Qubit® technologies.

2.5 Genotyping the *opaque-2* allele

Polymerase Chain Reaction (PCR) was carried out for *opaque-2* in-gene marker umc1066 according to Ren et al., 2018. Short sequence repeat (SSR) marker umc1066 first became a useful co-dominant polymorphism for QPM conversion in 2005, and Ren et al. successfully differentiated between QPM and popcorn *opaque-2* alleles with this marker (Babu et al., 2005). Hybrid verification of *o2o2* QPM-allele homozygosity also required QPM *opaque-2* allele differentiation, which was achieved by using primers for *opaque-2* flanking marker bnlg1200, also first described by Babu et al. (Babu et al., 2005). PCR conditions for marker bnlg1200 were to the same as marker umc1066 except annealing temperature of 55 ºC was used.

2.6 Trait Analysis

Preliminary prescreening of the 44 QPP hybrids for relative competitive assessment involved measuring the following traits: germination rate (Germination), days to pollination (DAP), rot/pest susceptibility (Rot), number of ears harvested per row out of 15 seeds planted (NEH), ear length (EL), number of kernel rows per ear (RPE), ear weight (or weight of ear's grain, WEG), 100-grain weight (100GW), kernel size (KS), kernel vitreousness (Vit), popability (PA), expansion volume (EV), flake type (FT), kernel color (KC), and amino acid profile of kernels and popped flakes in air, oil, and

microwaved conditions. Germination, DAP, Rot, and NEH were measured on all plants/ears in each plot. EL, RPE, WEG, 100GW, KS, Vitreousness, PA, EV, and FT were measured on five selected ears per row and averaged for one measurement per row. EL and RPE were measured prior to shelling. WEG, KS, Vitreousness, and 100-grain weight were measured after shelling but prior to pooling the five ears' kernels. One hundred grain weight has commonly replaced 1000-grain weight in popcorn research (Li et al., 2007; Li et al., 2008, Dar et al., 2018). Final traits (PA, EV, and FT) were measured after moisture equilibration for 6 weeks in a conditioning room set at 14% moisture. Following analysis of these traits, ten superior hybrids were selected for amino acid profiling.

Kernel Size was determined by counting the number of kernels in batches of 10-grams per ear, per row and averaging values. One-hundred grain weight was found through this estimate and appropriating the influence of each ear's value to the final average by Ear Weight. Vitreousness was determined through light-box screening and qualitatively scored on a 1-7 scale of complete opacity to complete vitreousness, as previously described (Vivek et al., 2008; Ren et al., 2018; Figure 2). Popability was measured by weighing one replication of 20 grams per row, counting the total number of kernels, and after popping, counting the number of unpopped kernels. Expansion volume was evaluated through popping in a domestic Orville Redenbacher Hot Air Popcorn Popper and measuring the total popped flake volume in a 1 liter cylinder. One batch of 20 grams of kernels per row was measured. Flake type was determined by evaluating one randomly selected batch of 20 grams of popped kernels and annotating flake type as

mushroom, unilateral, bilateral, or multilateral according to previously described terminology (Eldredge and Thomas, 1959; Sweley et al, 2011).

Free and protein-bound amino acid profiles were analyzed at the University of Missouri according to published procedures (Angelovici et al., 2013; Yobi and Angelovici, 2018). Acidic hydrolysis of protein-bound amino acids destroys tryptophan and cysteine, and confounds asparagine and aspartate (Asx) and glutamate and glutamine (Glx), but all free amino acids were recovered in native form (Tables 5, 6, 8-13). After determining the top ten hybrids, profiles from one replication of unpopped kernel powder per three rows per location (six samples) for each hybrid was quantified. Three kernels were ground and pooled for each replication, and all ground powder per row was used for UPLC-MSMS protein bound and free amino acid profiling. In addition to the ten best hybrids, biological replications of QPP inbred lines (two), original proprietary popcorn (four replications for Parents 1 and 2, two replications for Parents 3 and 4), QPM dent parents (four replications for CML154Q and K0326Y, two for Tx807), and B73 (four) were also analyzed for protein-bound and free amino acid relative content. Popped flakes were also measured for free- and protein-bound amino acid determination. Four replications of five hybrids and Popcorn Parents 1 and 2 were each air-popped, microwave-popped, and oil-popped (for a total of 12 popped samples per line), and flakes were frozen in liquid nitrogen and ground in a mortar and pestle to make a fine powder.

2.7 Statistical Analysis

2.7.1 QPP Inbred and Hybrid Analysis

The statistical model used for preliminary internal ranking of QPP hybrids is given by Equation 1:

$$y_{ijk} = \mu + \beta_i + \tau_j + (\beta\tau)_{ij} + \epsilon_{ijk} \quad (1)$$

Where y_{ijk} is the hybrid's response, μ is the overall mean, β_i is the environmental effect, τ_j is the treatment effect, $(\beta\tau)_{ij}$is the location*treatment interaction, and ϵ_{ijk} is the plot*treatment*block random effect, or error (Griffing, 1956; Addelman, 1969,). The treatment effect was considered random to estimate genetic values and Type II sums of squares was used to compute the Analysis of Variance to maintain proper degrees of freedom with missing hybrid data.

Relative values of mGCA, pGCA, and SCA were measured for each trait as shown theoretically by Equations 2, 3, 4, and 5 (Griffing 1956; Gardner, 1967):

$$y_{iklm} = \mu + g_k + g_l + s_{kl} + e_{iklm} \quad (2)$$

$$mGCA_k = y.k.. - \mu \quad (3)$$

$$pGCA_l = y..l. - \mu \quad (4)$$

$$SCA_{kl} = y_{.kl.} - mGCA_k - pGCA_l \quad (5)$$

Equation 1 was used sequentially with maternal, paternal, and hybrid treatments as random effects in ASReml-R software to estimate genetic values and standard errors (Butler, 2019). Genetic repeatability and maternal and paternal broad-sense heritabilities were calculated utilizing the genetic variance and phenotypic variance components as shown in Equation 6 (Isik et al., 2017):

$$Hybrid\ Repeatability\ or\ Inbred\ Broad - Sense\ Heritability = \frac{\tau_j}{\sigma_P} \quad (6)$$

$$\tau_j = Hybrid\ Effect\ (SCA)$$

$$\tau_j = Maternal\ Effect\ (mGCA)$$

$$\tau_j = Paternal\ Effect\ (pGCA)$$

All analysis was conducted using R® software, and the ASReml-R package was used to calculate mGCA, pGCA, SCA, co-variance, and variance of traits (Isik et al., 2017; Butler, 2019). The R-package 'GGally' was used to calculate trait correlations (Schloerke et al., 2018). R-packages 'lavaan', 'semPlot', 'OpenMx', 'tidyverse', 'knitr','kableExtra', and 'GGally' were used to conduct and visualize path analysis for comparative correlation values with EV as the main, independent variable with all variables excluding KS and DAP as dependent variables, and ear grain weight as a function of agronomic traits Germination, Rot, NEH, EL, NRE, 100GW, and Vit (Yves, 2012; Hunter, 2018; Schloerke et al., 2018; Sacha, 2019; Hao, 2019; Wickhan, 2019; Yihui, 2020). Tukey's Honest Significant Difference (HSD) method in R software was used to test significant differences of hybrid and parental mean trait values (R Core Team, 2018).

2.7.2 Index Selection: Adapted Rank of Sums

Selection indices are more commonly used to select inbred lines in recurrent breeding rather than ranking at the intermediate stage of hybrid selection (Hallauer and Eberhart, 1970; Johnson et al., 1988; Tardin et al., 2007; Marinho et al., 2014). This type of index requires heritability estimates coupled to repeatability to better gauge the genetic value of an inbred (Amaral Júnior et al., 2010; Lima et al., 2012; Marinho et al., 2014; De Azeredo et al., 2017; Da Luz et al., 2018). To further select the best QPP hybrids from the 44 continued crosses, a model was devised to prescreen and comparatively rank hybrids according to suggested genetic potential. The intrapopulation, relative hybrid ranking determined by the equation below reflects potential genetic value through

summing the products of estimated comparative phenotype and determined economic weight of each trait. Trait estimates served as prescreening comparations capable of effective, intrapopulation ranking of the 44 QPP hybrids rather than individual quantitative values through this model. Equation 7 also includes a measure of trait repeatability in each trait's summative ranking. For hybrid ranking, the heritability estimate was replaced with repeatability for suggested homogeneity of the hybrid, rather than heritable trait value.

$$X_h = \sqrt{\sum_{i=1}^{m} \left(\frac{y_{i,h}}{y_{i,max}} - 1 \right)^2 I_i \, (\sigma_{i,h} / \sigma_{i,max})} \tag{7}$$

In the equation, X_h is the final, continuous rank of hybrid 'h'; $y_{i,h}$ is 'h''s value of trait 'i'; $y_{i,max}$ is the superior value of trait 'i' across hybrids; and I_i is the selection intensity of trait 'i'. Germination rate, rot susceptibility, number of ears harvested per row, ear weight, 100-grain weight, vitreousness level, popability, and expansion volume were all considered important traits in intermediate selection. Not all traits were regarded as equally important in hybrid selection, so weighting values (selection intensities) were assigned on a scale of 0-1 that graded traits based on economic importance for a commercialize line. Popability and expansion volume were assigned the heaviest weight (0.85), followed by ear weight (0.80), 100-grain weight and germination rate (0.70), vitreousness and number of ears harvested (0.60), pest/rot susceptibility and ear length (0.50), and finally number of rows per ear (0.4). Days to pollination and kernel size traits were noted for other analyses but not considered for ranking. Traits with premium values not reflected as maximum were reconfigured. For example, the rot/pest susceptibility

values were subtracted from 1 (100% insusceptibility) and the differences were utilized. $\sigma_{i,h}$ is the standard deviation of trait 'i' from hybrid 'h' and $\sigma_{i,max}$ is the maximum standard deviation for trait 'i' across hybrids.

Final ranks were on a continuous scale with smallest values representing superior hybrids.

2.7.3 Pedigree Effect: Progression of Heterosis

The 44 QPP hybrids were separated into five categorical 'hybrid' levels according to their pedigrees (Table 1). Hybrids differentiated solely by single seed descent of the same QPM and popcorn lineage were considered 'pseudo-selfed'. Since inbred lines were backcrossed twice to the original popcorn parents, hybrids with the same popcorn lineage were conservatively considered 0-50% 'hybrid', while crosses with the same original QPM parent were considered closer to a true hybrid. Crosses with popcorn parents within the same heterotic group were categorized into 'same heterotic group: hybrids', and crosses between different popcorn heterotic groups were part of the 'complete hybrid' group. The statistical model used for variance analysis is shown by Equation 1 inputting treatment as the 'pedigree effect' on trait response. Analysis was conducted with Type II sums of squares in R® software and Tukey's HSD tests for significance (R Core Team, 2018).

3. Results

3.1 Verification of *o2o2* genotype in QPP Hybrid F_1 and F_2 kernels through PCR and SDS-PAGE analysis

Polymerase Chain Reaction (PCR) analysis of QPP inbred lines confirmed homozygous *opaque-2* introgression from dent parents. QPP Inbred lines 3, 9, 10, and 11 and their

parental pedigrees are shown (Figure 3). All inbreds were homozygous for the QPM *opaque-2* allele.

SDS-PAGE analyses of F1 and F2 kernels from the 44 selected QPP hybrids confirmed the consistent QPM proteome of modified, *o2o2* mutants (Figure 4). All semi-quantitative zein SDS-PAGE analysis revealed a substantial decrease of 22-kD α-zein accumulation, varied accumulation of 19 kD α-zein, and a uniform increase in 27-kD γ-zein accumulation compared to the original popcorn, mirroring the QPM zein protein profile (Figure 4A). Moreover, F_2 kernels showed a characteristic, although variable, relative increase in non-zein accumulation compared to the original popcorn parent indicative of increased lysine (Figure 4B). The seven random QPP hybrid kernels pictured represent the 28 kernels analyzed for zein and non-zein patterns. After ranking and selection of QPP hybrids, zein analysis of eight random kernels from elite hybrids showed the same pattern (decrease of 22-kD α-zein accumulation, varied accumulation of 19 kD α-zein, and a uniform increase in 27-kD γ-zein accumulation) (not shown). Moreover, protein-bound and free amino acid profiling of 10 select hybrids confirmed the general increase in lysine accumulation in the kernel endosperm co-validating the PCR and SDS-PAGE results of a rebalanced proteome due to introgression of the *opaque-2* recessive allele (Tables 5 and 6).

3.2 Agronomic and popcorn quality trait evaluation of QPP hybrids and original popcorn, QPM, and QPP inbreds

Superior agronomic performance was observed in all QPP hybrids compared to the six simultaneously grown inbred lines ($p < 0.01$; Figure 5). F_1 hybrid plants demonstrated significantly higher germination rates and number of ears harvested from 15 planted

seeds compared to QPP, Popcorn, and QPM inbreds (Figure 5A and 5B). Four traits out of the twelve analyzed, rot susceptibility, number of ears harvested, vitreousness, and 100-grain weight had a significant environmental interaction effects ($p < 0.01$). QPP hybrid ears were significantly longer than popcorn and QPM parents (Figure 5C). Hybrids averaged 46.6 grams per ear in grain weight, a significant improvement compared to QPP inbreds and popcorn parents (Figure 5D). Kernel sizes (as demonstrated by 100-grain weight) of all popcorn types were significantly smaller than QPM inbreds, while QPP hybrids exhibited slightly larger kernel size compared to QPP inbreds (Figure 5E). The original popcorn parents had significantly fewer number of kernel rows per ear (NRE) compared to QPM inbreds and QPP inbreds and hybrids averaged very similar NRE to QPM (Figure 5F). Flake expansion volume (EV) of QPP hybrids were on average lower than original popcorn parents (Figure 5G). QPP hybrids had a higher popability average than QPP inbreds and popability was not significantly different from the original popcorn parents (Figure 5H). These results suggest the successful selection of agronomic traits in QPP hybrids from QPM parents while sustaining popcorn quality traits from popcorn germplasm.

3.3 Phenotypic correlations and path analysis for agronomic and popcorn quality traits

Simple regression and path analysis of preliminary trait values suggested high covariances and correlations between multiple agronomic and popcorn traits (Figure 6). Charts along the downward diagonal of Figure 6A depict the range and generally normal distribution of each of the eight traits analyzed (Figure 6A). Dot plots under the diagonal plot trait values, as described in the column and row headings, on the x and y axis for

visualized regression and slope of response (Figure 6A). Values in replacement of dot plots indicate correlations derived from path analysis with EV as the independent variable and ear weight as a function of agronomic traits and vitreousness. Correlation coefficients positioned above the diagonal relate to traits as described in the column and row headings (Figure 6A) and were calculated by dividing the traits' covariance (above darkened diagonal in Figure 6B) by both traits' standard deviations (variances shown in diagonal, Figure 6B). Path analysis standardized coefficients and correlation coefficients complement each other in significance and trend, except for correlations between ear weight and Vit, EV and EL, and EV and number of ears harvested per row (Figure 6A). Negative coefficients were found between EV and 100-grain weight (-0.325 and -0.241), EV and ear weight (-0.232 and -0.241), and EV and number of rows per ear (-0.358 and -0.205) for phenotypic correlation and path analysis, respectively (Figure 6A). When agronomic traits were compared, high correlations between ear weight and ear length, ear weight and 100-grain weight, and 100-grain weight and ear length were calculated (Figure 6A). All three traits were evaluated to account for the possibility that kernel size and rot susceptibility could create variance in ear fill, but despite moderate occurrence of rot, strong correlations between these three traits were still observed. Additionally, though ear length variance was relatively large (10.63, Figure 6B), the trait conferred a high maternal heritability and hybrid repeatability estimate (0.432 and 0.716, respectively; Table 3 and Table 7). Vitreousness was slightly negatively correlated to 100-grain weight, ear weight, and number of rows per ear and positively correlated to EV (0.435 and 0.300, respectively) (Figure 6A). Path analysis revealed a significant, though small, positive correlation between EV and ear length (0.197) while phenotypic

correlation between vitreousness and ear length was insignificant (Figure 6A). This data supported the empirical findings that maintaining a high level of kernel vitreousness while improving popcorn agronomics, proposedly through ear length, lessened the negative side-effect on popcorn quality traits.

3.4 Pedigree analysis of QPP hybrids

Hybrids were categorically separated into five groups in order of increasing genetic diversity (Ren et al., 2018; Table 1 and Figure 7). All agronomic traits exhibited a similar trend of improvement from the pseudo-selfed lines to the complete-hybrid groups. 'Ears harvested per row' averages between categorical groups slowly inclined, and significant differences were found between all categories one step apart (Figure 7A). One hundred grain weight values exhibited a similar trend, except hybrids within the same QPM background had a slightly larger average than hybrids in the same heterotic group (Figure 7B). QPP hybrids from different heterotic groups averaged the highest ear length while categories involving the same popcorn background or heterotic pool notably decreased compared to the same QPM or different heterotic pool categories (Figure 7C). A dragging trend in similar popcorn genetics (backgrounds and heterotic pools) was also noticed in NRE (Figure 7D). Like EL, groups with the same popcorn background were significantly stunted in kernel row number, averaging almost the same as popcorn parental inbreds (11.78±0.809 and 12.11±0.928, respectively).

Principle Component Analysis of all trait data supported the validity of these categories and subsequent heterotic trend. A composite 96.56% of data variance was explained by the first two principle components (Figure 8). QPM parents K0326Y and CML154Q fell far from all other popcorn related lines and were clustered into the same group as other

inbreds. All ‘Same Popcorn Background’ hybrids fell in/near the inbred cluster (Figure 8). These components were determined predominantly by variances associated with a kernel size, ear weight, and maturity (Figure 8). Hybrids of the same heterotic group displayed a tight cluster separated completely from hybrids of different heterotic groups, though both overlapped with ‘Pseudo-self’ and ‘Same QPM Background’ clusters (Figure 8). Complete hybrids notably separated themselves from hybrids from the same heterotic pool due to heavier ear weight and longer ear length, while hybrids from the same heterotic group favored smaller, more popcorn-like kernel sizes and later maturity (Figure 8). Like Figure 7, progression in agronomic improvement, specifically in ear length, ear weight, and kernel size, was evident through PCA of the five genetically distinct categories of QPP hybrids (Figure 7, Figure 8).

3.5 QPP hybrid and inbred flake type analysis

Utilizing unilateral, bilateral, multilateral, and mushroom terminology (Sweley et al., 2011), all QPP inbreds and hybrids were categorized into one or two flake types (Table 2; Figure 9). Bilateral flake types were not observed across all hybrids (Table 2). Hybrids from maternal parents 5, 6, and 10 seemed to display either unilateral or mushroom flakes, in agreement with inbred morphology, while hybrids from maternal parent 6 had a more diverse morphology of mushroom or multilateral flakes (Figure 9; Table 2). Paternal parents 11 and 12 also exhibited a mushroom flake in all progeny with different degrees of uniformity, reflecting the flake type of the inbreds (Figure 9; Table 2). Hybrids involving Inbreds 3 and 4 also popped with mushroom flakes like the inbreds, though notably crosses 25 and 26 had uniform unilateral flakes, like Inbred 9. Out of the 22 crosses involving maternal lines 9 and 10, nearly half displayed uniformly unilateral

flakes (Table 2). In contrast, all hybrids from maternal Inbred 6 had mixed morphologies except for hybrid 19, which was multilateral (Table 2). Nine hybrids in all displayed some occurrence of multilateral flakes and the morphology was tested for association with high EV, but no correlation was found. Hybrids 23-26 exhibited uniformly unilateral flakes compared to Hybrids 34-37 that displayed near uniform mushroom morphology (Table 2). Half of hybrids from Inbreds 1 and 2 exhibited mushroom morphology though these inbreds had a multilateral morphology (Figure 9). Inbreds 11 and 12 exhibited the mushroom morphology successfully in almost all hybrids, including those with Inbred 9 as the maternal parent (Table 2). Before hybrid ranking and selection, it was determined that diversity in flake type would be maintained in the final list of chosen hybrids. Thus, after ranking and inbred analysis, final hybrids with two uniformly unilateral, two unilateral and multilateral mixed, and one mushroom morphology were chosen for continued analysis.

3.6 Novel hybrid ranking system identified top QPP hybrids

All relevant trait data was imputed into the ranking model as shown by Equation 7. After computation, each hybrid was assigned a final ranking number that was the composite of ten trait values (Figure 10). Hybrid 6 held the highest value (signifying the worst ranking of all hybrids), which was mostly due to its relatively poor germination (Figure 10). Hybrids 19, 20, 28, 38, 9, 8, 43, 30, 25, and 17 were identified as the top ten (Figure 10). Hybrids 19 and 20 ranked highest with minimal deviations from the maximum trait values in all traits. Hybrid 20 was slightly hindered by its lower EV, as was Hybrid 28's lower 100-grain weight. Hybrids 8, 25, and 32 had large rot values but they did not affect ear weight (Figure 10). Hybrids 30 and 25 were very similar in rank since Hybrid 30 had

a more inferior ear weight with minimal rot susceptibility. Hybrid 43, 44, 26, and 23 were hindered by expansion volume, which was more noteworthy for Hybrids 23 and 26 since they expanded unilaterally compared to Hybrids 43 and 44 which expanded in mushroom morphology (Table 2). Hybrid 17 ranked tenth, with a value predominantly composed of ear weight and ear length marks (Figure 10).

The summation of all preliminary evaluations enabled the holistic ranking of hybrids by overall genetic value, analyses akin to other selection indices. However, maintaining individual trait distinctions and extent of effect enabled a thorough understanding of hybrid rank. The top nine hybrids: 19, 20, 28, 38, 9, 8, 43, 30, 25, and hybrid 23 (lower due to EV) were chosen for amino acid profiling and further selection.

3.7 Assessment of top hybrids utilizing General and Specific Combining Ability Estimates

Hybrid analysis enabled maternal and paternal GCA values to be assigned according to offspring productivity. Maternal GCA values were only assigned for Inbreds 5, 6, 9, and 10, and paternal values were calculated for all QPP inbreds (Table 3). Due to inbred similarity in original pedigree (shown in Table 1), most combining ability values were similar for pairs of inbreds with the same QPM and popcorn parents. Trends were observed between the maternal pairs of Inbreds 5 and 6 and Inbreds 9 and 10. Ear weight maternal and paternal combining abilities were not used in downstream analysis due to large standard error and insignificant differences. mGCA estimates for Inbreds 9 and 10 (CML154Q x Popcorn Parent 1) were significantly higher than Inbreds 5 and 6 in agronomic traits ear length, number of rows per ear, and 100-grain weight (Table 3). These traits also had the highest maternal heritability values at 0.432, and 0.415 for EL,

and 100-grain weight respectively. Higher heritable values coupled to significant differences in maternal general combining ability values suggested that Inbreds 9 and 10 were superior maternal parents agronomically. Inbreds 5 and 6 held the highest expansion volume GCAs for all parents, though these values were considered insignificant. However, the trend in higher EV GCA values for these inbreds suggested that Inbreds 5 and 6 were strong paternal parents in popcorn quality traits, especially when considering they also held the highest popability pGCAs and paternal heritabilities were larger than maternal for both EV and popability, at 0.322 and 0.123, respectively (Table 3). Moreover, the heritability estimates for vitreousness varied substantially between maternal and paternal parents; with values of 0.024 and 0.445, respectively. Therefore, Inbreds 5 and 6 again stood out as premier paternal parents with significantly highest vitreousness pGCA values (Table 3). The combination of Inbreds 9 and 10 as maternal parents and Inbreds 5 and 6 as paternal parents suggested premier crosses, aiding the eventual selection of both Hybrids 28 and 38 rather than their reciprocals Hybrids 19 and 9 (Table 3). Hybrid 20 was favored over Hybrid 19 due to Inbred 10's larger popcorn quality trait pGCA value for Popability, which is highly correlated to EV, compared to Inbred 9 (Table 3).

Specific Combining Ability values, standard error, and genetic repeatability estimates were calculated for all QPP hybrids (Table 7). High standard errors for EV and ear weight in both general and specific combining ability estimates limited their direct use for QPP hybrid selection; however, calculated significant correlations between traits such as ear length and ear weight, and popability and EV, enabled discriminatory selection of elite hybrids utilizing more accurate inbred genetic values coupled to heritability and

repeatability estimates. The ranking system allowed for a direct, preliminary narrowing of best hybrids for further testing, after which heritability and repeatability estimates with standard error determined the reliability of combining ability values that guided final selection. Due to high heritability and low standard error, ear length and Vitreousness SCA values became the premier traits for final selection. Hybrids 20, 25, 28, 38, and 43 all exhibited positive EL SCAs and Hybrids 20, 28, 38, and 43 held positive Vitreousness SCAs.

3.8 Highly ranked QPP hybrids showed elevated lysine in raw and popped kernel flours

After the ten best hybrids were selected, flour from raw kernels and air, microwave, and oil popped flakes were analyzed for protein-bound and free amino acids. Principle Component Analysis of protein-bound raw kernel amino acid profiles suggested a major shift in the QPP proteome away from popcorn parents and toward QPM (Figure 11A). Genotypes were grouped into two main clusters. Cluster one was composed of popcorn parents (and B73 dent corn) and cluster two of QPP and QPM germplasm with the overlap of one genotype (QPP Inbred 9) (Figure 11A). CML154Q and K0326Y were grouped into cluster two and indistinguishable from QPP inbreds and hybrids (Figure 11A). QPP Inbreds 7 and 8 and QPM line Tx807 displayed a distinctive protein-bound amino acid profile compared to all other lines and formed cluster three, though too few points were available to calculate an ellipse (Figure 11A, Table 5). With histidine, methionine, and lysine as the exceptions, Inbreds 7 and 8 consistently had the highest protein-bound amino acid levels, though this trend did not hold with free amino acid values (Tables 5 and 6). Principle Component Analysis of free raw kernel amino acids

instead suggested a general distinction between QPP inbreds and QPP hybrids (Figure 12). Like the protein-bound analysis, Inbred 9 bordered the popcorn parent cluster, and K0326Y, Tx807, and QPP Inbreds 10, 8, and 6 overlapped with QPP hybrids (Figure 12). All other QPP Inbreds and CML154Q formed a separate group with characteristically high levels of proline, aspartate, glutamine, glutamine, and alanine (Figure 12).

To further confirm the homozygous introgression of the QPM *opaque-2* allele, free and protein-bound lysine levels in raw kernels were specifically compared between QPP hybrids and original QPM and popcorn parents (Figure 11B). Significant increases in QPP lysine levels compared to the original popcorn parents were observed in all hybrids (Figure 11B). K0326Y and CML154Q maintained slightly higher lysine levels than QPP hybrids, though not always significant (Figure 11B). QPP Hybrids 43, 20, and 38 had the highest protein-bound lysine levels (0.589, 0.558, and 0.552 g/100g respectively) compared to CML154Q and K0326Y (0.629 and 0.589 g/100g, respectively) (Figure 11B, Table 5). Overall, the ten tested QPP hybrids had 1.45 and 3.86 fold increases in raw kernel, protein-bound and free lysine content over popcorn parents, respectively (Tables 5 and 6). Specifically, the five selected hybrids for further analysis (Hybrids 20, 25, 28, 38, and 43) held 1.52 and 4.45 fold increases in protein-bound and free, raw kernel lysine levels, verifying the biofortification of the popcorn proteome to pattern that of QPM.

As pedigree analysis of agronomic traits revealed a manifestation of heterosis due to genetic diversity, raw kernel protein-bound lysine levels were compared between QPP hybrids and their inbred parents (Figure 11C). An additive effect was observed in all cases except Hybrid 38 (Figure 11C). Hybrid 38 and Inbred 10's lysine levels were

significantly larger than Inbred 5, suggesting a dominant heterotic effect in this singular case (Table 5). However, with nine out of ten parental pairs holding an additive effect, the trend suggests that lysine level in QPP crosses can be moderately predicted. Similar comparative analysis between parents and crosses were conducted on all protein-bound amino acids, and over-dominant trends, or the synergistic effect of a heterozygous state of alleles to confer a superior phenotype, in this case elevated amino acid abundance, in the hybrid compared to the parental inbreds, were noted for alanine, arginine, aspartate/asparagine, histidine, leucine, and methionine (Shapira and David, 2016). Additive and/or dominant trends were suggested in glutamate/glutamine, glycine, phenylalanine, serine, and isoleucine, and exclusively additive trends were identified in proline, threonine, and tyrosine (Table 5). Though verifying effects would require additional testing, consistent trends in particular amino acids suggest moderate predictability of hybrid amino acid levels according to inbred values and could guide selective breeding accordingly.

The five chosen QPP hybrids and two popcorn parents were popped using air, oil, and microwave methods to identify correlations in amino acid changes between ground powder and several different popping methods. QPP hybrids maintained higher lysine levels than popcorn parents across all popping methods, though protein-bound and free lysine levels decreased to different extents when kernels were popped (Figure 13). Air popping appeared to result in the least loss of protein-bound lysine, decreasing contents on average by ~0.15 g/100g lysine (Figure 13A, Tables 5 and 8). Values suggested that microwave and oil popping decreased protein-bound lysine content more than air popping, though confidence intervals overlap (Figure 13A, Tables 8-10).

To ascertain the consistency in lysine loss due to popping methods, correlation coefficients were calculated between all four treatments – raw powder and microwave, oil, and air popping, and a highly correlative trend in lysine loss was observed ($p < 0.05$; Figure 13A). With such a consistent decrease in protein-bound lysine due to popping, all other amino acids were examined for uniformity and extent of decline. Most protein-bound amino acid levels correlated with a coefficient higher than 0.700 between ground powder, air, microwave, and popped methods. Proline, threonine, and asparagine/aspartate's oil method correlations, isoleucine and serine's oil method correlations to air and microwave popping, and almost all correlations in glycine and valine levels were low. The amount of change varied by amino acid, commonly increasing in abundance after popping by air and microwave methods (ex. glycine, isoleucine, and leucine; Tables 5, 8, 9, and 10). Though levels changed by varying percentages depending on amino acid and method, high correlations between raw kernel and air and microwave popped flake protein-bound amino acid values suggest a consistent effect of popping on protein-bound amino acid level variations (Tables 5, 8-10). Like lysine levels, most QPP protein-bound amino acids supported a similar trend of insignificantly different amounts in air and microwave popping methods and slightly lower abundances with varying levels of significance in oil-popped flakes (Tables 5, 9, 10, 11). Though confidence intervals were wide across popping methods and genotypes, comparative analysis between QPP hybrids and popcorn parents suggested that popcorn germplasm held higher protein-bound serine, phenylalanine, methionine, alanine, tyrosine, isoleucine, leucine, and glutamate/glutamine levels than QPP, while QPP hybrids exhibited higher levels of histidine, arginine, asparagine/aspartate, and lysine

levels than popcorn parents (Table 5). Ground samples of QPP hybrids that were not tested in the popped state also exhibited superior lysine levels compared to popcorn parents, and high correlations between raw kernel and popping methods suggest that all hybrids are superior in lysine levels regardless of popping method employed, a trend further exemplified in free amino acid levels (Figure 9A, 9B, and Figure 14A).

Free amino acid analysis revealed that QPP hybrids had a higher abundance of free amino acids in all residues except serine and methionine compared to popcorn parents (Tables 6, 11, 12, and 13). Like protein-bound values, free amino acid levels suggested similar trends in declined abundance after all popping methods, with cysteine and threonine values as exceptions (Figure 14A, Tables 6, 11, 12, and 13). Like protein-bound residues, high correlations (>0.7) were observed between almost all popping methods and raw powder in free amino acid comparisons, offering further confidence that popping has a reliable, consistent effect on the proteome and amino acid fluctuations. Unlike protein-bound values, free amino acids suggested a uniform trend in decreased residue abundance due to all popping methods (except threonine and cysteine; Figure 13B and Figure 14A). On average, QPP hybrids sustained a 0.0087 g/100g loss of free lysine and popcorn germplasm sustained a 0.0023 g/100g loss when air popped, 72.3% and 74% respectively of the raw kernel free lysine level (Figure 13B, Tables 5,6,8, and 11). Since QPM conveys the characteristic increase of essential amino acids lysine and tryptophan, free tryptophan levels of QPP hybrids were examined and held significantly superior levels compared to popcorn parents and, like protein-bound lysine, most hybrids held insignificantly different levels of free tryptophan compared to QPM (Figure 14B).

4. Discussion

4.1 The popcorn market: future prospects

U.S. consumer trends veering toward a more health-consciousness and continually fast-paced lifestyle have correlatively increased with the popcorn market, which is expected to grow at an annual rate of 7.6% over the next three years (Dawande, 2018). Popcorn producers have responded with more detailed labeling describing caloric intake, offering all-natural, clean label options, and introducing more flavor options to the consumer (Mordor Intelligence, 2018). Successful dent by popcorn crosses have resulted in improved agronomics with enhanced flavor profiles of the popped flakes; however, maintaining popability and expansion volume remains a key challenge (Crumbaker et al., 1949; Johnson and Eldredge, 1953; Robbins and Ashman, 1984). In this study, the use of Quality Protein Maize varieties in QPM by popcorn crosses had a triplicate effect of improving popcorn agronomics, seed protein quality, and rapidly restoring popability in subsequent inbred lines due to their selectively high level of vitreous endosperm (Figure 1, Ren et al., 2018).

4.2 Improved agronomics of Quality Protein Popcorn hybrids

Multiple QPP inbreds with different pedigrees were maintained throughout breeding to enable hybrid production (Table 1). Though inbreds have elevated lysine levels due to the successful introgression of the *opaque-2* allele and adequate popability, poor agronomics due to inbreeding depression, a common phenomenon in maize, disqualified the lines' capability for commercialization as inbreds. Once hybridized, we clearly observed agronomic heterosis in QPP crosses that increased overall ear weight while maintaining popcorn-like kernels (vitreous and small). QPP hybrids had a significantly higher germination rate, number of harvested ears, ear length, number of rows per ear,

and ear weight compared to the original popcorn parental inbred lines (Figure 3). Comparing QPP inbreds to popcorn inbreds, QPP inbreds had significantly longer ears and more kernel rows per ear, though 100-grain weight and ear weight were insignificantly different. Since original popcorn hybrids weren't required in this preliminary pre-screening, it cannot be certainly ascertained if QPP hybrids are superior in agronomics compared to original popcorn hybrids. The main aim of our Quality Protein Popcorn breeding program, the improvement in protein quality in QPP inbreds and hybrids, was able to be tested and confirmed at this point in our study. However, the selection of agronomic traits from the original QPM parent and kernel traits from the original popcorn parent suggests agronomically superior popcorn varieties, an assumption that will be tested in the upcoming field season.

Multiple previous maize breeding experiments have found correlations between plant, ear, and kernel agronomic traits (Yousuf and Saleem, 2001; Ross, 2002; Malik et al., 2005; Rafiq et al., 2010) . Similar to the correlations observed in our field trials, other studies have observed highly positive associations between overall grain yield, ear weight, 100-grain weight, number of rows per ear, and ear length, while other studies have suggested insignificant or negative correlations between some of these traits (Dass et al., 1990; Djordjevic and Ivanovic, 1996; Mandefro, 1998; Vasic et al., 2001; Hadji, 2004; Li et al., 2007; Yusuf, 2010; Bekel and Rao, 2014; Tulu, 2014; Ribeiro et al., 2016). Though conflicting results as to the nature and extent of agronomic correlations are not difficult to find in the literature, our study supported the prevailing notion of moderately positive correlations between ear and yield traits. Likewise, correlations found in this study between expansion volume and agronomic traits were negative, as has

been observed multiple times (Brunson, 1937; Dofing et al., 1991; Ziegler and Ashman, 1994; Pereira and Amaral Júnior, 2001; Daros et al., 2002; Li et al., 2002; Li et al., 2007; Dhliwayo; 2008; Li et al., 2008; Li et al., 2009). The genetic repeatability estimate for 100-grain weight was found at 0.683, a similar estimate to that found previously (Spaner et al., 1992). Likewise, the genetic repeatability estimate for EV was 0.582, in agreement with previous studies suggesting heritabilities of 0.61, 0.59, and 0.58 (Vasic et al., 2001; Coimbra et al., 2002; Table 7). The correlation and heritability agreement between our values and those previously observed provided confidence that, despite the occurrence of high variance on few traits, values were suitable for evaluation and downstream analysis and QPP hybrid selection (Table 3, Table 7, Figure 4). High correlations and heritabilities between ear weight and ear length coupled to strong correlations with 100-grain weight suggest that future trait analysis may only require measuring one value. The measurement of ear length as a representative agronomic trait in small-scale breeding analysis may be practical and efficient, especially considering the high genetic repeatability and low standard error observed in this study. Moreover, the prevailing, significant negative relationships between popcorn quality traits and all other agronomic traits suggests that selecting for EL and vitreousness may be a tangible, successful option to improve dent by popcorn cross agronomics while maintaining popcorn quality traits.

4.3 QPP hybrid evaluation and ranking

In our approach, we hypothesized that the preliminary screening of hybrids would provide adequate information to simultaneously estimate inbred and hybrid general and specific combining abilities and improve our hybrid ranking and intermediate selection through evaluating both hybrid and inbred potential. The elucidation of parental values

proved to be valuable when our ranking system's best hybrids held very similar pedigrees. To maintain germplasm diversity in future stages of selection, representative hybrids from similar crosses were chosen based on parental breeding values. As shown in Table 3, maternal parents 9 and 10 held higher agronomic combining abilities while paternal parents 5 and 6 suggested superior popcorn quality trait combining abilities. These values aided in determining the final selection of Hybrids 28 and 38 over their reciprocals Hybrids 19 and 9, respectively. We also recognized that the use of hybrid phenotypes to suggest inbred potential did not account for poor agronomics due to inbred depression. QPP Inbreds 7 and 8 have characteristically poor seed set and slightly retained dent kernel phenotype. However, both inbreds performed well as paternal parents for Hybrids 17 and 30 and no QPP hybrid displayed a dent kernel phenotype. The utilization of hybrid analysis for inbred potential enabled the superior hybrid expression of inferior inbred lines like Inbreds 7 and 8. The high ranking of Hybrids 17 and 30 demonstrated this advantage. In other commonly used breeding selection methods, such as recurrent selection, these inferior inbreds would have been selected against in the first year of the original selection cycle (Allard, 1960).

With analysis and selection of the best QPP hybrids as the primary goal in this analysis, we also explored the basic and applied aspects of heterosis within our 44 hybrids with respect to their genetic relationships. The pedigrees and probable genetic architectures of each QPP inbred line is well understood (Table 1). Hybrids with the same popcorn and QPM parental lines were named 'Pseudo-selfed' to describe the only available interaction of the same QPM and popcorn genomes. A double back-cross of the popcorn parent suggests an 87.5:12.5 ratio of popcorn:QPM genome in the BC_2 lines. Five generations

of selfing and marker-assisted and phenotypic selection of QPM genes and QPM and popcorn traits also warrants the probable homozygosity of a majority of the introgressed QPM genome, at minimum surrounding the *opaque-2* gene on Chromosome 7 and essential *o2* modifiers, when related lines are crossed (Holding et al., 2008; Holding et al., 2011; Babu et al., 2015). Thus, Hybrids 5, 16, 31, and 42 were categorically grouped as 'Pseudo-selfed' to describe the limited genetic diversity and interaction (Table 1). The hybrids with the 'Same Popcorn Background' were assumed to have more similar genetic composition than inbreds with the 'Same QPM Background' since inbreds were back-crossed twice to the original popcorn parent (Ren et al., 2018). Hybrids without similarity in either popcorn or QPM parents were further subdivided into 'Same Popcorn Heterotic Pool' and 'Different Heterotic Pool' categories. Popcorn Parents 2 and 3 are from the same heterotic pool, thus Hybrids 3, 4, 10, 11, 14, 15, 21, and 22 were categorized as hypothetically lesser in heterotic capacity than the rest of the hybrids interacting from different pools. Overall, these five groups of hybrids were tested for significant differences in agronomic trait values, and we observed a gradual trend in improved agronomics as groups became more genetically diverse (Figure 5). The most notable example of this gradual, step-wise trait improvement was observed in the number of ears harvested per row, followed by 100-grain weight (Figure 5A and 5B). The increased grain weight for QPP hybrids in different heterotic groups compared to hybrids in the same QPM background is more meaningful in light of inbred comparison, in that one hundred grain weight values for QPM inbreds were significantly higher than all popcorn related lines (Figure 5B, Figure 3E). This comparison demonstrated the efficacy of heterotic group delineation (Figure 3E, Figure 5B). The significant improvement in

ear length of hybrids with the same QPM background was surprising since QPM inbreds exhibited the shortest ears across all lines planted, and it may be an effect of extraneously improved plant agronomics in QPM dent corn backgrounds compared to popcorn backgrounds (Figure 5C; Figure 3C). The significant drag in ear length and number of kernel rows per ear in popcorn related lines attested to the primary selection of expansion volume over the course of popcorn breeding rather than agronomic capacity, and significant improvement in these traits was observed once lines were hybridized from different heterotic groups. Overall, this empirical trend supports the theory that heterosis is manifest on a genetic basic and the degree of expression is largely determined by genetic relatedness of the parents (Moll et al., 1965; Reif et al., 2003; Reif et al., 2005; Springer and Stupar, 2007; Fu et al., 2014). However, this progression of improvement was only observed for agronomic traits. Expansion volume and popability values in more popcorn-related lines were superior to those of unrelated pedigrees. Additionally, lysine contents of QPP crosses compared to those of their respective parents suggested an additive effect (Figure 8C). Though the underlying causes of these heterotic patterns have yet to be elucidated, grouping hybrids and observing this agronomic trend aided our eventual selection of hybrids to favor the 'complete hybrid' group.

Overall, these genetic analyses were used alongside a tailored ranking system for QPP hybrid selection. While selection indices are more commonly used for recurrent inbred selection, it was evident that a model was needed for our hybrid analysis. Such a model could properly manipulate the genetic potentials of multiple traits into a single sum that could accurately represent hybrid value (Tardin et al., 2007; Marinho et al. 2014). The ranking system utilized is similar to a Rank Summation Index in which each trait is

evaluated across hybrids, ranked independently, and then summed for a final ranking value (Mulamba and Mock, 1978, Figure 6). In our model, the economic value of each trait was partitioned through selection intensity coefficients and the genetic value was imputed through trait value and standard deviation (Table 4). This allowed for both an overall hybrid rank and the partitioning of rank value by trait, a distinction from other ranking systems (Figure 7). This simple model agreed well with concurrent analyses of our hybrids' genetic potential and elite hybrids were narrowed quickly. Due to Inbreds 9 and 10 having superior maternal agronomic capabilities, Hybrids 28 and 38 were chosen for continued analysis instead of their reciprocals. Hybrid 20 was also selected since it ranked well and the agronomic pGCAs for Inbred 10 were high. Hybrid 43 came from a relatively more diverse cross (Inbred 10 x Inbred 11), and notably had a consistent mushroom flake type (Figure 6). Popcorn hybrid flake types are commonly classified as either mushroom or butterfly (Eldredge and Thomas, 1959). Butterfly hybrid seed are commonly selected for packaging and can further be classified as unilateral, bilateral, or multilateral depending on the number and symmetry of flake branching, while popped mushroom hybrids are preferred as marketable products due to the minimized breakage during coating and packaging (Eldredge and Thomas, 1959; Sweley et al., 2011). This distinction in popped flake morphology compared to the other elite hybrids made Hybrid 43 a top contender for further analysis. Finally, to sustain diversity, Hybrids 30, 25, and 17 were considered for advancement. During this portion of analysis the relatively lower broad-sense heritability estimates, or the proportion of total phenotypic variance due to additive, dominant, and epistatic genetic effects, for inbred lines contrasted with higher repeatability estimates for SCA. Due to the use of hybrids to estimate inbred heritability

including non-additive effects, it is reasonable that SCA estimates had higher genetic repeatability and lower standard error. Moreover, since hybrids were being evaluated, all genetic effects were considered applicable for selection and SCA values became paramount in the selection of elite hybrids (Table 7). The highest repeatability estimates were identified for ear length and ear weight, though ear weight had a very high standard error. Both of these agronomic traits estimated high SCA values for Hybrid 25 compared to Hybrids 17 and 30, albeit not significant (Table 7). Expansion Volume SCAs for Hybrids 17 and 30 were superior to Hybrid 25 (0.582 repeatability with high standard error), but Hybrid 25 held a significantly better 100-grain weight (0.683 repeatability) and significantly larger kernel size (0.676 repeatability) compared to these two hybrids (Table 7). Hybrids 17 and 30 also included Inbreds 7 and 8 as paternal parents; QPP inbreds that were difficult to advance due to low inbred grain fill and sustained dent kernel phenotype. Hybrid 25 received low index sums for all traits except rot susceptibility, a less valuable trait outweighed by other highly-correlative traits to grain yield. Therefore, Hybrid 25 was ultimately selected for continued analysis. Other top hybrids had notable SCA values in agronomic and popcorn quality traits. Hybrids 20 and 28 held positive 2.6 and 2.7 (cm) values for SCA in ear length, Hybrid 43 had the highest SCA value for number of kernel rows per ear (2.265 rows, 0.673 repeatability), and Hybrids 20, 28, and 38 all had significantly large SCA values for expansion volume, estimated at 50.11, 48.94, and 57.98 mL/20g, respectively (Table 7). Due to superior agronomics and confirmed quality protein, as further described, Hybrids 20, 25, 28, 38, and 43 were chosen for continued analysis.

4.4 Elevated lysine content in QPP Hybrids across popping methods

In conjunction with hybrid selection through agronomic and popping evaluations, ten hybrids were chosen for amino acid profiling of free and protein-bound amino acids in the kernel. Previous temporal studies on maize endosperm protein quality have observed that lysine and tryptophan amino acid levels differentially decrease during kernel maturity with high variability between genetic backgrounds (Sethi et al., 2020). However, tryptophan and lysine levels within a genetic background correlate in relative abundance (Hernandez and Bates, 1969; Krivanek et al., 2007; Olakojo et al., 2007). Therefore, acidic hydrolysis, which destroys tryptophan, was conducted for protein-bound lysine determination. All free amino acids including tryptophan were recovered and measurable. Principle Component Analyses on protein-bound and free amino acid data demonstrated that the QPP proteome imitated that of QPM rather than the genetically dominating popcorn background (Figure 8A and Supplementary Figure 4). Genetic repeatability estimates including both additive and non-additive effects were calculated per genotype for raw kernel protein-bound amino acids. Eight out of the sixteen amino acids had high repeatability estimates above 0.700 (excluding isoleucine at 0.693), including lysine, histidine, leucine, methionine, and phenylalanine essential amino acids. The high repeatability measurement for lysine validated downstream selection for elevated levels. Ground raw kernel powder of the ten best QPP hybrids revealed an average 1.45 fold increase in protein-bound lysine, and the five selected QPP hybrids exhibited an average 1.52 fold increase in protein-bound lysine compared to popcorn germplasm (Table 5). These fold changes of increased lysine were similarly observed by Ren et al. with QPP inbreds, ranging from a 1.45-2.0 fold increase in the amino acid abundance compared to original popcorn inbreds (Ren et al., 2018). The

Food and Agriculture Organization of the United Nations recommends a 5.8% lysine requirement in total protein for children ages 2-5 for optimum health. During QPM hybrid production, QPM inbred pools conferred 2.7-4.5% lysine in total protein, an improvement from 1.6-2.6% in normal maize and considered an acceptable standard for 'Quality Protein' Maize. In this study, protein-bound lysine accounted for ~4.65% of total protein in QPP hybrids compared to ~2.65% in popcorn inbreds and surpassed the previously cited range for QPM breeding pools (Vasal, 2002; Krivanek et al., 2006; Table 5).

Additionally throughout CIMMYT's breeding of QPM, researchers understood the necessity of monitoring the lysine and tryptophan content of raw, whole grain flour and consumable products such as nixtamal, masa, and tortillas. After quantification, researchers found an overall significant decrease in tryptophan and both significant and insignificant losses of lysine in all consumable products (Vasal et al., 1986). However, this trend was general to all tested maize lines and QPM was legitimized as effective in conferring elevated lysine and tryptophan levels in the cooked, consumable products (Ortega et al., 1986). Since popcorn is consumed by humans after popping, popped flake amino acid levels were of paramount importance to evaluate and measurements are sparse in the literature. The last available amino acid profile of oil- and air- popped popcorn was in 1991 (Cutrufelli, 1991). Popping effect on amino acid content, correlations between raw kernel flour and that of popped flakes, and specific effect of each popping mechanism have remained unexplored. Analysis on popped flakes revealed a general trend in free amino acid level decrease, while protein-bound amino acid fluctuations were dependent on the residue. Histidine, isoleucine, leucine, lysine,

methionine, phenylalanine, threonine, and valine are considered essential amino acids because they are not synthesized by the human body in adequate amounts for maintained human health (Wu, 2009). After popping by air or microwave methods, all quantified essential amino acids except lysine and methionine increased in protein-bound abundance compared to raw kernel flour while oil-popped flakes decreased the abundance of all protein-bound amino acids, though confidence intervals overlapped (Tables 5, 8-10). These results suggest that air and microwave popping may not affect amino acid composition or abundance as severely as oil popped methods. Furthermore, protein-bound lysine was the only essential amino acid to significantly decrease after popping (Tables 5, 8-10). With lysine already the most limiting amino acid in maize grain, this observation reinforced the requirement for elevated lysine in the popcorn kernel to convey higher abundance in the popped flake (Alan, 2009). The increase in both lysine and tryptophan abundance compared to popcorn parents, maintained before and after popping by various methods, ultimately validated the proteomic biofortification of the Quality Protein Popcorn endosperm in its raw and popped form. On average, QPP air popped flakes offered more lysine than original popcorn parent raw kernel flour and approximately two times more lysine than original parent air popped flakes. In context, the recommended intake of lysine is ~30 milligrams per kilogram of body weight per day, which converts to approximately 2.108 grams per day for a 68 kilogram (150 pound) individual (Elango et al, 2009). Microwavable popcorn packets use ~47 grams of popcorn kernels per bag. When air popped, one bag of QPP hybrids would fulfill ~8.6% of lysine daily dietary requirement while original popcorn parents would only satisfy ~4.3% (Tables 8 and 11).

With these raw and popped kernel amino acid values, we are confident that QPP hybrids are successfully yielding the characteristic *opaque-2* endosperm proteome while maintaining popability and improving popcorn agronomics. As introgressing dent germplasm into popcorn has been previously difficult, we suggest a prerequisite phenotype of highly vitreous dent endosperm for future dent by popcorn crosses that aim to restore and maintain popcorn quality traits. This phenotype was key for rapid restoration of QPP popability. Once at the inbred stage, hybrid production and analysis of QPP lines was necessary to improve agronomics. The integration of inbred and hybrid analysis proved helpful in the final determination of our elite QPP hybrids and is transferable to various other breeding programs involved in hybrid testing and selection.

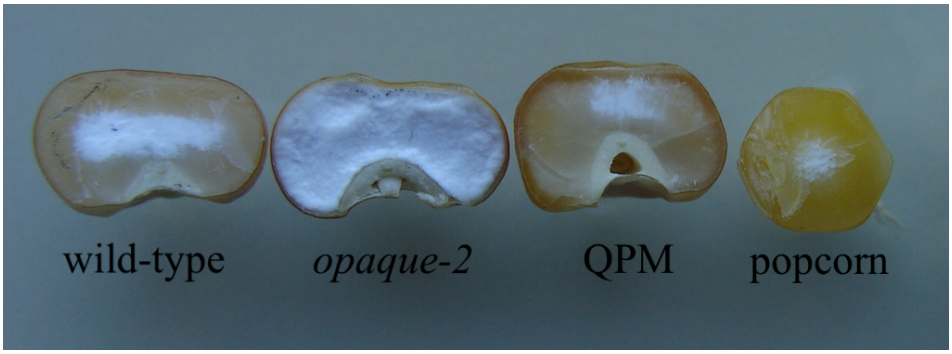


FIGURE 1 | Comparative Endosperm Vitreousness in Dent Corn and Popcorn Backgrounds. Wild-type, *opaque-2,* and modified *opaque-2* maize kernels are from dent backgrounds. QPM has a more vitreous endosperm, like popcorn, than other dent germplasm. Popcorn has very little chalky endosperm and a round kernel morphology, determinant characteristics for popping.

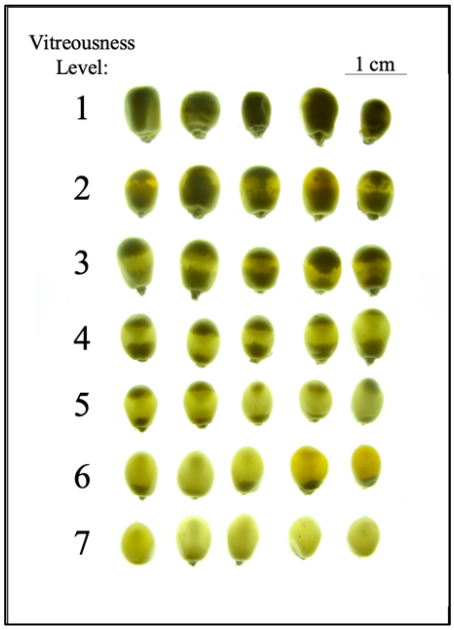


Figure 2 | Popcorn kernel endosperm vitreousness scale. Ten grams of kernels were randomly selected from each row of the 2019 field and scored on a continuous scale of 1-7, with a rank of '1' being nearly complete opacity and '7' as completely vitreous.

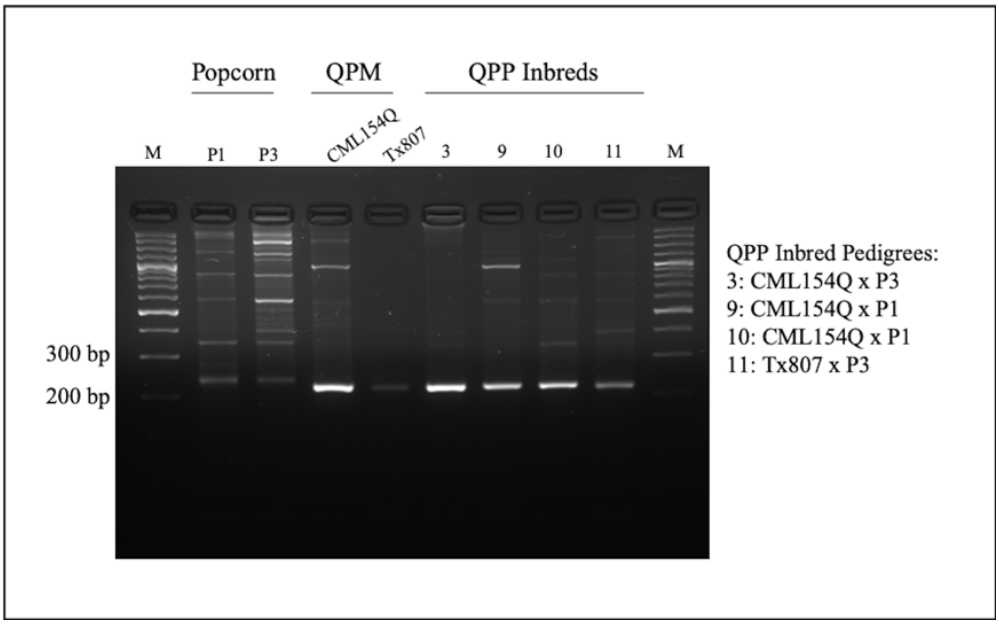


Figure 3 | DNA-based marker aided verification of *o2o2* genotype in parental inbreds. All QPP inbred parents were genotyped with *opaque-2* in-gene marker umc1066 and/or flanking marker bnlg1200. As shown, popcorn parents encode a differentiated, wild-type *opaque2* allele while QPM parents have a lower band. All QPP inbreds shown are crosses between Popcorn Parent 1, Popcorn Parent 3, and CML154Q and Tx807. All inbreds displayed the alike lower band to QPM parents.

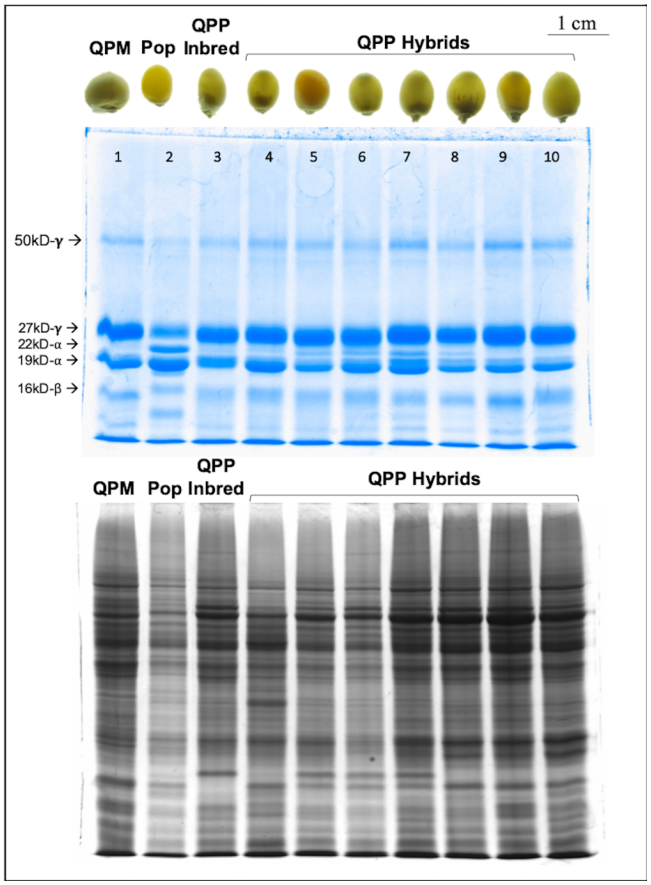


FIGURE 4 | SDS-PAGE gel of Random QPP Hybrids Verifying *o2o2* Genotype. Semi-quantitative zein and non-zein extractions of random QPP hybrid kernels displayed QPM-patterned proteomes. **(A)** QPP kernels 4-10 displayed a near complete knock-down of 22kd-α zein synthesis and uniformly increased synthesis of the 27kd-**γ** zein, confirming the maintenance of *o2o2* genotype from previously established inbreds. **(B)** Kernels 1 (CML154Q) and 3 (QPP Inbred 10) displayed an overall increase in non-zein production compared to Kernel 2 (Popcorn Parent 1). Random QPP hybrid kernels also displayed this trend, suggesting heightened lysine levels in the kernel due to the selected mutation. PCR verification of *o2o2* genotype in QPP inbreds is shown in **Figure 3.**

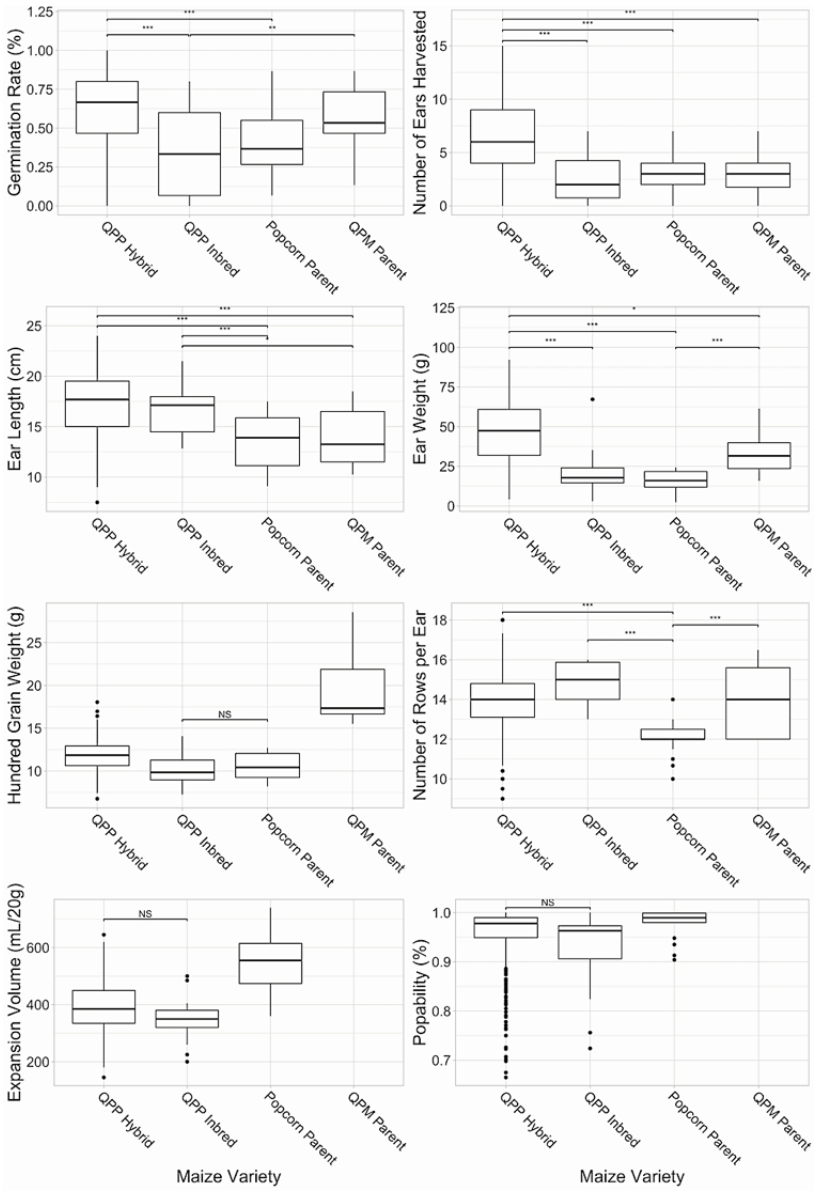
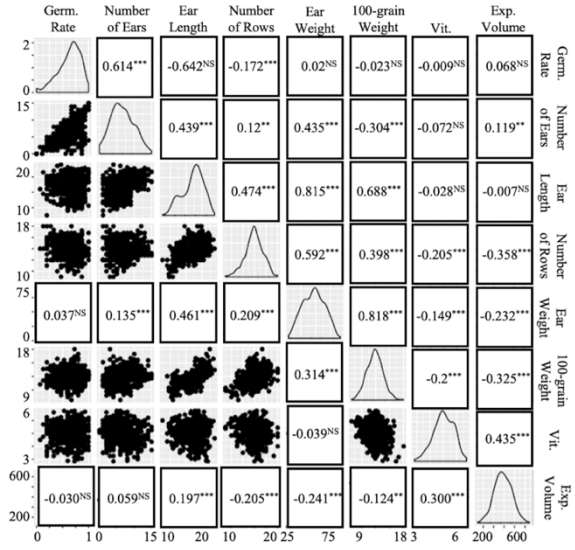


FIGURE 5 | Comparison of QPP Hybrids and Inbreds in Agronomic and Popcorn Quality Traits. Six agronomic and two popcorn quality traits were compared between QPP hybrids and QPP, popcorn, and QPM inbreds. **(A)** Germination rate, **(B)** Number of ears harvested from single rows, **(C)** Ear lengths, **(D)** Ear weight, **(E)** Hundred grain weight, **(F)** Number of kernel rows per ear, **(G)** Expansion volume, and **(H)** Popability were compared. Popping traits were not available for QPM dent inbreds. Significant differences were noted at the $p < 0.001$, 0.001, and 0.01 levels as '***', '**', and '*', respectively. 'NS' denoted non-significant comparisons between groups if all other comparisons were significant. Whisker length signify range of values, boxes signify upper and lower quartiles, and the horizontal line denotes average value.



Germ. Rate (%)	Number of Ears	Ear Length (cm)	Number of Rows	Ear Weight (g)	100-grain Weight (g)	Vit. Level	Exp. Volume (mL/20g)	
0.039	0.357	-0.047	-0.057	0.018	-0.007	0.001	1.273	Germ. Rate
	9.952	4.457	0.526	24.067	1.643	-0.147	33.276	Number of Ears
		10.626	2.392	48.096	3.850	-0.070	-1.900	Ear Length
			2.369	16.283	1.058	-0.232	-45.05	Number of Rows
				324.08	25.335	-2.039	-333.8	Ear Weight
					2.957	-0.269	-45.30	100-grain Weight
						0.576	27.139	Vit. Level
							6762.5	Exp. Volume

FIGURE 6 | Correlations and Covariances of Agronomic and Popping Traits. High covariances and correlations were observed between multiple agronomic traits. **(A)** Agronomic and Popping Trait Correlations. Diagonal line graphs show normality of trait data. Traits correlate according to x- and y- axis labels. Dot plots under the diagonal show simple regression of traits in x-, y- columns and rows. Standardized values in replacement of dot plots under diagonal were obtained by using a path analysis. Values above the diagonal are Pearson's Correlation Coefficients of gridded, corresponding traits. Levels of significance: $p < 0.0001$ '***', $p < 0.001$ '**', $p < 0.05$ 'NS'. **(B)** Agronomic and Popping Trait Variances and Covariances. Covariances of traits according to row and column labeling in gridded fashion are shown above the shaded diagonal. Trait variance is described in shaded diagonal are in trait units shown on horizontal labels.

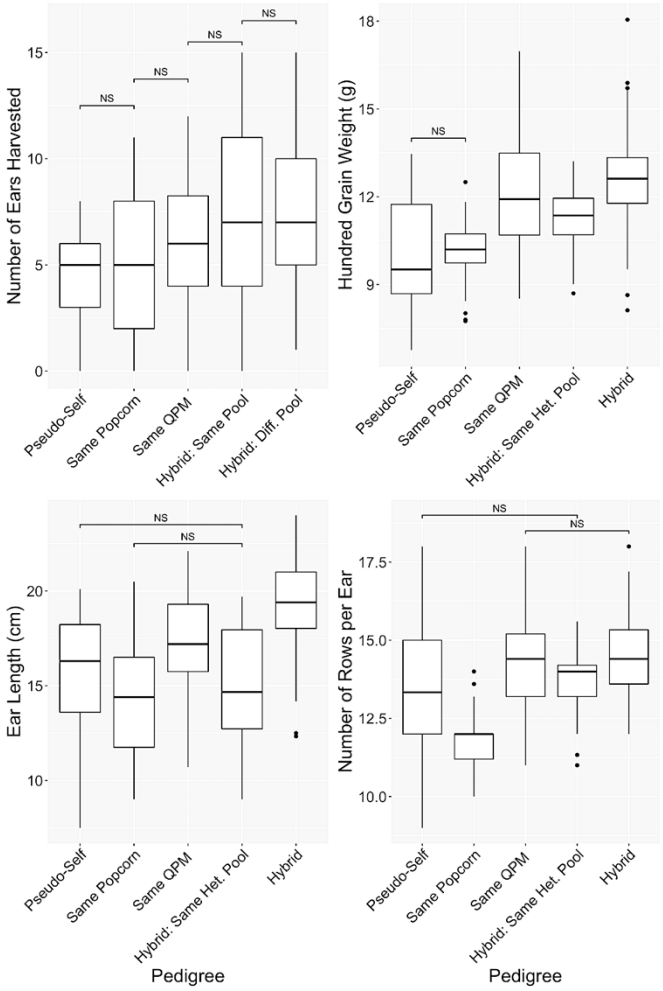


FIGURE 7 | Manifestation of hybrid vigor through pedigree analysis. Pedigree-based categorical grouping of hybrids for agronomic comparison. In order of increasing genetic diversity, hybrids were sorted into 'Pseudo-self', 'Same Popcorn', 'Same QPM', 'Hybrid: Same Het. Pool', and 'Hybrid' categories. Traits analyzed were (A) Number of ears harvested per row, (B) 100-grain weight (g), (C) Ear length (cm), and (D) Number of rows per ear. 'NS' denoted non-significant comparisons between groups with all other comparisons as significant. Whisker length signify range of values, boxes signify upper and lower quartiles, and the horizontal line denotes average value.

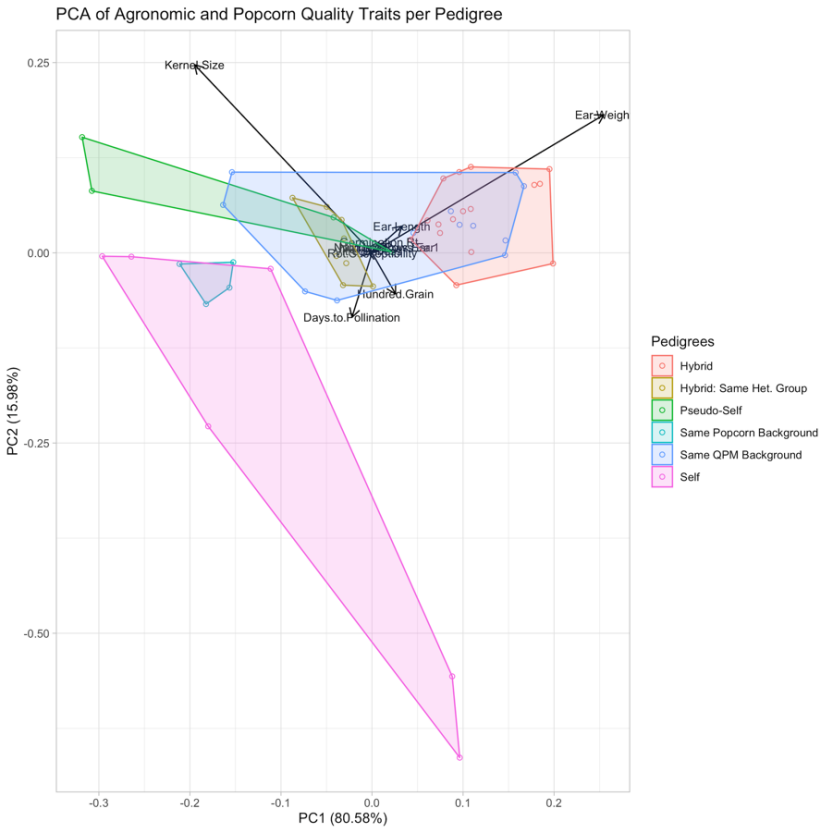


Figure 8 | Principle Component Analysis of QPP Hybrids, Inbreds, QPM, and Popcorn Parents Grown in 2019 fields. Principle Component scores (PC1 and PC2) from each variable are described as text in plot. Six clusters of pedigree categories (Self, Pseudo-self, Same Popcorn Background, Same QPM Background, Hybrid: Same Het. Group, and Hybrid) were observed.

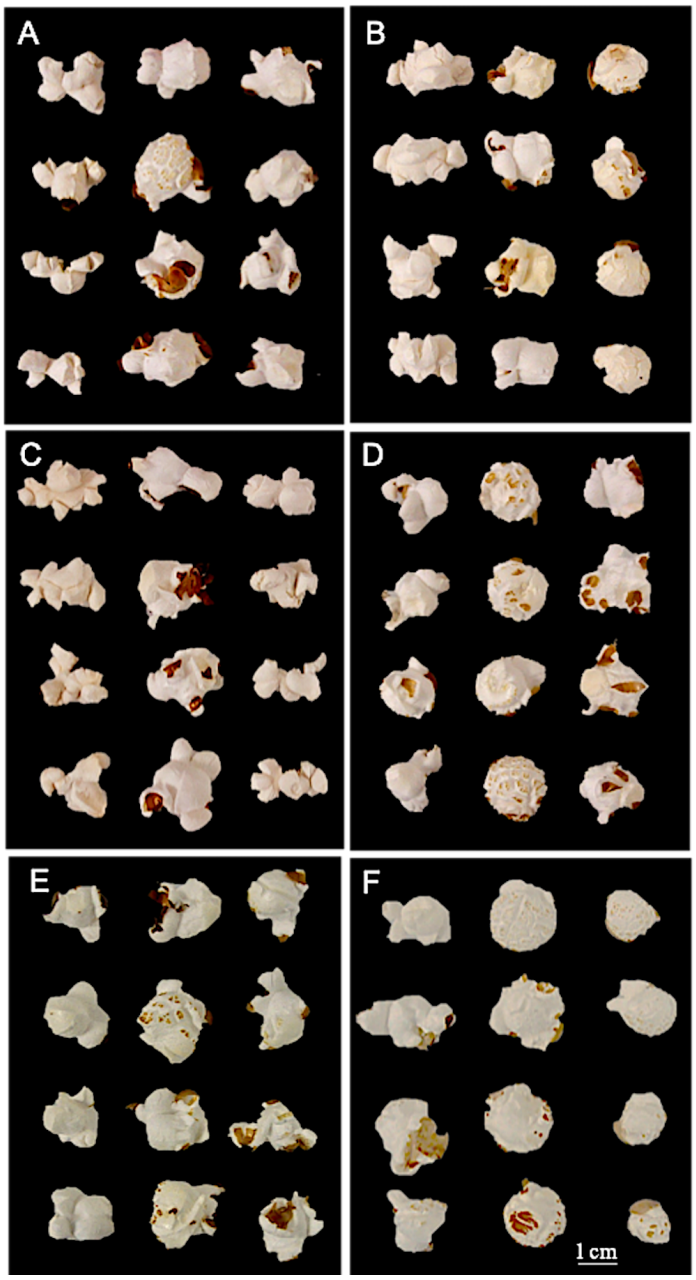


FIGURE 9 | Inbred and Hybrid Flake Morphology. **(A)** First column: maternal parent 6 (bilateral morphology); Second column: Hybrid 20; Third column: paternal parent 10. **(B)** First column: maternal parent 9; Second column: Hybrid 25 (unilateral morphology); Third column: paternal parent 3. **(C)** First column: maternal parent 9; Second column: Hybrid 28 (multilateral morphology); Third column: paternal parent 6. **(D)** First column: maternal parent 10; Second column: Hybrid 34 (mushroom morphology); Third column: paternal parent 1. **(E)** First column: maternal parent 10; Second column: Hybrid 38; Third column: paternal parent 5. **(F)** First column: maternal parent 10; Second column: Hybrid 43; Third column: paternal parent 11.

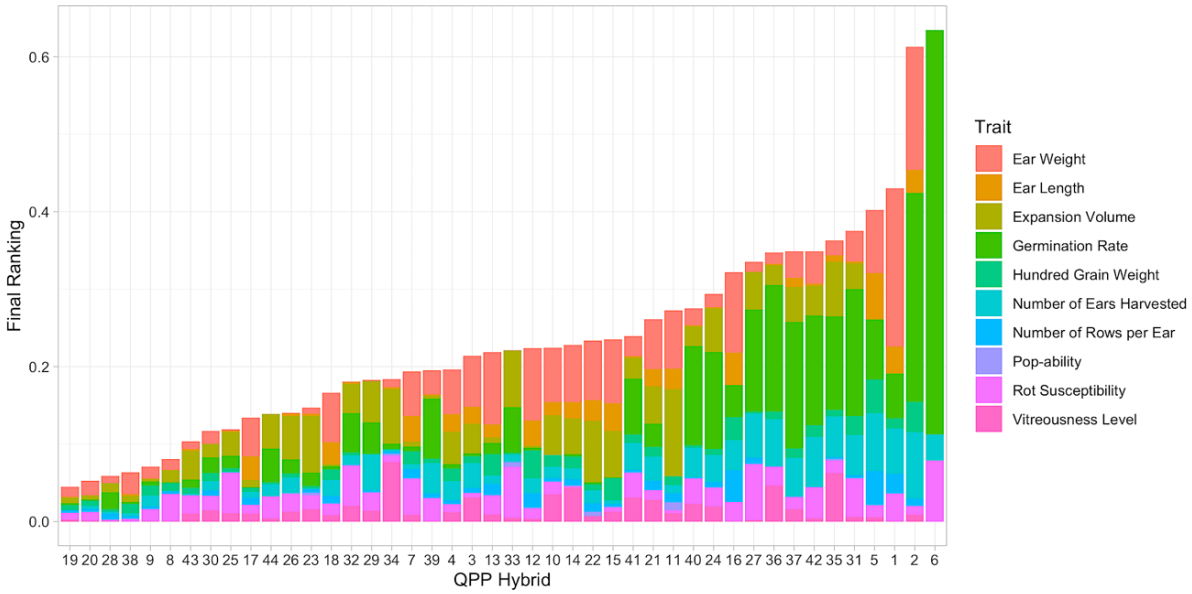


FIGURE 10 | Categorized Results from Hybrid Ranking Model. Elite hybrids determined from the Ranking Model are listed from left to right as summed ranking value increases. Lower score indicates less distance from maximum trait value, i.e., Hybrid 19 ranked best compared to all hybrids. Stacked bars represent individual trait influence on each hybrid's overall rank.

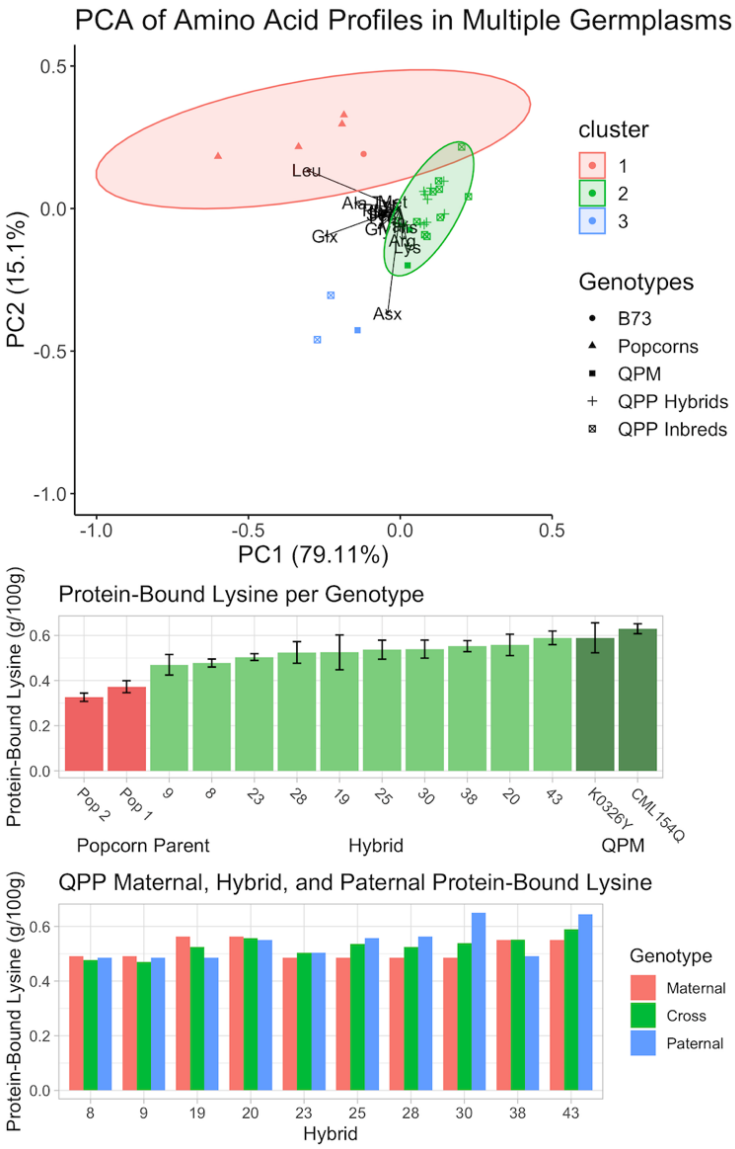


FIGURE 11 | Analysis of protein-bound amino acid composition in various genotypes in flour from raw kernels. **(A)** Principle Component Analysis of protein-bound amino acids in ground powder of B73, QPP Inbreds, QPP Hybrids, Popcorn, and QPM germplasms. Various shapes represent different germplasms. **(B)** Protein-bound lysine (g/100g) of two popcorn parents, two QPM parents, and 10 QPP hybrids with standard deviation error bars. **(C)** Protein-bound lysine (g/100g) of QPP hybrids and respective maternal and paternal parents. Standard errors are not shown and available in **Table 5 (end of chapter).**

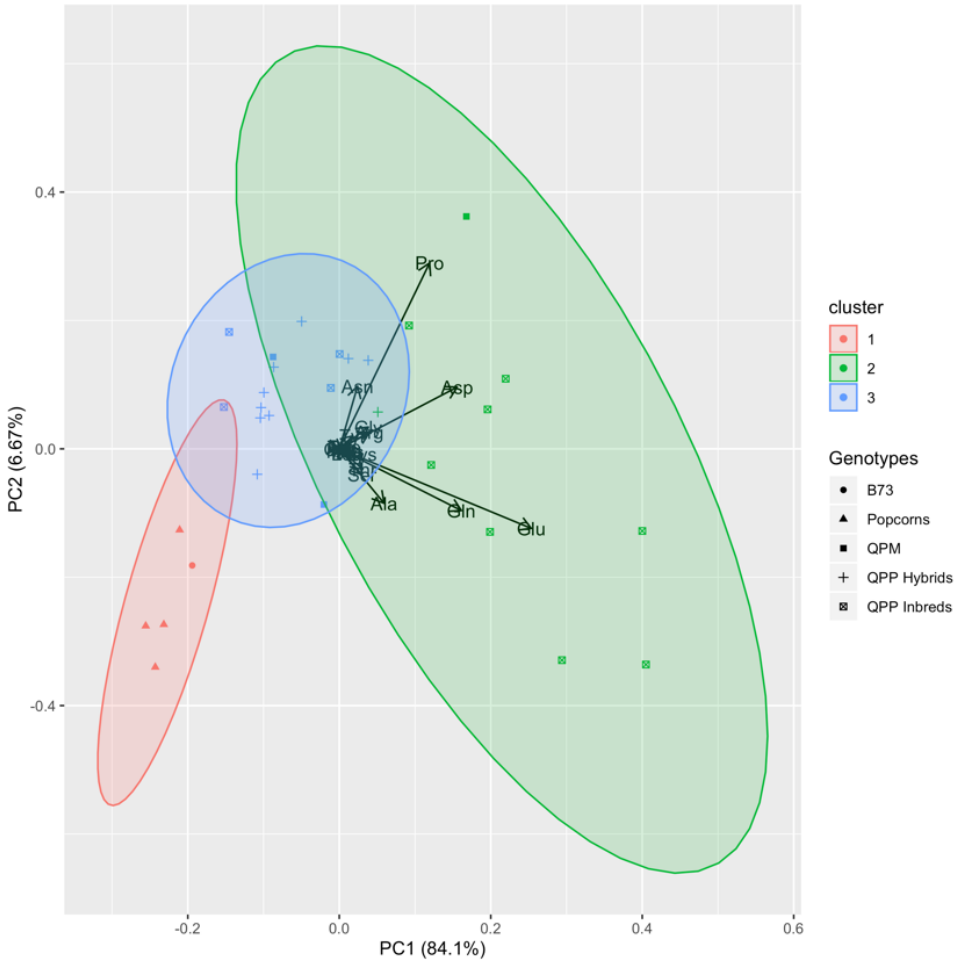


Figure 12 | Principle Component Analysis of Free Amino Acids from raw Kernel Flour in Multiple Germplasms. All amino acids were available for quantification in free form. Three clusters arose from the data; one of popcorn parents (red), one of QPP hybrids (blue), and one of QPP inbreds (green). Inbreds were characterized with higher proline, aspartate, glutamate, and glutamine levels. QPP hybrids overlapped with both clusters though most overlay occurred between QPP Inbreds and Hybrids. QPM inbreds were present in both QPP inbred and hybrid clusters.

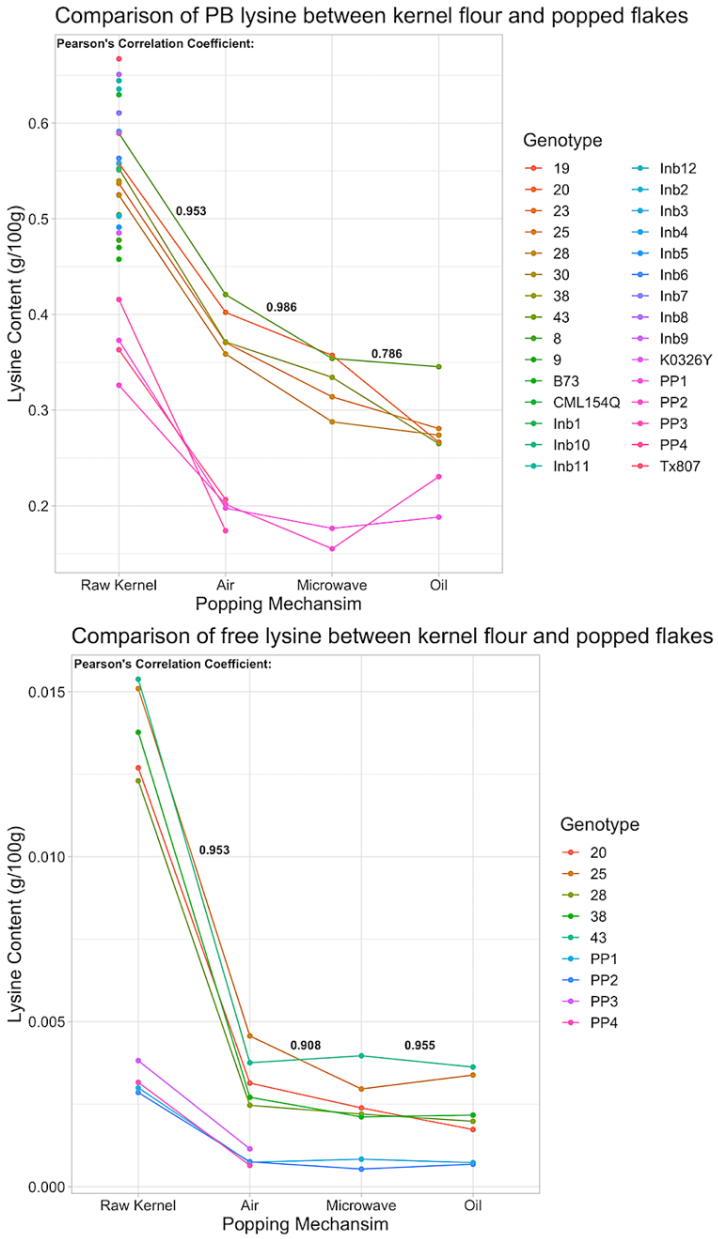


FIGURE 13 | Protein-bound and free lysine content of QPP Hybrids, Inbreds, QPM, and Popcorn Germplasm in raw kernel and popped flakes. **(A)** Protein-bound lysine content (g/100g) in various germplasm samples under air, microwave, or oil popping conditions compared to raw kernel powder. Points along vertical 'Raw Kernel' axis are lysine levels from germplasm that was not popped. **(B)** Free lysine (g/100g) in multiple germplasm samples under air, microwave, or oil popping conditions compared to raw kernel powder. Correlation Coefficients between protein-bound and free lysine levels in raw kernel and air popped flakes, air popped flakes and microwaved flakes, and microwaved flakes and oil popped flakes were calculated and are in respective positions in bold. Genotypes with solely a numbered label signify QPP hybrids, QPP Inbreds are named 'Inb' preceding inbred number, and 'PP1'- 'PP4' represent 'Popcorn Parent 1-4', respectively.

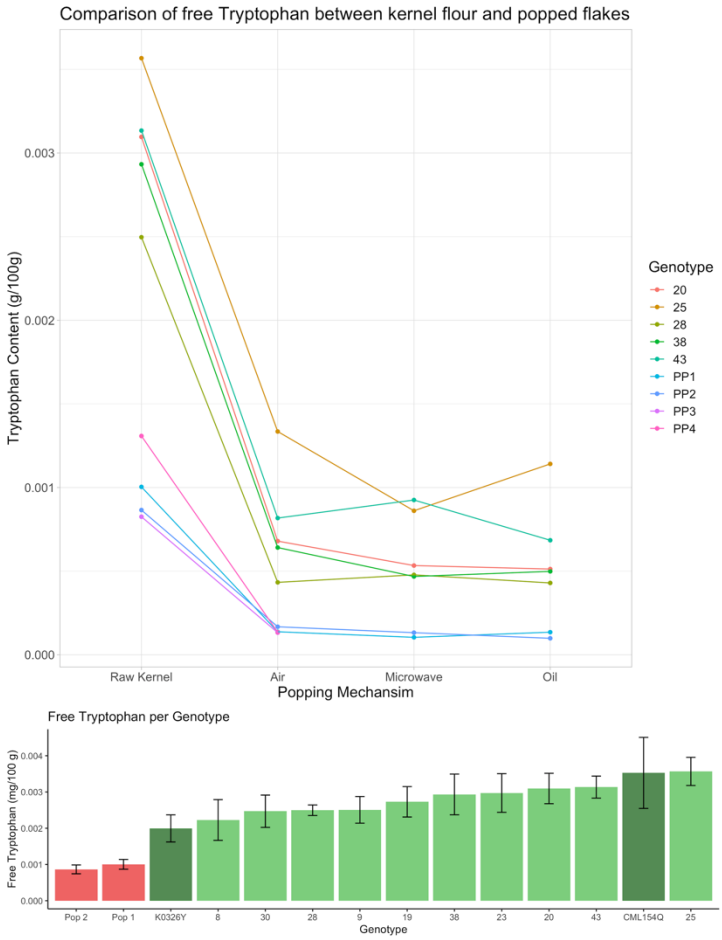


Figure 14 | Free Tryptophan Values and Effect of Popping Methods. **(A)** Alike to protein-bound and free lysine, free tryptophan values from raw kernel flour decreased at a similar rate when popped by multiple methods and correlation coefficients were high (range of 0.882 – 0.992). **(B)** All QPP hybrids (light green) held larger raw kernel flour free-tryptophan values than popcorn parents (red) and potentially QPM parents (dark green). At minimum, QPP hybrids were insignificantly different in free tryptophan content than QPM parents.

		CML154Q x Popcorn Parent 2		*CML154Q x Popcorn Parent 3*		*K0326Y x Popcorn Parent 2*		*K0326Y x Popcorn Parent 4*		*CML154Q x Popcorn Parent 1*		*Tx807 x Popcorn Parent 3*	
	Inbred	1	2	3	4	5	6	7	8	9	10	11	12
K0326Y x Popcorn Parent 2	5	1	2	3	4		5	6	7	8	9	10	11
	6	12	13	14	15	16		17	18	19	20	21	22
CML154Q x Popcorn Parent 1	9	23	24	25	26	27	28	29	30		31	32	33
	10	34	35	36	37	38	39	40	41	42		43	44

Pseudo-selfed
Same QPM Background
Same Popcorn Background
Same Popcorn Heterotic Pool
Different Popcorn Heterotic Pool

TABLE 1 | Depiction of Inbred Lines, Hybrids, and Pedigrees. Maternal parents shown in left two columns with pedigree history and Inbred number. Paternal parents shown horizontally in top two rows with pedigree history and Inbred number. Forty-four produced hybrids depicted as gridded squares and categorized by color according to pedigree.

Inbred Parents	1	2	3	4	5	6	7	8	9	10	11	12
5	*S,u*	*U,m*	*S*	*S,u*		*U*	*U*	*S,u*	*U*	*S,u*	*S*	*U,m*
6	*S,u*	*U,m*	*S,u*	*S,u*	*U,s*		*M,s*	*U,s*	*M*	*U,m*	*U,s*	*S*
9	*U*	*U*	*U*	*U*	*M*	*U,m*	*U*	*U,s*		*U*	*U,s*	*S*
10	*S*	*S*	*S,u*	*S,u*	*U*	*U*	*U*	*U*	*U,m*		*S*	*S,u*

TABLE 2 | Flake Morphologies in Hybrid Popped Flakes. One sample of 20 grams of popped kernels were examined and flake types assigned for each hybrid. 'S' is Mushroom morphology. 'U' is Unilateral morphology. 'M' is multilateral morphology. Capital lettering suggests the prevailing flake type, while lower-case suggests a secondary flake type, if applicable.

	Inbred	Germination Rate (%)	Days to Pollinating	Rot Susceptibility (%)	Number of Ears Harvested Per Rows	Ear Length (cm)	Number of Rows per Ear	Ear Weight (g)	Kernel Size (#/10g)	Vitreousness Level	Hundred Grain Weight (g)	Exp. Volume (mL/20g)	Pop-ability (%)
mGCA	**5**	0.012	0.915	0.020	-0.653	-1.419	-0.680	-9.033	7.407	0.110	-1.030	25.788	0.002
	6	0.114	0.647	0.064	0.635	-1.715	-0.897	-10.186	6.089	0.055	-0.764	30.836	0.006
	9	-0.038	-0.708	-0.075	0.374	1.950	0.465	11.841	-7.732	-0.048	1.084	-35.859	-0.010
	10	-0.088	-0.854	-0.010	-0.356	1.184	1.112	7.378	-5.764	-0.118	0.709	-20.765	0.003
Standard Error		0.006	0.753	0.004	0.393	2.82	0.758	105.377	51.430	0.015	0.911	966.98	0.000
Heritability		0.163	0.123	0.059	0.049	0.432	0.358	0.448	0.368	0.024	0.415	0.173	0.026
pGCA	**1**	0.048	0.220	0.000	0.519	-1.283	-1.235	-3.867	-0.135	-0.148	0.051	18.245	-0.009
	2	-0.122	1.554	0.000	-1.293	-0.334	-0.776	-5.470	2.646	-0.315	-0.258	4.426	-0.008
	3	0.013	-0.914	0.000	0.208	-0.215	0.658	2.234	-1.423	-0.682	0.074	-25.857	0.001
	4	0.010	0.736	0.000	0.070	-1.066	0.837	1.537	0.669	-0.281	-0.134	-28.950	-0.005
	5	-0.006	-0.139	0.000	-0.150	-0.023	-0.982	-4.466	5.214	0.798	-0.519	53.808	0.014
	6	-0.050	0.300	0.000	-0.499	-0.042	-0.888	-4.474	4.136	0.698	-0.500	72.878	0.018
	7	-0.131	0.674	0.000	-1.689	0.401	0.267	3.179	-6.910	-0.404	0.989	-26.492	0.004
	8	0.048	1.105	0.000	0.346	-0.611	-0.307	-4.625	5.485	-0.266	-0.765	25.927	0.003
	9	0.039	-1.315	0.000	0.697	1.330	0.561	4.611	-2.976	0.578	0.297	9.362	0.009
	10	0.023	-1.126	0.000	0.306	1.670	0.523	2.197	-0.583	0.494	0.027	33.080	0.020
	11	0.075	-0.688	0.000	0.795	-0.343	0.920	3.512	-1.237	-0.531	0.067	-57.287	-0.009
	12	0.054	-0.407	0.000	0.691	0.515	0.422	5.632	-4.887	0.059	0.671	-79.139	-0.039
Standard Error		0.003	0.473	0.00	0.401	0.447	0.298	11.77	9.07	0.119	0.146	947.81	0.0001
Heritability		0.117	0.138	0.000	0.092	0.124	0.274	0.086	0.115	0.445	0.119	0.322	0.123

TABLE 3 | maternal and paternal General Combining Abilities and Broad-Sense Heritability of all Traits. mGCA and pGCA values for all traits are listed as columns with broad-sense heritability estimates shown in gray. All combining ability estimates are in units according to trait calculated.

Trait	*Weight Value (I_i)*
Germination Rate (%)	0.7
Days to Pollination (days)	0
Pest/Rot Susceptibility	0.5
Number of Ears Harvested	0.6
Ear Length (cm)	0.5
Number of Rows per Ear	0.4
Ear Weight (g)	0.8
Kernel Size	0
100-Grain Weight	0.7
Vitreousness	0.6
Pop-ability	0.85
Expansion Volume (mL/g)	0.85

TABLE 4 | Relative trait weighting values for ranking model. Traits were ranked according to economic value with scores ranging from 0-1 in increasing importance. Popcorn quality traits were ranked highest followed by yield and agronomic traits. Number of Days to Pollination was not used to determine rank since it held minimal economic value. Kernel size was a repetitive measure of 100-grain weight and was not used to rank hybrids.

Genotype	Ala	Arg	Asx	Glx	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
Popcorn Parent 1	1.101±0.052	0.358±0.023	0.793±0.056	2.751±0.075	0.952±0.08	0.452±0.014	0.647±0.044	2.084±0.123	0.372±0.026	0.257±0.021	0.71±0.014	1.267±0.058	0.692±0.03	0.589±0.049	0.461±0.034	0.557±0.02
Popcorn Parent 2	0.963±0.126	0.362±0.025	0.664±0.047	2.449±0.201	0.886±0.115	0.394±0.015	0.58±0.059	1.852±0.177	0.326±0.018	0.194±0.02	0.652±0.051	1.1±0.069	0.638±0.061	0.5±0.042	0.379±0.035	0.498±0.032
Popcorn Parent 3	0.996±0.012	0.354±0.002	0.685±0.006	2.446±0.048	0.804±0.009	0.385±0.015	0.563±0.004	1.848±0.042	0.415±0.015	0.196±0.004	0.667±0.027	1.216±0.003	0.658±0	0.591±0.034	0.413±0.037	0.5±0
Popcorn Parent 4	1.385±0.035	0.402±0.017	0.861±0.002	3.145±0.189	1.222±0.035	0.458±0.017	0.802±0.02	2.617±0.171	0.363±0.049	0.236±0.009	0.817±0.05	1.321±0.077	0.832±0.04	0.628±0.038	0.574±0.008	0.662±0.012
B73	0.92±0.041	0.46±0.02	0.735±0.041	2.369±0.108	0.924±0.07	0.439±0.017	0.554±0.041	1.65±0.096	0.457±0.033	0.209±0.007	0.636±0.025	1.078±0.054	0.64±0.023	0.523±0.031	0.373±0.006	0.538±0.028
CML154Q	0.754±0.058	0.57±0.033	1.098±0.133	2.233±0.161	0.93±0.029	0.541±0.026	0.509±0.051	1.187±0.173	0.629±0.022	0.153±0.007	0.557±0.037	1.183±0.102	0.586±0.04	0.548±0.04	0.311±0.034	0.6±0.007
K0326Y	0.751±0.107	0.585±0.057	0.955±0.185	2.16±0.213	0.866±0.125	0.576±0.024	0.487±0.047	1.257±0.141	0.589±0.066	0.166±0.013	0.545±0.05	1.168±0.088	0.611±0.069	0.543±0.062	0.307±0.023	0.603±0.052
Tx807	0.874±0.109	0.632±0.046	1.496±0.026	2.447±0.097	0.99±0.032	0.571±0.019	0.609±0.059	1.56±0.231	0.667±0.002	0.15±0.009	0.663±0.046	1.132±0.08	0.689±0.053	0.604±0.049	0.351±0.016	0.63±0.023
QPP Inbred 1	0.674±0.05	0.401±0.045	0.967±0.026	2.247±0.043	0.876±0.104	0.527±0.031	0.448±0.034	1.166±0.115	0.503±0.018	0.122±0.011	0.503±0.033	1.12±0.032	0.518±0.024	0.483±0.009	0.302±0.034	0.53±0.042
QPP Inbred 2	0.57±0.033	0.489±0.046	0.869±0.151	1.828±0.158	0.724±0.076	0.486±0.015	0.378±0.027	0.919±0.112	0.502±0.044	0.111±0.005	0.446±0.064	0.98±0.05	0.485±0.042	0.43±0.051	0.251±0.011	0.488±0.031
QPP Inbred 3	0.722±0.071	0.449±0.038	0.985±0.208	2.258±0.011	0.797±0.052	0.504±0.037	0.457±0.062	1.052±0.16	0.557±0.06	0.133±0.005	0.49±0.073	1.014±0.003	0.53±0.065	0.531±0.047	0.274±0.012	0.538±0.051
QPP Inbred 4	0.67±0.066	0.454±0.001	0.908±0.053	2.33±0.044	0.935±0.036	0.486±0.017	0.424±0.031	0.969±0.172	0.591±0.04	0.14±0.006	0.463±0.013	1.059±0.085	0.521±0.033	0.523±0.005	0.293±0	0.527±0.014
QPP Inbred 5	0.599±0.045	0.427±0.011	0.8±0.014	2.07±0.083	0.803±0.027	0.523±0.014	0.414±0.05	1.073±0.135	0.491±0.008	0.11±0.004	0.476±0.063	1.131±0.049	0.504±0.042	0.467±0.028	0.273±0.052	0.52±0.015
QPP Inbred 6	0.684±0.089	0.448±0.036	0.801±0.011	2.016±0.02	0.803±0.071	0.546±0.012	0.47±0.034	1.128±0.055	0.563±0.047	0.104±0.005	0.514±0.062	1.167±0.017	0.539±0.033	0.497±0.046	0.309±0.023	0.564±0.041
QPP Inbred 7	1.045±0.031	0.546±0.024	1.512±0.033	2.847±0.074	1.13±0.105	0.539±0.016	0.62±0.013	1.674±0.024	0.61±0.007	0.127±0	0.698±0	1.211±0.029	0.696±0.033	0.622±0.005	0.386±0.039	0.641±0.002
QPP Inbred 8	0.951±0.087	0.638±0.017	1.27±0.021	2.603±0.158	1.033±0.091	0.606±0.023	0.656±0.068	1.726±0.251	0.65±0.02	0.154±0.002	0.692±0.102	1.367±0.075	0.73±0.114	0.646±0.058	0.392±0.074	0.717±0.036
QPP Inbred 9	0.617±0.065	0.424±0.055	0.64±0.007	1.766±0.132	0.782±0.009	0.512±0.037	0.414±0.026	1.034±0.103	0.485±0.018	0.106±0.014	0.451±0.034	1.027±0.093	0.485±0.046	0.461±0.027	0.274±0.015	0.525±0.038
QPP Inbred 10	0.643±0.023	0.454±0.066	0.785±0.039	1.906±0.115	0.867±0.018	0.525±0.026	0.48±0.022	1.155±0.05	0.551±0.016	0.132±0.006	0.534±0.04	1.045±0.035	0.523±0.022	0.471±0.034	0.327±0.022	0.561±0.038
QPP Inbred 11	0.636±0.016	0.496±0.064	0.888±0.149	2.049±0.041	0.736±0.071	0.49±0.004	0.436±0.001	1.046±0.028	0.644±0.04	0.131±0.005	0.484±0.022	1.112±0.02	0.527±0.017	0.584±0.011	0.296±0.018	0.541±0.012
QPP Inbred 12	0.843±0.021	0.488±0.032	0.88±0.056	2.311±0.06	0.87±0.035	0.535±0.006	0.49±0.001	1.258±0.026	0.635±0.015	0.141±0	0.548±0.023	1.192±0.018	0.603±0.009	0.596±0.001	0.353±0.012	0.605±0.001
QPP Hybrid 8	0.634±0.048	0.457±0.029	0.805±0.101	1.911±0.049	0.826±0.121	0.562±0.049	0.417±0.026	1.114±0.063	0.477±0.017	0.125±0.015	0.495±0.028	1.055±0.065	0.505±0.031	0.472±0.023	0.316±0.033	0.521±0.024
QPP Hybrid 9	0.667±0.039	0.444±0.057	0.865±0.087	1.972±0.083	0.856±0.103	0.535±0.028	0.451±0.027	1.202±0.063	0.469±0.045	0.128±0.019	0.525±0.026	1.051±0.039	0.52±0.033	0.483±0.027	0.309±0.026	0.529±0.029
QPP Hybrid 19	0.708±0.12	0.498±0.088	0.868±0.204	2.005±0.23	0.828±0.117	0.577±0.047	0.474±0.074	1.192±0.185	0.525±0.077	0.126±0.013	0.536±0.071	1.097±0.063	0.548±0.07	0.496±0.069	0.322±0.022	0.563±0.06
QPP Hybrid 20	0.73±0.065	0.513±0.058	0.981±0.137	2.128±0.117	0.946±0.045	0.582±0.037	0.512±0.036	1.327±0.082	0.558±0.047	0.138±0.017	0.571±0.026	1.136±0.057	0.591±0.031	0.54±0.033	0.331±0.012	0.583±0.04
QPP Hybrid 23	0.67±0.049	0.469±0.021	1.001±0.199	2.073±0.163	0.85±0.054	0.534±0.014	0.457±0.028	1.165±0.083	0.504±0.014	0.124±0.006	0.519±0.036	1.082±0.022	0.532±0.023	0.476±0.018	0.299±0.018	0.532±0.013
QPP Hybrid 25	0.624±0.014	0.517±0.04	0.895±0.135	1.998±0.074	0.839±0.084	0.548±0.03	0.421±0.011	1.03±0.032	0.536±0.042	0.132±0.012	0.474±0.012	1.059±0.05	0.513±0.035	0.482±0.024	0.293±0.016	0.542±0.024
QPP Hybrid 28	0.666±0.072	0.493±0.072	0.839±0.169	2.041±0.242	0.813±0.143	0.57±0.035	0.474±0.053	1.223±0.154	0.524±0.047	0.119±0.021	0.529±0.071	1.126±0.088	0.559±0.064	0.51±0.051	0.286±0.045	0.565±0.052
QPP Hybrid 30	0.705±0.065	0.509±0.041	0.813±0.094	2.048±0.098	0.817±0.01	0.542±0.035	0.472±0.025	1.214±0.106	0.539±0.039	0.143±0.022	0.534±0.029	1.121±0.076	0.557±0.04	0.528±0.034	0.307±0.024	0.562±0.029
QPP Hybrid 38	0.71±0.053	0.528±0.035	0.964±0.171	2.061±0.148	0.9±0.116	0.555±0.029	0.478±0.04	1.217±0.106	0.552±0.024	0.137±0.014	0.54±0.037	1.107±0.061	0.558±0.048	0.527±0.03	0.29±0.032	0.571±0.038
QPP Hybrid 43	0.702±0.067	0.51±0.046	0.97±0.186	2.073±0.131	0.819±0.087	0.52±0.017	0.474±0.022	1.175±0.072	0.589±0.03	0.149±0.019	0.533±0.033	1.109±0.044	0.546±0.023	0.55±0.04	0.301±0.022	0.55±0.013

Table 5: Protein-Bound Amino Acid Values (g/100g) in Raw Kernel Flour. Protein-bound amino acid values of sixteen amino acids are recorded. Aspartate and asparagine (Asx), glutamine and glutamate (Glx), Serine, and Tryptophan are destroyed during acidic hydrolysis, the procedure used for amino acid quantification. Standard deviations were calculated by two-six biological replications, dependent on genotype. Lysine levels are shaded in gray.

	Ala	Arg	Asn	Asp	Gln	Glu	Gly	His	Ile	Leu
Popcorn Parent 4	0.0054±0.0008	0.0059±0.0004	0.0235±0.0027	0.004±0.0002	0.0001±0	0.0047±0.0002	0.0015±0.0003	0.0024±0.0003	0.0004±0.0001	0.0005±0
Popcorn Parent 3	0.0187±0.0011	0.0063±0.0003	0.0211±0	0.0101±0.0023	0.0031±0	0.0132±0.0037	0.0064±0.0003	0.0045±0	0.0007±0	0.001±0.0002
Popcorn Parent 1	0.0046±0.0016	0.0052±0.0001	0.0407±0.0034	0.0084±0.0023	0.0081±0.0061	0.0146±0.0034	0.0057±0.001	0.0045±0.0012	0.0005±0	0.0005±0
Popcorn Parent 2	0.0023±0.0004	0.0074±0.0015	0.0268±0.0024	0.0046±0.0013	0.0023±0.0005	0.0173±0.0025	0.0059±0.0013	0.0022±0.0005	0.0001±0	0.0002±0
B73	0.0072±0.0023	0.0072±0.0012	0.0295±0.0021	0.0155±0.0056	0.0035±0.001	0.0316±0.0031	0.0089±0.002	0.0029±0.001	0.0006±0.0001	0.0007±0.0001
CML154Q	0.0205±0.0068	0.0396±0.0019	0.0713±0.0078	0.1241±0.0088	0.0958±0.0366	0.1199±0.0146	0.0308±0.0011	0.0132±0.0026	0.0033±0.0013	0.0065±0.0032
K0326Y	0.0101±0.005	0.0295±0.0093	0.0704±0.0137	0.0711±0.0325	0.015±0.0153	0.0433±0.0237	0.0235±0.0073	0.0119±0.0045	0.0029±0.0026	0.0031±0.0027
QPP Inbred 1	0.0274±0.0198	0.0283±0.0004	0.0676±0.0059	0.1289±0.0022	0.099±0.0761	0.1588±0.053	0.0335±0.0005	0.0087±0.0019	0.0027±0.0024	0.0061±0.0046
QPP Inbred 10	0.0046±0.0003	0.0113±0.0014	0.0486±0.0012	0.029±0.0022	0.0047±0.0006	0.031±0.0016	0.0103±0.0004	0.0055±0.0019	0.001±0	0.0008±0
QPP Inbred 11	0.0339±0.0201	0.0417±0.0046	0.0443±0.0113	0.1151±0.0011	0.0787±0.0373	0.1855±0.0048	0.0434±0.0014	0.0101±0.002	0.002±0.0006	0.0055±0.0036
QPP Inbred 12	0.0931±0.0423	0.0346±0.0035	0.0422±0.0065	0.116±0.0058	0.0677±0.0228	0.1715±0.0277	0.0483±0.0062	0.0108±0.0004	0.0045±0	0.0133±0.0019
QPP Inbred 2	0.0152±0.0023	0.0292±0.0011	0.0682±0.0118	0.1067±0.0203	0.0511±0.0093	0.1248±0.0098	0.0335±0.0009	0.0078±0.0019	0.0008±0.0003	0.0023±0.0004
QPP Inbred 3	0.05±0.024	0.036±0.0009	0.0598±0.012	0.1332±0.0213	0.1658±0.0847	0.2007±0.0464	0.034±0.0019	0.0195±0.0006	0.0014±0.0005	0.006±0.0047
QPP Inbred 4	0.0532±0.0036	0.0409±0.0002	0.0548±0.008	0.1637±0.0131	0.1724±0.0445	0.2373±0.0174	0.0343±0.0002	0.019±0.0076	0.0029±0.0006	0.0102±0.0004
QPP Inbred 5	0.0176±0.0103	0.0336±0.0028	0.0598±0.0035	0.1047±0.0027	0.0819±0.0834	0.1398±0.0637	0.0315±0.0016	0.0114±0.0022	0.0013±0.0004	0.0034±0.0019
QPP Inbred 6	0.0114±0.0032	0.0354±0.0032	0.0442±0.0032	0.0766±0.0129	0.0227±0.0256	0.0949±0.0627	0.0352±0.0169	0.0093±0.002	0.002±0.0006	0.0021±0.0011
QPP Inbred 7	0.0637±0.0134	0.0551±0.0017	0.0667±0.0004	0.1561±0.0068	0.1082±0.0229	0.292±0.0241	0.0486±0.0003	0.0201±0.0004	0.0041±0.0003	0.0093±0.0011
QPP Inbred 8	0.0146±0.0035	0.0528±0.0065	0.0648±0.0049	0.0931±0.0379	0.0084±0.0092	0.0907±0.0473	0.0393±0.0082	0.0149±0.0027	0.001±0.0002	0.0012±0.0005
QPP Inbred 9	0.0041±0.0001	0.0182±0.0011	0.0369±0.0071	0.0214±0.0091	0.0051±0.0004	0.0362±0.0023	0.0151±0.0102	0.0048±0.0007	0.0015±0	0.0011±0
Tx807	0.0134±0.0011	0.0344±0.0004	0.0954±0.0035	0.1004±0.0065	0.0217±0.0085	0.0898±0.0075	0.0321±0.0007	0.0145±0.0014	0.0015±0.0002	0.0033±0.0004
Hybrid 19	0.0066±0.0011	0.0247±0.0058	0.0636±0.0114	0.0676±0.0189	0.0064±0.0054	0.0508±0.0228	0.0232±0.0062	0.0075±0.0022	0.0005±0.0001	0.0009±0.0004
Hybrid 20	0.0089±0.002	0.0235±0.0046	0.0704±0.0096	0.0721±0.0102	0.0075±0.0073	0.0535±0.0234	0.0251±0.0057	0.0077±0.0013	0.0006±0.0001	0.0008±0.0001
Hybrid 23	0.014±0.0036	0.0186±0.0054	0.0698±0.0079	0.1053±0.0158	0.0517±0.0423	0.0932±0.0262	0.0306±0.0011	0.0083±0.0032	0.001±0.0004	0.0028±0.0015
Hybrid 25	0.0167±0.009	0.0243±0.002	0.0629±0.0089	0.1068±0.0349	0.0702±0.0704	0.0932±0.0484	0.0279±0.0061	0.009±0.0023	0.0013±0.001	0.0036±0.0032
Hybrid 28	0.007±0.0014	0.0201±0.0059	0.0614±0.0081	0.0692±0.0253	0.0137±0.0173	0.0532±0.0281	0.0226±0.0071	0.0071±0.0015	0.0005±0	0.0009±0.0005
Hybrid 30	0.012±0.0044	0.0131±0.006	0.0576±0.0142	0.0688±0.0322	0.0214±0.0262	0.0648±0.036	0.0227±0.0098	0.0073±0.0022	0.0006±0.0001	0.0013±0.0008
Hybrid 38	0.0071±0.0023	0.0213±0.0081	0.0703±0.0122	0.0624±0.0226	0.0079±0.0089	0.0513±0.0222	0.0241±0.0074	0.0075±0.0024	0.0005±0.0001	0.0009±0.0002
Hybrid 43	0.0121±0.0033	0.0265±0.0063	0.0693±0.0124	0.0809±0.0207	0.0399±0.0433	0.0928±0.0404	0.0292±0.0049	0.0087±0.0021	0.0009±0.0001	0.0015±0.0005
Hybrid 8	0.0063±0.0012	0.0216±0.0043	0.0605±0.0081	0.0677±0.0215	0.0112±0.0101	0.0484±0.0261	0.0218±0.0081	0.0062±0.0016	0.0003±0	0.0008±0.0002
Hybrid 9	0.0068±0.0029	0.0171±0.0069	0.0635±0.0061	0.0646±0.0149	0.014±0.0198	0.0519±0.0223	0.0242±0.0045	0.0059±0.0019	0.0004±0.0001	0.0008±0.0004

Table 6: Free Amino Acid Values (g/100g) in Raw Kernel Flour. Free amino acid values of all twenty amino acids are recorded. Standard deviations were calculated by two-six biological replications, dependent on genotype.

	Lys	Met	Phe	Pro	Ser	Trp	Thr	Tyr	Val	Cys
Popcorn Parent 4	0.0031±0.0005	0.0001±0	0.0006±0	0.0138±0.0009	0.0011±0.0001	0.0013±0	0.0008±0.0001	0.0024±0.0001	0.0011±0.0001	0.0001±0
Popcorn Parent 3	0.0038±0.0005	0.0002±0	0.002±0.0003	0.052±0.0046	0.0077±0.0004	0.0008±0	0.002±0	0.0032±0.0002	0.0015±0	0.0001±0
Popcorn Parent 1	0.0029±0.0001	0.0002±0.0001	0.0008±0.0001	0.0135±0.0073	0.0022±0.0008	0.001±0.0001	0.0009±0.0002	0.0021±0.0002	0.0011±0.0001	0.0001±0
Popcorn Parent 2	0.0028±0.0005	0.0001±0	0.0005±0.0001	0.0043±0.0031	0.0027±0.0012	0.0008±0.0001	0.0008±0.0002	0.0017±0.0002	0.0007±0	0.0001±0
B73	0.0029±0.0005	0.0004±0.0001	0.0011±0.0001	0.0393±0.0102	0.0021±0.0006	0.001±0.0002	0.0007±0.0002	0.0049±0.0024	0.0015±0.0001	0.0001±0
CML154Q	0.0266±0.0058	0.0029±0.0015	0.0094±0.0033	0.1691±0.0681	0.0151±0.009	0.0035±0.0009	0.0113±0.0051	0.0304±0.0082	0.0094±0.0032	0.0005±0
K0326Y	0.0214±0.01	0.0007±0.0007	0.0027±0.0019	0.0814±0.0286	0.0111±0.0081	0.0019±0.0003	0.0047±0.0031	0.0096±0.003	0.0045±0.0032	0.0002±0
QPP Inbred 1	0.0162±0.0071	0.0035±0.0032	0.0053±0.0034	0.1297±0.0423	0.0155±0.0108	0.0033±0.0024	0.0191±0.0156	0.0128±0.0063	0.0076±0.0041	0.0005±0.0003
QPP Inbred 10	0.006±0.0003	0.0001±0	0.0008±0	0.1015±0.0115	0.0009±0.0001	0.0025±0.0001	0.0018±0.0004	0.0047±0.0006	0.0017±0.0001	0.0001±0
QPP Inbred 11	0.0177±0.0028	0.0007±0.0005	0.0048±0.0006	0.1554±0.0323	0.0126±0.0046	0.0027±0.0011	0.0166±0.0017	0.0121±0.0017	0.0053±0.0022	0.0008±0.0001
QPP Inbred 12	0.0315±0.0079	0.0036±0.0007	0.0048±0	0.1205±0.0086	0.0335±0.006	0.002±0.0001	0.0317±0.0042	0.0092±0.0005	0.0116±0.0023	0.0011±0.0002
QPP Inbred 2	0.0123±0.0003	0.001±0.0008	0.0029±0.0009	0.1267±0.0177	0.0071±0.0023	0.0026±0.0006	0.0065±0.0002	0.0098±0.0029	0.0034±0.0005	0.0003±0
QPP Inbred 3	0.0226±0.0027	0.0016±0.0005	0.0055±0.0021	0.0986±0.0713	0.0238±0.0147	0.0043±0.0005	0.021±0.003	0.0174±0.0041	0.007±0.0027	0.0005±0.0001
QPP Inbred 4	0.0302±0.0037	0.0021±0.0006	0.0068±0.0001	0.1509±0.0013	0.0255±0.0052	0.0034±0.0005	0.0286±0.0059	0.0182±0.0017	0.0111±0.0015	0.0009±0.0001
QPP Inbred 5	0.0155±0.0064	0.0018±0.0012	0.0039±0.0016	0.1041±0.0349	0.0105±0.0043	0.0023±0.0003	0.0085±0.0032	0.0085±0.0024	0.0052±0.0022	0.0003±0.0001
QPP Inbred 6	0.0141±0.0055	0.0007±0.0003	0.0024±0.001	0.1116±0.0352	0.0058±0.0003	0.0027±0.0004	0.0049±0.0021	0.007±0.0015	0.0043±0.0022	0.0004±0.0002
QPP Inbred 7	0.0486±0.0072	0.0055±0.0007	0.0066±0.0011	0.112±0.0118	0.0384±0.0033	0.0044±0.0004	0.0344±0.0006	0.0217±0.0002	0.0156±0.0001	0.0004±0.0002
QPP Inbred 8	0.0257±0.0079	0.0008±0.0008	0.0022±0.0016	0.0809±0.0173	0.0052±0.0031	0.0032±0.0006	0.0059±0.0046	0.0101±0.004	0.0026±0.0017	0.0004±0.0003
QPP Inbred 9	0.0089±0.0019	0.0001±0	0.0011±0.0004	0.0853±0.0183	0.0014±0.0008	0.0017±0.0001	0.0021±0.0002	0.005±0.0004	0.0023±0	0.0001±0
Tx807	0.0254±0.0013	0.0006±0.0001	0.0054±0.0001	0.0385±0.006	0.0107±0.0011	0.0028±0.0002	0.0112±0.0015	0.0134±0.0014	0.005±0.0005	0.0004±0
Hybrid 19	0.0116±0.0025	0.0002±0	0.0019±0.0008	0.0717±0.0161	0.0028±0.0016	0.0027±0.0004	0.0035±0.0022	0.0065±0.0015	0.0019±0.0005	0.0001±0
Hybrid 20	0.0126±0.0016	0.0002±0	0.0021±0.0006	0.078±0.023	0.0028±0.0015	0.003±0.0004	0.0037±0.0015	0.0087±0.0024	0.0022±0.0003	0.0001±0
Hybrid 23	0.0115±0.004	0.0006±0.0004	0.0047±0.0023	0.1035±0.0246	0.0078±0.005	0.0029±0.0005	0.0096±0.0056	0.0122±0.0035	0.0041±0.0015	0.0003±0.0001
Hybrid 25	0.015±0.005	0.0007±0.0007	0.0049±0.0032	0.0957±0.0256	0.0096±0.0095	0.0035±0.0003	0.0094±0.0081	0.0166±0.0087	0.0051±0.0034	0.0003±0.0001
Hybrid 28	0.0123±0.0024	0.0002±0	0.0017±0.0011	0.0686±0.0188	0.0027±0.0016	0.0024±0.0001	0.0033±0.0023	0.0076±0.0029	0.0021±0.0006	0.0001±0
Hybrid 30	0.0085±0.0025	0.0002±0.0001	0.0023±0.0013	0.1095±0.0128	0.0038±0.0032	0.0024±0.0004	0.005±0.0039	0.0121±0.0049	0.0026±0.0014	0.0002±0
Hybrid 38	0.0137±0.0033	0.0001±0	0.0017±0.0011	0.0673±0.0102	0.0025±0.0015	0.0029±0.0005	0.0032±0.0015	0.0089±0.0033	0.0019±0.0003	0.0002±0
Hybrid 43	0.0153±0.0048	0.0003±0.0001	0.0034±0.0013	0.106±0.0285	0.0042±0.0023	0.0031±0.0003	0.0055±0.0036	0.0143±0.0037	0.003±0.0007	0.0002±0.0001
Hybrid 8	0.0096±0.0016	0.0001±0	0.0021±0.0014	0.0655±0.0176	0.0023±0.0014	0.0022±0.0005	0.0032±0.0016	0.006±0.0028	0.0015±0.0002	0.0002±0
Hybrid 9	0.0096±0.0036	0.0002±0.0001	0.0016±0.0008	0.0505±0.0172	0.0026±0.0015	0.0025±0.0003	0.0038±0.0022	0.0063±0.0021	0.002±0.0006	0.0002±0

Table 6 Continued: Free Amino Acid Values (g/100g) in Raw Kernel Flour. Free amino acid values of all twenty amino acids are recorded. Standard deviations were calculated by two-six biological replications, dependent on genotype.

	Hybrid	Germination Rate	Days to Pollinating	Rot Susceptibility	Number of Ears Harvested	Ear Length	Number of Rows per Ear	Ear Weight	Kernel Size	Vitreousness Level	Hundred Grain Weight	Pop-Ability	Expansion Volume
	1	-0.037	2.076	-0.022	-1.142	-3.261	-1.841	-18.283	10.831	0.855	-1.686	0.016	94.932
	2	-0.270	2.976	0.024	-4.236	-1.642	-2.027	-25.041	16.319	0.395	-2.060	0.005	99.905
	3	0.140	-1.004	0.053	0.081	-1.501	-0.167	-5.694	2.639	-0.595	-0.528	-0.002	-8.857
	4	0.097	1.163	0.036	0.297	-1.666	-0.034	-3.716	3.886	-0.025	-0.727	0.002	-17.113
	5	-0.078	3.825	0.028	-2.221	-4.573	-2.370	-29.536	27.055	0.316	-2.879	0.017	112.069
	6	-0.510	1.422	-0.013	-5.603	-2.622	-1.195	-2.570	-1.981	-0.074	0.049	0.006	3.366
	7	0.104	2.304	-0.049	1.089	-2.909	-0.581	-14.350	16.520	-0.106	-2.071	0.010	52.473
	8	0.160	-1.797	-0.047	1.808	2.376	0.546	9.909	-2.967	0.903	0.270	0.010	28.098
	9	0.128	-0.472	0.019	0.801	2.345	0.621	8.096	-1.805	0.819	0.111	0.020	71.737
	10	0.202	-0.810	0.020	0.945	-1.237	0.223	-6.276	5.986	-0.908	-0.947	-0.003	-59.179
	11	0.197	0.175	0.067	1.089	-1.711	-0.997	-7.679	0.038	-0.166	-0.245	-0.059	-100.851
	12	0.114	2.418	0.022	0.153	-4.015	-2.575	-23.254	10.553	0.382	-1.495	0.006	85.890
	13	0.061	3.368	-0.010	0.657	-1.715	-1.782	-19.941	9.818	0.198	-1.264	0.015	53.259
	14	0.135	-0.852	0.078	0.585	-1.687	-0.293	-7.342	2.226	-1.077	-0.491	-0.004	-18.292
	15	0.165	0.517	0.063	1.376	-3.159	0.116	-9.107	9.699	-0.209	-1.124	-0.008	-10.429
	16	-0.027	3.140	-0.004	-1.142	-3.597	-2.476	-29.301	31.190	0.791	-3.200	0.022	119.700
	17	0.162	1.962	0.027	1.017	-2.324	-0.533	-8.613	-0.029	-0.077	-0.210	0.013	28.491
	18	0.129	2.342	0.013	0.009	-2.745	-0.925	-16.935	15.242	0.043	-1.838	0.021	74.095
	19	0.141	-2.677	0.035	2.168	2.127	-0.225	9.178	-6.284	0.668	0.716	0.015	35.934
	20	0.157	-2.487	0.028	1.520	2.637	0.204	6.771	-5.455	0.717	0.710	0.019	50.114
	21	0.046	-0.624	0.030	0.009	-1.884	-0.298	-6.388	1.070	-0.642	-0.267	-0.003	-29.693
	22	0.139	-0.244	0.120	0.585	-1.528	-0.645	-4.402	-2.503	0.044	0.265	-0.038	-56.820
	23	0.040	-1.042	0.002	1.664	0.943	-0.725	12.595	-10.644	-0.262	1.591	-0.032	-30.873
	24	-0.168	-0.092	-0.013	-0.782	1.795	-0.287	9.770	-6.910	-0.378	0.970	-0.013	-34.411
	25	0.051	-1.156	-0.075	1.880	1.637	1.413	16.628	-7.374	-0.191	0.937	-0.001	-39.129
	26	0.029	0.479	-0.055	0.081	1.068	1.838	17.377	-8.543	-0.436	1.094	-0.010	-54.068
	27	-0.138	-1.427	-0.101	-0.830	2.318	-0.661	7.919	-8.657	0.646	1.153	-0.003	-8.464
	28	0.030	-2.826	0.072	1.376	2.702	-0.586	9.367	-6.365	0.929	0.668	0.014	48.935
	29	-0.037	-0.320	-0.023	-0.494	3.106	0.492	16.712	-15.752	-0.652	2.548	-0.012	-76.084
	30	0.055	0.897	-0.001	0.729	2.061	-0.362	4.791	-2.810	-0.246	0.176	-0.009	2.151
	31	-0.205	-0.814	-0.075	-1.286	1.223	0.849	-6.119	4.893	0.091	-0.657	0.018	-14.754
	32	-0.006	-0.510	-0.079	0.801	1.141	1.718	18.423	-9.479	-0.355	1.244	-0.014	-69.400
	33	-0.054	-0.890	-0.113	0.873	3.313	1.427	18.797	-9.928	0.213	1.740	-0.046	-109.500
	34	0.093	-2.753	0.034	1.520	1.175	0.204	11.252	-11.778	-1.359	1.912	-0.017	-67.435
	35	-0.153	0.061	0.016	-0.998	0.505	0.920	8.333	-5.632	-1.206	0.859	-0.028	-76.084
	36	-0.268	-0.966	-0.025	-1.646	1.139	1.787	7.619	-4.281	-0.635	0.503	0.014	-24.976
	37	-0.247	0.669	0.017	-1.430	-0.497	1.475	2.915	-2.226	-0.224	0.206	0.003	-21.830
	38	0.144	-2.411	0.065	1.448	1.603	0.151	5.027	-4.594	1.098	0.312	0.023	57.977
	39	-0.121	-0.481	-0.020	-0.782	1.752	0.140	1.746	-2.834	0.956	0.154	0.022	68.198
	40	-0.185	0.061	-0.036	-1.933	1.563	1.101	4.903	-9.084	-0.349	1.252	0.012	-26.155
	41	-0.079	-1.194	-0.017	-0.351	1.420	0.693	5.212	-4.896	-0.545	0.426	-0.009	-15.933
	42	-0.169	0.061	-0.039	-1.646	0.571	1.405	-0.890	-1.759	0.310	0.150	0.002	-26.155
	43	0.084	-1.139	-0.017	1.592	1.000	2.265	11.506	-3.534	0.006	0.360	-0.006	-56.820
	44	-0.048	-0.928	-0.036	0.369	2.752	1.998	20.589	-9.862	0.340	1.316	0.013	-34.018
Standard Error		0.006	0.772	0.002	0.731	1.17	0.366	41.73	23.967	0.0874	4.444	0.0001	862.378
Genetic Repeatability		0.566	0.465	0.078	0.345	0.716	0.673	0.728	0.676	0.684	0.683	0.201	0.582

Table 7: Specific Combining Ability (SCA) and genetic repeatability estimates for all recorded traits. Specific Combining Ability and genetic repeatability estimates were found with ASReml-R software. High SCAs were noted in elite hybrids, shaded in gray. High repeatabilities were calculated for ear length and ear weight.

	Ala	Arg	Asx	Glx	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
Popcorn Parent 4	1.254± 0.144	0.365± 0.061	0.807± 0.057	3.085± 0.087	1.159± 0.076	0.451± 0.022	0.783± 0.073	2.48± 0.194	0.206± 0.043	0.193± 0.029	0.809 ±0.033	1.291± 0.091	0.773± 0.074	0.6± 0.071	0.486 ±0.053	0.66± 0.044
Popcorn Parent 3	1.056± 0.024	0.31± 0.024	0.7± 0.031	2.591± 0.092	0.909± 0.018	0.402± 0.009	0.659± 0.029	2.006± 0.117	0.173± 0.017	0.196± 0.005	0.698± 0.018	1.168± 0.06	0.667± 0.023	0.547± 0.034	0.445± 0.031	0.555± 0.018
Popcorn Parent 1	1.248± 0.037	0.379± 0.022	0.795± 0.026	3.077± 0.165	1.089± 0.016	0.499± 0.017	0.755± 0.053	2.388± 0.148	0.197± 0.013	0.259± 0.035	0.788± 0.025	1.386± 0.054	0.783± 0.048	0.616± 0.027	0.515± 0.043	0.65± 0.026
Popcorn Parent 2	0.991± 0.1	0.295± 0.021	0.662± 0.04	2.58± 0.139	1.003± 0.107	0.391± 0.018	0.642± 0.038	1.978± 0.152	0.201± 0.028	0.182± 0.015	0.707± 0.035	1.146± 0.052	0.642± 0.047	0.484± 0.054	0.425± 0.024	0.527± 0.029
QPP Hybrid 20	0.752± 0.052	0.514± 0.038	0.852± 0.122	2.234± 0.188	0.862± 0.139	0.552± 0.024	0.54± 0.038	1.342± 0.099	0.402± 0.024	0.128± 0.006	0.603± 0.04	1.128± 0.05	0.59± 0.032	0.527± 0.029	0.322± 0.037	0.628± 0.036
QPP Hybrid 25	0.709± 0.066	0.494± 0.078	0.973± 0.219	2.24± 0.171	0.936± 0.127	0.553± 0.054	0.501± 0.047	1.164± 0.086	0.37± 0.065	0.135± 0.023	0.532± 0.043	1.113± 0.079	0.556± 0.066	0.508± 0.039	0.294± 0.045	0.609± 0.061
QPP Hybrid 28	0.872± 0.079	0.558± 0.072	0.829± 0.119	2.57± 0.229	0.935± 0.151	0.614± 0.027	0.622± 0.051	1.581± 0.164	0.358± 0.061	0.137± 0.012	0.672± 0.069	1.297±0.0 67	0.653± 0.05	0.571± 0.041	0.39± 0.061	0.708± 0.048
QPP Hybrid 38	0.713± 0.03	0.553± 0.057	0.778± 0.11	2.189± 0.103	1.282± 0.137	0.644± 0.05	0.517± 0.025	1.359± 0.058	0.371± 0.04	0.117± 0.011	0.555± 0.029	1.26± 0.049	0.554± 0.04	0.519± 0.031	0.338± 0.025	0.617± 0.035
QPP Hybrid 43	0.758± 0.087	0.63± 0.155	0.915± 0.248	2.348± 0.32	1.292± 0.096	0.641± 0.094	0.546± 0.086	1.326± 0.175	0.42± 0.077	0.148± 0.025	0.559± 0.058	1.269± 0.121	0.599± 0.099	0.571± 0.068	0.357± 0.044	0.651± 0.097

Table 8: Protein-Bound Amino Acid Levels (g/100g) in Air Popped Flakes. Protein-bound amino acid values of sixteen amino acids in air popped flakes are recorded. Aspartate and asparagine (Asx), glutamine and glutamate (Glx), Serine, and Tryptophan are destroyed during acidic hydrolysis, the procedure used for amino acid quantification. Only five QPP hybrids and four popcorn parents were tested with air popping. Standard deviations were calculated by four biological replications.

	Ala	Arg	Asx	Glx	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
Popcorn Parent 1	1.314± 0.085	0.336± 0.039	0.838± 0.091	3.185± 0.264	1.101± 0.122	0.502± 0.04	0.823± 0.091	2.555± 0.285	0.176± 0.024	0.274± 0.025	0.821± 0.071	1.431± 0.115	0.776± 0.059	0.612± 0.068	0.537± 0.08	0.679± 0.066
Popcorn Parent 2	0.967± 0.054	0.249± 0.031	0.614± 0.073	2.485± 0.163	0.843± 0.092	0.38± .027	0.603± 0.062	1.917± 0.123	0.155± 0.014	0.171± 0.01	0.668± 0.047	1.098± 0.056	0.593± 0.058	0.451± 0.041	0.374± 0.043	0.506± 0.044
QPP Hybrid 20	0.849± 0.052	0.504± 0.073	0.872± 0.079	2.479± 0.154	0.911± 0.14	0.582± 0.042	0.608± 0.063	1.517± 0.165	0.357± 0.078	0.148± 0.025	0.662± 0.064	1.22±0 .087	0.632± 0.06	0.565± 0.059	0.353± 0.042	0.682± 0.063
QPP Hybrid 25	0.748± 0.044	0.466± 0.041	0.831± 0.084	2.325± 0.056	0.982± 0.127	0.568± 0.021	0.524± 0.033	1.255± 0.045	0.313± 0.011	0.138± 0.016	0.569± 0.017	1.138± 0.057	0.558± 0.035	0.524± 0.025	0.31±0 .022	0.64±0 .035
QPP Hybrid 28	0.715± 0.075	0.506± 0.062	0.688± 0.119	2.195± 0.168	1.196± 0.017	0.658± 0.031	0.511± 0.06	1.345± 0.121	0.287± 0.038	0.117± 0.012	0.527± 0.063	1.2±0. 034	0.537± 0.059	0.502± 0.051	0.32±0 .056	0.596± 0.056
QPP Hybrid 38	0.695± 0.054	0.558± 0.059	0.788± 0.108	2.099± 0.136	1.197± 0.034	0.593± 0.061	0.503± 0.028	1.268± 0.115	0.334± 0.031	0.118± 0.012	0.53±0 .033	1.163± 0.096	0.545± 0.044	0.509± 0.034	0.317± 0.034	0.591± 0.034
QPP Hybrid 43	0.726± 0.077	0.577± 0.024	0.869± 0.114	2.276± 0.222	1.253± 0.054	0.627± 0.06	0.527± 0.046	1.301± 0.139	0.353± 0.074	0.139± 0.022	0.538± 0.05	1.216± 0.078	0.548± 0.049	0.532± 0.019	0.331± 0.045	0.622± 0.062

Table 9: Protein-Bound Amino Acid Levels (g/100g) in Microwaved Popped Flakes. Protein-bound amino acid values of sixteen amino acids in microwave-popped flakes are recorded. Aspartate and asparagine (Asx), glutamine and glutamate (Glx), Serine, and Tryptophan are destroyed during acidic hydrolysis, the procedure used for amino acid quantification. Only five QPP hybrids and two popcorn parents were tested with air popping. Standard deviations were calculated by four biological replications.

	Ala	Arg	Asx	Glx	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
Popcorn Parent 1	0.897± 0.195	0.267± 0.021	0.601± 0.083	2.269± 0.389	0.773± 0.172	0.364± 0.044	0.529± 0.114	1.751± 0.358	0.188± 0.022	0.179± 0.046	0.625± 0.102	1.067± 0.176	0.575± 0.101	0.459± 0.077	0.38±0. 089	0.48±0. 076
Popcorn Parent 2	0.855± 0.146	0.296± 0.073	0.635± 0.158	2.187± 0.422	0.745± 0.187	0.351± 0.076	0.548± 0.136	1.662± 0.338	0.23±0. 082	0.152± 0.033	0.63±0. 116	0.969± 0.17	0.571± 0.115	0.454± 0.099	0.354± 0.108	0.503± 0.116
QPP Hybrid 20	0.611±0 .078	0.379± 0.025	0.672± 0.101	1.8± 0.24	0.77± 0.08	0.474± 0.05	0.428± 0.046	1.076± 0.154	0.266± 0.018	0.098± 0.007	0.487± 0.06	0.945± 0.103	0.475± 0.064	0.414± 0.035	0.269± 0.018	0.519± 0.05
QPP Hybrid 25	0.543± 0.051	0.363± 0.097	0.686± 0.12	1.78± 0.192	0.645± 0.098	0.481± 0.059	0.391± 0.047	0.927± 0.078	0.28±0. 05	0.097± 0.01	0.425± 0.049	0.894± 0.089	0.426± 0.06	0.398± 0.037	0.239± 0.038	0.503± 0.058
QPP Hybrid 28	0.565± 0.105	0.485± 0.135	0.586± 0.088	1.675± 0.224	1.014± 0.151	0.499± 0.036	0.396± 0.058	0.989± 0.151	0.273± 0.027	0.088± 0.014	0.434± 0.062	0.935± 0.083	0.433± 0.053	0.399± 0.027	0.245± 0.039	0.488± 0.053
QPP Hybrid 38	0.62±0. 088	0.487± 0.013	0.677± 0.055	1.807± 0.324	1.117±0 .107	0.529± 0.098	0.437± 0.057	1.123± 0.238	0.264± 0.016	0.102± 0.012	0.481± 0.056	0.983± 0.225	0.496± 0.05	0.448± 0.041	0.29±0. 067	0.529± 0.054
QPP Hybrid 43	0.662± 0.023	0.567± 0.061	0.823± 0.191	2.007± 0.155	1.141± 0.076	0.589± 0.048	0.466± 0.021	1.152± 0.059	0.345± 0.077	0.117±0 .006	0.493± 0.022	1.089± 0.079	0.509± 0.042	0.495± 0.048	0.26±0. 016	0.561± 0.034

Table 10: Protein-Bound Amino Acid Levels (g/100g) in Oil Popped Flakes. Protein-bound amino acid values of sixteen amino acids in oil-popped flakes are recorded. Aspartate and asparagine (Asx), glutamine and glutamate (Glx), serine, and tryptophan are destroyed during acidic hydrolysis, the procedure used for protein-bound amino acid quantification. Only five QPP hybrids and two popcorn parents were tested with air popping. Standard deviations were calculated by four biological replications.

	Ala	Arg	Asn	Asp	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Trp	Thr	Tyr	Val	Cys
Popcorn Parent 4	0.000806 ±0.00028	0.00121± 0.000196	0.00133± 0.00111	0.000792 ±0.0003	0±0	0.000194 ±0.00010	0.000823 ±0.00041	0.000599 ±0.00015	0.0000868 ±0.00000 173	0.0000288 ±0.00004 98	0.000642 ±0.00024	0±0	0.000109 ±0.00000 218	0.00137± 0.000914	0.000278 ±0.00006 62	0.000135 ±0.00000 269	0.000211 ±0.00009 61	0.0000791 ±0.00006 85	0.0000257 ±0.00004 45	0.0000539 ±0.00004 67
Popcorn Parent 3	0.00119± 0.0000386	0.00128± 0.000163	0.0014±0. 000284	0.00331± 0.000461	0±0	0.000795 ±0.00015 1	0.00196± 0.0019	0.00113± 0.0000851	0.000085 ±0.00000 0498	0.000114 ±0.00005	0.00115± 0.000369	0±0	0.00025± 0.0000634	0.00996± 0.00355	0.000705 ±0.00016 2	0.000132 ±0.00000 0775	0.00155± 0.000955	0.000313 ±0.00006 9	0.0000759 ±0.00000 0445	0.0000522 ±0.00004 52
Popcorn Parent 1	0.000764 ±0.00022	0.00149± 0.00017	0.00472± 0.000973	0.0022±0. 00037	0±0	0.00067± 0.000207	0.00146± 0.000794	0.000742 ±0.00013 3	0.0000666 ±0.00004 44	0.0000667 ±0.00004 45	0.000738 ±0.00009 11	0.0000247 ±0.00004 95	0.000111 ±0.00000 172	0.00258± 0.000852	0.000337 ±0.00010 7	0.000138 ±0.00000 213	0.000141 ±0.00004 13	0.000122 ±0.00000 189	0.0000596 ±0.00003 97	0.0000817 ±0.00000 126
Popcorn Parent 2	0.000512 ±0.00012	0.00132± 0.000171	0.00365± 0.00201	0.00199± 0.000666	0±0	0.0012±0. 000591	0.00115± 0.00059	0.000657 ±0.00021	0.0000647 ±0.00004	0.0000864 ±0.00000 123	0.000753 ±0.00021	0±0	0.000109 ±0.00000 155	0.00192± 0.00114	0.000519 ±0.00012	0.000168 ±0.00006 46	0.000137 ±0.00003 81	0.000239 ±0.00000 34	0.0000575 ±0.00003 84	0.0000798 ±0.00000 114
QPP Hybrid 20	0.00235± 0.000557	0.00993± 0.00437	0.0277±0. 00573	0.0305±0. 0109	0±0	0.0076±0. 00541	0.00361± 0.00244	0.00206± 0.000355	0.00011± 0.0000469	0.000152 ±0.00007 94	0.00314± 0.000935	0.0000242 ±0.00004 84	0.000518 ±0.00038	0.0296±0. 0126	0.000697 ±0.00034	0.00068± 0.000239	0.00645± 0.00226	0.00201± 0.000953	0.000487 ±0.00012 2	0.000221 ±0.00007 29
QPP Hybrid 25	0.00536± 0.00225	0.00833± 0.00149	0.0337±0. 00797	0.0581±0. 013	0.0000712 ±0.00014	0.015±0.0 0615	0.00647± 0.00183	0.00318± 0.000803	0.000321 ±0.00023	0.0015±0. 00132	0.00457± 0.00112	0.000244 ±0.00017	0.00245± 0.00147	0.0421±0. 0159	0.00458± 0.0044	0.00133± 0.000274	0.0103±0. 00426	0.00936± 0.00542	0.0021±0. 00165	0.000237 ±0.00011
QPP Hybrid 28	0.00177± 0.000798	0.00675± 0.00361	0.0197±0. 00976	0.0265±0. 0174	0±0	0.00412± 0.00562	0.00207± 0.00263	0.00186± 0.000655	0.0000427 ±0.00005	0.000129 ±0.00008 27	0.00247± 0.000967	0±0	0.000348 ±0.00041	0.0199±0. 0161	0.000636 ±0.00043	0.000433 ±0.00042	0.00411± 0.00427	0.00148± 0.00159	0.000287 ±0.00026	0.000198 ±0.00015
QPP Hybrid 38	0.00234± 0.000545	0.00481± 0.0026	0.0244±0. 00603	0.034±0.0 107	0±0	0.00415± 0.00179	0.00256± 0.00184	0.00176± 0.000496	0.0000867 ±0.00000 114	0.000109 ±0.00004 39	0.00271± 0.000944	0±0	0.000465 ±0.00029	0.0267±0. 0123	0.000607 ±0.00023	0.000641 ±0.00017	0.00688± 0.0029	0.00165± 0.000541	0.000465 ±0.00011	0.000279 ±0.00015
QPP Hybrid 43	0.00402± 0.0017	0.00738± 0.00441	0.0259±0. 0146	0.0376±0. 0218	0.0000735 ±0.00009 45	0.011±0.0 105	0.00467± 0.00392	0.00234± 0.000952	0.000198 ±0.00017	0.000441 ±0.00060	0.00376± 0.00228	0.000125 ±0.00019	0.00127± 0.00116	0.0525±0. 0181	0.00178± 0.00193	0.000817 ±0.00040	0.0137±0. 00617	0.00432± 0.00275	0.00104± 0.00109	0.000649 ±0.00044

Table 11: Free Amino Acid Levels (g/100g) in Air Popped Flakes. Free amino acid values of all twenty amino acids are recorded. As shown, all free amino acids substantially decline in abundance after popping. The five elite QPP hybrids and four popcorn parents were popped by air. Standard deviations were calculated by four biological replications of each genotype.

	Ala	Arg	Asn	Asp	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Trp	Thr	Tyr	Val	Cys
Popcorn Parent 1	0.0009482463±0.0002365559	0.0017418 48±0.0004 865403	0.0052037 587±0.002 0342478	0.0022507 094±0.000 7099502	0±0	0.0003721 632±0.000 1004849	0.0018393 288±0.000 8881289	0.0007413 003±0.000 1857273	0.0000224 9914±0.00 00449982	0.0000224 6062±0.00 00449212	0.0008330 781±0.000 3289226	0.0000242 224±0.000 04844481	0.0001111 534±0.000 00282410	0.0037565 672±0.001 4144277	0.0002834 253±0.000 06064005	0.0001042 685±0.000 06952621	0.0002011 72±0.0000 8390191	0.0000925 0559±0.00 00616826	0.0000598 1031±0.00 00398814	0.0000815 2643±0.00 000207136 3
Popcorn Parent 2	0.0004314 31±0.0001 754707	0.0011925 1±0.00022 72991	0.0009401 704±0.000 2164053	0.0007320 231±0.000 1078965	0±0	0.0001904 881±0.000 00271307	0.0005427 041±0.000 6295066	0.0005418 658±0.000 1880436	0±0	0.0000638 5784±0.00 00425865	0.0005316 34±0.0001 5499558	0±0	0.0001069 351±0.000 00152305	0.0006483 133±0.000 1851235	0.0002383 546±0.000 04142166	0.0001322 075±0.000 00188299	0.0000771 1189±0.00 00010982	0.0000882 0921±0.00 00588263	0±0	0.0000784 3248±0.00 000111709
QPP Hybrid 20	0.0026273 338±0.000 5253412	0.0052710 28±0.0009 995258	0.0236457 545±0.004 3510332	0.0333104 174±0.006 6603816	0±0	0.0024168 42±0.0013 0196	0.0022047 871±0.001 5269503	0.0019492 611±0.000 1685865	0.0001074 379±0.000 04478807	0.0002569 593±0.000 09933126	0.0023867 759±0.000 2357523	0±0	0.0006469 428±0.000 2638239	0.0241894 477±0.007 442605	0.0007720 446±0.000 2609134	0.0005334 992±0.000 1889074	0.0047557 81±0.0017 10336	0.0027793 87±0.0006 441274	0.0004977 861±0.000 1606601	0.0001584 233±0.000 06633965
QPP Hybrid 25	0.0045106601±0.0042004423	0.0061511 39±0.0019 706678	0.0261335 466±0.007 1172068	0.0394566 982±0.013 7240549	0.0000475 3136±0.00 00548916	0.0028958 854±0.001 870876	0.0029132 709±0.001 264521	0.0023100 025±0.000 7745874	0.0001692 952±0.000 2281432	0.0008056 195±0.000 959592	0.0029598 845±0.000 9597532	0.0001446 011±0.000 1659447	0.0014719 689±0.001 014932	0.0242898 97±0.0042 562222	0.0028388 085±0.003 157153	0.0008609 623±0.000 3166468	0.0049458 29±0.0015 69736	0.0056358 69±0.0036 78759	0.0010996 31±0.0010 51773	0.0001375 024±0.000 03958659
QPP Hybrid 28	0.0022881 962±0.000 4643949	0.0031461 65±0.0009 619635	0.0175810 897±0.004 551998	0.0250493 416±0.007 6961613	0±0	0.0018119 86±0.0006 98582	0.0027110 223±0.002 8351934	0.0016151 557±0.000 4456378	0.0000657 1855±0.00 00438915	0.0001755 595±0.000 61256886	0.0022014 835±0.000 7000656	0.0000252 8983±0.00 00505796	0.0005790 401±0.000 6761225	0.0138389 265±0.004 8636911	0.0008664 472±0.000 8493801	0.0004778 727±0.000 1751826	0.0031735 39±0.0015 45485	0.0017192 9±0.00111 8404	0.0004122 344±0.000 2708196	0.0001418 729±0.000 04030057
QPP Hybrid 38	0.0020741 728±0.000 7131781	0.0035095 22±0.0014 794624	0.0231666 703±0.008 1097997	0.0265255 607±0.012 4497166	0±0	0.0021857 841±0.001 273317	0.0013758 317±0.001 0168604	0.0015060 134±0.000 4030024	0.0000856 4974±0.00 00016095	0.0001074 94±0.0000 6178895	0.0021156 913±0.000 5635579	0±0	0.0004596 332±0.000 2973852	0.0155259 675±0.006 0488209	0.0005322 905±0.000 3029079	0.0004681 73±0.0001 38271	0.0036566 42±0.0016 46911	0.0015146 08±0.0009 443404	0.0002495 89±0.0001 314453	0.0001388 724±0.000 04101482
QPP Hybrid 43	0.0054818223±0.003 260012	0.0063161 67±0.0027 925839	0.0264189 24±0.0116 961777	0.0374209 989±0.022 0457084	0.0002341 987±0.000 4683974	0.0086145 505±0.012 31544	0.0031117 14±0.0032 355611	0.0026129 592±0.001 4820626	0.0001697 118±0.000 1180368	0.0004661 569±0.000 4436924	0.0039654 384±0.002 1068802	0.0001680 829±0.000 2261091	0.0013354 26±0.0009 928845	0.0406693 458±0.025 2109382	0.0021879 253±0.002 17686	0.0009253 966±0.000 5606712	0.0093394 66±0.0069 71784	0.0046027 06±0.0030 60331	0.0010957 81±0.0011 52142	0.0000391 1748±0.00 004517136

Table 12: Free Amino Acid Levels (g/100g) in Microwave Popped Flakes. Free amino acid values of all twenty amino acids in microwave-popped flakes are recorded. As shown, all free amino acids substantially decline in abundance after popping. The five elite hybrids and two popcorn parents were popped with microwave and oil popping methods. Standard deviations were calculated by four biological replications of each genotype.

	Ala	Arg	Asn	Asp	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Trp	Thr	Tyr	Val	Cys
	0.000605	0.0013612	0.0075552	0.0020541		0.0008027	0.0010705	0.0006586		0.0000651	0.0007262	0.0001959	0.0001093	0.0032155	0.0002608	0.0001351	0.0001193	0.0001199	0.0000775	0.0000801
	5715±0.0	82±0.0003	73±0.0013	6±0.00047		696±0.000	21±0.0008	571±0.000		3198±0.00	074±0.000	22±0.0001	286±0.000	498±0.000	39±0.0000	667±0.000	118±0.000	18±0.0000	3404±0.00	88±0.0000
Popcorn Parent 1	00089926	79367	26479	04377	0±0	1185045	517239	1731321	0±0	00434933	1781827	364582	00315079	9254667	3583853	00389543	0796212	03455971	00022345	02310975
	0.000478	0.0015033	0.0041520	0.0010354		0.0007145	0.0012058	0.0006785	0.0000213	0.0000633	0.0006798		0.0001071	0.0009911	0.0004248	0.0000985	0.0001731	0.0001164	0.0000378	0.0000588
	017±0.00	35±0.0002	39±0.0024	01±0.0004		323±0.000	14±0.0003	01±0.0001	6319±0.00	0407±0.00	674±0.000		172±0.000	009±0.001	041±0.000	6362±0.00	916±0.000	347±0.000	238±0.000	331±0.000
Popcorn Parent 2	02365074	000403	50013	524599	0±0	3522831	073448	855328	00427264	00422092	1704428	0±0	00188884	300758	2161146	00657193	07290276	09468833	04367861	03924577
	0.001770	0.0050627	0.0197823	0.0273530		0.0043415	0.0027494	0.0015724	0.0000662	0.0002200	0.0017294	0.0000253	0.0006376	0.0137838	0.0006703	0.0005126	0.0027059	0.0019717	0.0003926	0.0001214
	4579±0.0	79±0.0028	65±0.0078	54±0.0097		193±0.002	15±0.0012	698±0.000	9293±0.00	694±0.000	353±0.000	7585±0.00	837±0.000	543±0.005	452±0.000	073±0.000	333±0.001	565±0.000	669±0.000	359±0.000
QPP Hybrid 20	00349768	149051	90235	435045	0±0	6988147	870895	3871151	00442062	09025278	6078962	00507517	2970466	7840178	3229067	2085285	501073	7426972	1716898	04748471
	0.004269	0.0057931	0.0278972	0.0430361	0.0001874	0.0093543	0.0049239	0.0023030	0.0002992	0.0013379	0.0033823	0.0001440	0.0019076	0.0333032	0.0032996	0.0011411	0.0074640	0.0062570	0.0013339	0.0001618
	0561±0.0	4±0.00098	33±0.0049	81±0.0113	414±0.000	635±0.002	67±0.0020	685±0.000	667±0.000	67±0.0010	71±0.0005	558±0.000	603±0.001	687±0.011	147±0.002	68±0.0002	906±0.001	388±0.002	8±0.00069	139±0.000
QPP Hybrid 25	00699833	02109	86444	80583	2641226	863602	015564	4049921	141909	49088	977175	1663501	067472	3730244	578605	281071	153801	253185	8569	119189
	0.001688	0.0042970	0.0185201	0.0237715		0.0033776	0.0017557	0.0014256	0.0001063	0.0001698	0.0019808		0.0004548	0.0207593	0.0005275	0.0004295	0.0034245	0.0018163	0.0003982	0.0000978
	0979±0.0	51±0.0006	59±0.0033	48±0.0121		91±0.0020	29±0.0017	385±0.000	339±0.000	036±0.000	56±0.0004		921±0.000	688±0.008	476±0.000	147±0.000	118±0.002	766±0.001	46±0.0000	0602±0.00
QPP Hybrid 28	00271718	235101	25288	309901	0±0	479588	553255	3213544	04304956	06898908	819294	0±0	3536305	3449688	2563255	1246762	788864	34109	9454382	00747207
	0.001578	0.0031190	0.0228559	0.0251230		0.0047721	0.0030079	0.0015691	0.0000640	0.0001278	0.0021699	0.0000242	0.0005911	0.0148574	0.0006148	0.0004987	0.0033889	0.0016209	0.0003803	0.0001575
	6804±0.0	74±0.0011	92±0.0083	42±0.0129		435±0.003	02±0.0017	532±0.000	2349±0.00	75±0.0001	272±0.000	6179±0.00	391±0.000	978±0.005	385±0.000	46±0.0002	995±0.001	766±0.001	437±0.000	181±0.000
QPP Hybrid 38	00485819	731056	87709	98166	0±0	3670578	312362	5538586	00426869	095665	7644188	00485236	4997597	8599485	4543317	962318	750523	019124	2540361	06357525
	0.003149	0.0078645	0.0268852	0.0304050	0.0000248	0.0067119	0.0037956	0.0018990	0.0001101	0.0002207	0.0036252	0.0000253	0.0008606	0.0378674	0.0009561	0.0006847	0.0090652	0.0030706	0.0006703	0.0004283
	2882±0.0	14±0.0051	91±0.0115	05±0.0152	1154±0.00	031±0.006	33±0.0026	164±0.000	307±0.000	845±0.000	118±0.002	3277±0.00	107±0.000	777±0.026	96±0.0011	551±0.000	053±0.006	223±0.002	602±0.000	841±0.000
QPP Hybrid 43	01029297	672403	74059	503882	00496231	3318403	524559	8719656	08499491	2247013	5363583	00506655	7323513	2502169	45673	34193	734026	085509	5682421	3642795

Table 13: Free Amino Acid Levels (g/100g) in Oil Popped Flakes. Free amino acid values of all twenty amino acids in oil-popped flakes are recorded. As shown, all free amino acids substantially decline in abundance after popping. The five elite hybrids and two popcorn parents were popped with microwave and oil popping methods. Standard deviations were calculated by four biological replications of each genotype.

References

Addelman, S. (1969). The Generalized Randomized Block Design. The American Statistician. 23, 35-36.

Adunola, P.M. (2017). Introgression of Opaque-2 Gene into the Genetic Background of Popcorn using Marker Assisted Selection. [Master's Thesis]. [Akure, (Nigeria)]: Federal University of Technology, Akure

Alan L.K. (2009). "Enhancement of Amino Acid Availability in Corn Grain," in Molecular Genetic Approaches to Maize Improvement - Biotechnology in Agriculture and Forestry, ed. A.L Kriz and B.A. Larkins (Berlin, Germany: Springer), 79-89.

Allard, R.W., (1960). Principles of plant breeding. New York: John Wiley & Sons, Inc.

Amaral Júnior, A.T., Freitas Júnior, S.P., Rangel R.M., Pena, G.F., Riberio, R.M., Morais, R.C., Schuelter A.R. (2010). Improvement Of A Popcorn Population Using Selection Indexes From A Fourth Cycle Of Recurrent Selection Program Carried Out In Two Different Environments. Genetics And Molecular Research 9:1. 340-347. doi:10.4238/vol9-1gmr702

Angelovici R., Lipka A. E., Deason N., Gonzalez-Jorge S., Lin H., Cepela J. (2013). Genome-wide analysis of branched-chain amino acid levels in arabidopsis seeds. Plant Cell. 25, 4827–4843. doi:10.1105/tpc.113.119370

Babu B. K., Agrawal P., Saha S., Gupta H. (2015). Mapping QTLs for opaque2 modifiers influencing the tryptophan content in quality protein maize using genomic and candidate gene-based SSRs of lysine and tryptophan metabolic pathway. Plant Cell Rep. 34, 37–45. doi: 10.1007/s00299-014-1685-5

Babu R., Nair S. K., Kumar A., Venkatesh S., Sekhar J. C., Singh N. N. (2005). Two-generation marker-aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). Theor. Appl. Genet. 111, 888–897. doi: 10.1007/s00122-005-0011-6

Babu R., Prasanna B. (2014). "Molecular breeding for quality protein maize (QPM)," in Genomics of Plant Genetic Resources, eds. R. Tuberosa, A. Graner, E. Frison (Dordrecht, Netherlands: Springer), 489–505.

Bekele, A. and Rao, T. N. (2014) Estimates of heritability, genetic advance and correlation study for yield and it's attributes in maize (Zea mays L.). Journal of Plant Science. 2(1),1-4. Doi: 10.11648/j.jps.20140201.11

Brunson, A.M. (1937). "Popcorn Breeding," in Specialty corns, ed. A.R. Hallauer (New York, NY: CRC Press), 189–223.

Butler, D. (2019). asreml: Fits the Linear Mixed Model. R package version 4.1.0.110. www.vsni.co.uk

Coan, M.M.D., Pinto, R.J.B., Kuki, M.C., Amaral Júnior, A.T., Figueiredo, A.S.T., Scapim, C.A., Warburton, M. (2019). Inheritance Study for Popping Expansion in Popcorn vs. Flint Corn Genotypes. Agron. J. 111, 2174.

Coimbra, R.R., Miranda, G.V., Viana, J.M.S., Cruz, C.D., Murakami, D.M., Souza, L.V., Fidelis, R.R. (2002). Estimation Of Genetic Parameters And Prediction Of Gains For DFT1-Ribeirão Popcorn Population. Crop Breeding And Applied Biotechnology 2:1, 33-38. doi:10.12702/1984-7033.v02n01a05

Crumbaker, D. E., Johnson, I., Eldredge, J. (1949). Inheritance of popping volume and associated characters in crosses between popcorn and dent corn. Agron. J. 41, 207–212. doi: 10.2134/agronj1949.00021962004100050009x

Cutrufelli, R. (1991). Composition of Foods: Snacks and Sweets: Raw, Processed, Prepared. Washington, D.C.: United States Department of Agriculture: Human Nutrition Information Service.

Da Luz, P.B., Dos Santos A.A.B. Ambrosio, V.C., Neves, L.G., Tavares, A.R. (2018). Selection Of Indexes To Evaluate The Genetic Variability Aiming Ornamental Use Of Peppers Accessions. Ornamental Horticulture 24:1, 7-11. doi:10.14295/oh.v24i1.1109

Dar, Z.A., Lone, A.A., Alie, B.A., Ahangar, M.A., Ali, G., Abidi, I., Gazal, A., and Lone, R.A. (2018). Combining ability analysis for yield and yield contributing traits in Popcorn (Zea mays everta L.) under temperate conditions. Journal of Pharm. And Phyto. 7:1, 361-366.

Daros, M., Amaral Júnior., A.T., Pereira, M.G., (2002). Genetic gain for grain yield and popping expansion in full-sib recurrent selection in popcorn. Crop Breeding and Applied Biotechnology. 2, 339-344.

Dass, S., Singh, M., Schtiya, H.L., (1990). Genetic variability and correlation among some quantitative and quality traits in maize. Agricultural Science Digest 10:4), 189-193.

Dawande, Rushikesh. (2018). Popcorn Market by Type (Microwave Popcorn and Ready-to-eat Popcorn) and End User (Household and Commercial) - Global Opportunity Analysis and Industry Forecast, 2017-2023. Portland: Allied Market Research.

De Azeredo, A.A.C., Bhering, L.L., Brasileiro, B.P., Cruz, C.D., Silveira, L.C.I., Oliveira, R.A., Bespalhok Filho, J.C., and Daros, E. (2017). Comparison Between Different Selection Indices In Energy Cane Breeding. Genetics And Molecular Research 16:1. doi:10.4238/gmr16019535

Dhliwayo T. (2008) Genetic Mapping and Analysis of Traits Related to Improvement of Popcorn. [Ph.D. thesis]. [Ames, (IA)]: Iowa State University

Djordjevic, S.J., Ivanovic, R.M., (1996). Genetic analysis for stalk lodging resitance in narrow-base maize synthetic population ZP514. Crop Science 36, 909-913.

Dofing S.M., Thomas-Compton, M.A., Buck, .J.S. (1990). Genotype × popping method interaction for expansion volume in popcorn. Crop Sci. 30, 62–65.

Dofing, S. M., ĎCroz-Mason N., Thomas-Compton, M. A. (1991). Inheritance of Expansion Volume and Yield in Two Popcorn ⨯ Dent Corn Crosses. Crop Sci. 31,715-718. doi:10.2135/cropsci1991.0011183X003100030035x

Elango, R., Ball, R.O., Pencharz, P.B. (2009). Amino acid requirements in humans: with a special emphasis on the metabolic availability of amino acids. Amino Acids 37:19. doi: 10.1007/s00726-009-0234-y.

Eldredge, J.C., and Thomas, W.I. (1959). Popcorn… Its production, processing, and utilization. Bulletin P: Vol 7: Bulletin P127, Article 1.

Erazo-Barradas, M. (2009). Evaluation of Popping Expansion Traits in a Maize (Zea mays L.) Population. [Master's thesis] [Ames (IA)]: Iowa State University.

Fu, D., Xiao, M., Hayward, A., Fu, Y., Liu, G., Jiang, G, Zhang, H. (2014). Utilization Of Crop Heterosis: A Review. Euphytica. 197:2, 161-173. doi:10.1007/s10681-014-1103-7

Geetha K., Lending C. R., Lopes M. A., Wallace J. C., Larkins B. A. (1991). *opaque-2* modifiers increase γ-zein synthesis and alter its spatial distribution in maize endosperm. Plant Cell. 3, 1207–1219.

Gevers H. O., Lake J. K. (1992). "Development of modified *opaque-2* maize in South Africa," in Quality Protein Maize, ed. E. T. Mertz (Saint Paul, MN: AACC), 49–78.

Griffing, B. (1956). Concept of general and specific combining ability in relation to diallel crossing systems. Australian J. Biol. Sci. 9, 463–93.

Hadji, T. (2004). Combining ability analysis for yield and yield related traits in quality protein maize inbred lines. [Master's thesis]. [Dire Dawa, (Ethiopia)]: Haramaya University

Hallauer, A.R., and Eberhart, S.A. (1970). Reciprocal full-sib selection. Crop Sci. 10, 315-316

Hao, Z. (2019). kableExtra: Construct Complex Table with 'kable' and Pipe Syntax. R package version 1.1.0. https://CRAN.R-project.org/package=kableExtra

Hernandez, H.H., and L.S. Bates. (1969). A modified method for rapid tryptophan analysis in maize. CIMMYT Research Bulletin no. 13. Mexico, D.F.: CIMMYT.

Hunter, M. D. (2018). State space modeling in an open source, modular, structural equation modeling environment. Structural Equation Modeling, 25(2), 307-324. doi:10.1080/10705511.2017.1369354

Holding, D. R. (2014). Recent advances in the study of prolamin storage protein organization and function. Front. Plant Sci. 5:276. doi: 10.3389/fpls.2014.00276

Holding, D. R., Hunter, B. G., Chung, T., Gibbon, B.C., Ford, C.F., Bharti, A.K. (2008). Genetic analysis of opaque2 modifier loci in quality protein maize. Theor. Appl. Genet. 117, 157–170. doi: 10.1007/s00122-008-0762-y

Holding, D. R., Hunter, B.G., Klingler, J.P., Wu, S., Guo, X., Gibbon, B. C. (2011). Characterization of opaque2 modifier QTLs and candidate genes in recombinant inbred lines derived from the K0326Y quality protein maize inbred. Theor. Appl. Genet. 122, 783–794. doi: 10.1007/s00122-010-1486-3

Hoseney R., Zeleznak K., Abdelrahman, A. (1983). Mechanism of popcorn popping. J. Cereal Sci. 1, 43–52. doi: 10.1002/anie.201402040

Isik, F., Holland, J.B., Christian, M., and Hüsnü, D. (2017). Genetic Data Analysis For Plant And Animal Breeding. Cham, Switzerland: Springer International Publishing.

Johnson, B.E., Dauer, J.P., Gardner, C.O. (1988). A model for determining weights of traits in simultaneous multitrait selection. Appl. Math. Modelling. Vol 12, August.

Johnson, I., and Eldredge, J. (1953). Performance of recovered popcorn inbred lines derived from outcrossed to dent corn. Agron. J. 45, 105-110. doi: 10.2134/agronj1953.00021962004500030005x

Kantety, Ramesh V., Zeng, X., Bennetzen, J.L., Zehr, B.E. (1995). Assessment Of Genetic Diversity In Dent And Popcorn (Zea Mays L.) Inbred Lines Using Inter-Simple Sequence Repeat (ISSR) Amplification. Molecular Breeding 1, 365-373. doi:10.1007/bf01248414

Kostadinovic M., Ignjatovic-Micic D., Vancetovic J., Ristic D., Bozinovic S., Stankovic G. (2016). Development of high tryptophan maize near isogenic lines adapted to temperate regions through marker assisted selection-impediments and benefits. PLoS One. doi: 10.1371/journal.pone.0167635

Krishna M., Reddy S. S., Satyanarayana, S. D. (2017). Marker-assisted breeding for introgression of *opaque-2* allele into elite maize inbred line BML-7. Biotech. 7,165.

Krivanek, A.F., Groote, H.D., Gunaratna, N.S., Diallo, A.O., Friesen, D. (2006). Breeding and disseminating quality protein maize (QPM) for Africa. African Journal of Biotechnology. 6:4, 312-324.

de Lima, V.J., do Amaral Júnior, A.T., Kamphorst, S.H., Bispo, R.B., Leite, J., de Oliveira Santos, T., Schmitt, K., Chaves, M.M., de Oliveira, U.A., dos Santos, P.H., Gonçalves, G.M.B., Khan, S., and Guimarães, L.J.M. (2019). Combined Dominance and Additive Gene Effects in Trait Inheritance of Drought-Stressed and Full Irrigated Popcorn. Agronomy. 9, 782. doi:10.3390/agronomy9120782

Li Y. L., Dong Y. B., Niu S. Z., Cui D. Q. (2009). Identification of QTL for popping characteristics using a BC_2F_2 population and comparison with its F2:3 population in popcorn. Agric. Sci. China. 8,137–143. doi: 10.1016/S1671-2927(09)60020-1

Li Y., Dong Y., Niu S., Cui D. (2007). QTL for popping characteristics in popcorn. Plant Breed. 126, 509–514. doi: 10.1111/j.1439-0523.2007.01372.x

Li, Y. L., Dong Y., Niu, S., Cui, D., Wang, Y., Liu, Y., Wei, M., Li, X. (2008). Identification Of Agronomically Favorable Quantitative Trait Loci Alleles From A Dent Corn Inbred Dan232 Using Advanced Backcross QTL Analysis And Comparison With The F2:3 Population In Popcorn. Molecular Breeding. 21,1-14. doi:10.1007/s11032-007-9104-z

Li, Y.L., Lu, F.Y., Du, Z.W., Wu, S.W., Han, C.P. (2002). Preliminary report on improvement result to popcorn using normal corn germplasm and backcross. Acta Agriculturae Boreali-Sinica. 17, 37–43.

Lima, L.K., Ramalho, M.A.P., Abreu, A.F.B. (2012). Implications of the progeny x environment interaction in selection index involving characteristics of the common bean. Genet. Mol. Res 11:4, 4093-4099. doi: http://dx.doi.org/10.4238/2012.September.19.5

Liu, H., Shi J., Sun C., Gong H., Fan X., Qiu F. (2016). Gene duplication confers enhanced expression of 27-kDa γ-zein for endosperm modification in quality protein maize. Proc. Natl. Acad. Sci. U.S.A. 113, 4964–4969. doi: 10.1073/pnas.1601352113

Liu, H., Huang, Y., Li, X., Wang, H., Ding, Y., Kang, C., Sun, M., Li, F., Wang, J., Deng, Y., Yang, X., Huang, X., Gao, X., Yuan, L., An, D., Wang, W., Holding, D.R., Wu, Y. (2019). High frequency DNA rearrangement at qγ27 creates a novel allele for Quality Protein Maize breeding. Commun Bio 2, 460. doi: 10.1038/s42003-019-0711-0.

Mafra, G.S., Amaral Junior, A.T.D., Vivas, M., Santos, J.S.D., Guimarães, A.G., Pena, G.F. (2018). The combining ability of popcorn S7 lines for Puccinia polysora resistance purposes. Bragantia. 77, 519–526.

Malik, S.I., Malik, H.N., Minhas, N.M., Munir, M. (2004). General and Specific Combining Ability in Maize. Int. J. Agri. Biol. 6:5.

Mandefro, N. (1998). Heterosis, combining ability and correlation in 8 x 8 diallel crosses of drought tolerant Maize (Zea mays L.) populations. [Master's thesis] [Dire Dawa (Ethiopia)]: Haramaya University

Marinho, C.D., Gravina, G.A., Sebastião, L.C., Almeida, N.C., Daher, R.F., Brasileiro, B.P., Paula, T.O.M, Amaral Júnior, A.T. (2014). Indexes in the comparison of pre-commercial genotypes of common bean. Ciência Rural, 44:7, 1159-1165. doi: 10.1590/0103-8478cr20121155

Mbuya K., Nkongolo K., Kalonji-Mbuyi A. (2011). Nutritional analysis of quality protein maize varieties selected for agronomic characteristics in a breeding program. Int. J. Plant Breed. Genet. 5, 317–327. doi: 10.3923/ijpbg.2011.317.327

Mertz E. T., Bates L. S., Nelson O. E. (1964). Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science. 145,279–280. doi: 10.1126/science.145.3629.279

Moll, R.H., Lonnquist, J.H., Fortuno, J.V., Johnson, E.C. (1965). The Relationship of Heterosis and Genetic Divergence in Maize. Genetics 52, 139-144.

Mordor Intelligence. (2018). POPCORN MARKET – GROWTH, TRENDS AND FORCAST (2020-2025). Hyderabad, India: Mordor Intelligence.

Mulamba, N.N., and Mock, J.J. (1978). Improvement of yield potential of the Eto Blanco maize (Zea mays L.) population by breeding for plant traits. Egypt. J. Gen. Cytol. 7, 40-51.

Olakojo, S.A., Omueti, O., Ajomale, K., and Ogunbodede, B.A., (2007). Development of Quality Protein Maize: Biochemical and Agronomic Evaluation. Tropical and Subtropical Agroecosystems. 7, 97-104.

Ortega, E.I., Villegas, E., and Vasal, S.K. (1986). A Comparative Study of Protein Changes in Normal and Quality Protein Maize During Tortilla Making. Cereal Chem. 65:5, 466-451.

Panda A. K., Raju M. V., Rao S. V., Lavanya G., Pradeep Kumar Reddy E., Shyam Sunder G. (2010). Replacement of normal maize with quality protein maize on performance, immune response and carcass characteristics of broiler chickens. Asian Aust. J. Anim. Sci. 23, 1626–1631. doi: 10.5713/ajas.2010.10036

Pereira, M.G., Amaral Júnior, A.T. (2001). Estimation of genetic components in popcorn based on the nested design. Crop Breeding and Applied Biotechnology. 1, 3-10.

Prasanna B. M., Vasal S. K., Kassahun B., Singh N. N. (2001). Quality protein maize. Curr. Sci. 81, 1308–1319.

R Core Team. (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rafiq, C.M., Rafique, M., Hussain, A., and Altaf, M. (2010). Studies on Heritability, Correlation and Path Analysis in Maize (Zea Mays L.) J. Agric. Res. 48:1.

Reif, J.C., Hallauer, A.R., and Melchinger, A.E. (2005). Heterosis and Heterotic Patterns in Maize. Maydica 50, 215-223.

Reif, J.C., Melchinger, A.E., Xia, X.C., Warburton, M.L., Hoisington, D.A., Vasal, S.K., Beck, D., Bohn, M., Frisch, M. (2003). Use of SSRs for establishing heterotic groups in subtropical maize. Theor Appl Genet. 107:5, 947–957.

Ren, Y., Yobi, A., Marshall, L., Angelovici, R., Rodriguez, O., Holding, D. (2018). Generation and Evaluation of Modified Opaque-2 Popcorn Suggests a Route to Quality Protein Popcorn. Front. Plant Sci. 9, 1803. doi: 10.3389/fpls.2018.01803

Ribeiro, L.P, Teodoro, P.E., Corrêa, C.C.G., Oliveira, E.P., da Silva, F.A., Torres, F. E. (2016). Correlations And Genetic Parameters In Maize Hybrids. Bioscience Journal. 32:1, 48-54. doi:10.14393/bj-v32n1a2016-29388.

Robbins, W. A., and Ashman, R.B. (1984). Parent-offspring popping expansion correlations in progeny of dent corn x popcorn and flint corn x popcorn crosses. Crop Sci. 24, 119-121. doi: 10.2135/cropsci1984.0011183X002400010027x

Ross, A. (2002). Genetic analysis of ear length and correlated traits in maize. [Ph.D. thesis].[Ames, (IA)]: Iowa State University

Sacha, E. (2019). semPlot: Path Diagrams and Visual Analysis of Various SEM Packages' Output. R package version 1.1.2. https://CRAN.R-project.org/package=semPlot

Senhorinho, H.J.C., Coan, M.M.D., Marino, T.P., Kuki, M.C., Pinto, R.J.B., Scapim, C.A., and Holland, J.B. (2019). Genomic-Wide Association Study of Popping Expansion in Tropical Popcorn and Field Corn Germplasm. Crop Sci. 59, 2007-2019. doi:10.2135/cropsci2019.02.0101

Sethi, M., Kumar, S., Sign, A., and Chaudhary, D.P. (2020). Temporal profiling of essential amino acids in developing maize kernel of normal, *opaque-2* and QPM germplasm. Physiol Mol Biol Plants. 26, 341-351. https://doi.org/10.1007/s12298-019-00724-x

Schloerke, B., Crowley, J., Cook, D., Briatte, F., Marbach, M., Theon, E., Elberg, A., Larmarange, J. (2018). GGally: Extension to 'ggplot2'. R package version 1.4.0. https://CRAN.R-project.org/package=GGally

Shapira, R., and David, L. (2016). Genes with a Combination of Over-Dominant and Epistatic Effects Underlie Heterosis in Growth of Saccharomyces cerevisiae at High Temperature. Front. Genetics. 7:72. doi: 10.3389/fgene.2016.00072

Sofi P., Wani S. A., Rather A., Wani S. H. (2009). Quality protein maize (QPM): genetic manipulation for the nutritional fortification of maize. J. Plant Breed. Crop Sci. 1, 244–253.

Spaner, D., Mather, D.E., Hamilton, R.I. (1992). Genetic and agronomic evaluation of short-season Quality Protein Maize. Can. J. Plant Sci. 72, 1171-1181.

Sprague, G.F. and Dudley, J.W. (1988). Corn and Corn Improvement. Madison: America Society of Agronomy.

Springer, N. M., and Stupar, R. M. (2007). Allelic Variation And Heterosis In Maize: How Do Two Halves Make More Than A Whole?. Genome Research. 17:3, 264-275. doi:10.1101/gr.5347007.

Surender M., Reddy, S. S., Sudarshan, M. R., Durgarani, C. V. (2014). Gene specific SSR marker for detection of OPAQUE-2 alleles for conservation of non-QPM lines for QPM development. Res. J. Agric. Sci. 5, 666–669.

Sweley, J.C., Rose, D.J., and Jackson, D.S. (2011). Composition and Sensory Evaluation of Popcorn Flake Polymorphisms for a Select Butterfly-Type Hybrid. Cereal Chemistry. 88, 321-327.

Tardin, F.D., Pereira, M.G., Gabriel, A.P.C., Amaral Júnior, A.T., Souza Filho, G.A. (2007). Selection Index And Molecular Markers In Reciprocal Recurrent Selection In Maize. Crop Breeding And Applied Biotechnology. 3, 225-233. doi:10.12702/1984-7033.v07n03a01

Tulu, B.N. (2014). Correlation and path coefficients analysis studies among yield and yield related traits of quality protein maize (QPM) inbred lines. International Journal of Plant Breeding and Crop Science. 1:2, 006-017.

Vasal, S.K. (2002). Quality Protein Maize: Overcoming the Hurdles. Journal of Crop Production. 6:1-2,193-227 doi: 10.1300/J144v06n01_11

Vasic, N., Ivanovic, M., Peternelli, L.A., Jockovic, D., Stojakovic, M., and Bocanski, J. (2001). Genetic Relationships between Grain Yield and Yield Components in a Synthetic Maize Population and their Implications in Selection. Acta Agronomica Hungarica 49:4, 337-342. doi:10.1556/aagr.49.2001.4.4.

Vivek, B., Krivanek, A., Palacios-Rojas, N., Twumasi-Afriyie, S., and Diallo, A.O. (2008). Breeding Quality Protein Maize (QPM): Protocols for Developing QPM Cultivars. Texcoco: CIMMYT

Wallace J. C., Lopes M. A., Paiva E., Larkins B. A. (1990). New methods for extraction and quantitation of zeins reveal a high content of gamma-zein in modified *opaque-2* maize. Plant Physiol. 92, 191–196. doi: 10.1104/pp.92.1.191

Wickham et al., (2019). Welcome to the tidyverse. Journal of Open Source Software, 4(43), 1686, https://doi.org/10.21105/joss.01686

Wu, G. (2009). Amino acids: metabolism, function, and nutrition. Amino Acids. 37, 1-17. doi: 10.1007/s00726-009-0269-0.

Wu Y., Holding D. R., Messing J. (2010). γ-zeins are essential for endosperm modification in quality protein maize. Proc. Natl. Acad. Sci. U.S.A. 107, 12810–12815. doi: 10.1073/pnas.1004721107

Yihui, X. (2020). knitr: A General-Purpose Package for Dynamic Report Generation in R. R package version 1.28.

Yobi, A., and Angelovici, R. (2018). A High-Throughput Absolute-Level Quantification of Protein-Bound Amino Acids in Seeds. Curr Protoc Plant Biol. 3:4 doi: 10.1002/cppb.20084.

Yusuf, M. (2010). Genetic Variability And Correlation In Single Cross Hybrids Of Quality Protein Maize (Zea Mays L.). African Journal Of Food, Agriculture, Nutrition And Development. 10:2. doi:10.4314/ajfand.v10i2.53358.

Yousuf, M. and Saleem, M. (2001). Correlation Analysis of S1 Families of Maize for Grain Yield and its Components. Int. J. of Agri. Biol. 3:4, 387-388.

Yves, R. (2012). lavaan: An R Package for Structural Equation Modeling. Journal of Statistical Software, 48(2), 1-36. URL http://www.jstatsoft.org/v48/i02/.

Zhou, Q., Shi, Q.L., and Dong, Y.B. (2016). Effect of *opaque-2* gene on grain, quality, and popping characteristics of popcorn. J. Henan Agric. Sci. 45, 24-28.

Ziegler, K.E., and Ashman, B. (1994). “Popcorn”, in Specialty corns, ed. A.R. Hallauer (New York, NY: CRC Press), 189-223.

CHAPTER 3: FINAL SELECTION OF ELITE QUALITY PROTEIN POPCORN (QPP) HYBRIDS FIT FOR COMMERCIALIZATION USING THE 2020 RANKING SYSTEM

1. Introduction

Popcorn [Zea mays L. ssp. everta (Sturt.) Zhuk] is a type of flint corn characterized by its ability to expand and form light flakes under high heat. Popcorn has been enjoyed as a direct-to-consumer product in the United States for more than a century, and in 2013 the popcorn industry revitalized from a two-decade retailing plateau owing to growing consumer demand for a healthier, innovative snack food option and increased diversity in the popcorn market (Smith et al., 2004; Topping, 2011; Mordor Intelligence, 2018). Intraspecies crosses between dent maize (Zea mays var. indentata) and popcorn, one avenue for increasing diversity in the popcorn germplasm pool, have shown to enhance popcorn's agronomic fitness and flavor profile at the cost of deficient popcorn quality traits such as popability and expansion volume (Robbins and Ashman, 1984; Sprague and Dudley, 1988; Dofing et al., 1991; Ziegler and Ashman, 1994). To negate these undesired side effects, a study in 2018 described an inter-subspecies breeding program crossing highly vitreous dent Quality Protein Maize (QPM) varieties with proprietary popcorn lines to produce highly vitreous, high lysine Quality Protein Popcorn (QPP) BC_2F_5 inbred lines (Ren et al., 2018). Concurrent to rapidly restoring popcorn traits, these unique popcorn inbred lines carried the *opaque-2* homozygous recessive mutation and conferred a 1.5-2 fold increase in kernel endosperm lysine levels compared to the original popcorn parents (Ren et al., 2018). This proof-of-concept study supported the positive correlation between kernel endosperm vitreousness, the hard and translucent

endosperm phenotype, and popcorn quality traits, a previously published but majorly unexplored concept (Hoseney et al., 1983; Matz, 1984; da Silva et al., 1993; Smith et al., 2004; Babu et al., 2006). Methods involved in this study included a phenotypic assessment of vitreousness, genotypic marker-assisted-selection for the *opaque-2* allele, and proteomic evaluation through endosperm protein extraction and SDS-PAGE (Ren et al., 2018). Though modifier genes conferring vitreousness in *opaque-2* carrying lines still remain largely unknown, a 2016 study confirmed that the over-expression of the 27-kd γ-zein maize endosperm storage protein, a known requirement for restoring vitreousness in *opaque-2* carrying lines, was due to a genetic duplication of the 27-kd γ-gene (Liu et al., 2016). Since the rest of the endosperm modifier genes are unspecified (though genetic locations have been postulated), phenotypic evaluation of vitreousness and zein profiling still serve as the best means for selecting vitreousness, and consequently popping traits, in an *opaque-2* background (Holding et al., 2008, 2014; Wu et al., 2010; Parsons et al., 2020).

To further develop this proof-of-concept intraspecies breeding program, twelve BC_2F_5 QPP inbreds were hybridized in the summer of 2018 to produce 132 QPP F_1 seed. After initial observation, 44 QPP crosses were selected for further pre-screening analysis of agronomic, protein, and popcorn quality traits. In the summer of 2019, these 44 crosses were grown in multiple locations and fourteen traits were evaluated for selection of five superior BC_2F_5 QPP hybrids (Parsons et al., 2020). Quantitative positive correlations between popcorn expansion volume, popability, and endosperm vitreousness were measured, and the results further emphasized the preliminary requirement for highly vitreous dent parents in a successful popcorn by dent maize subspecies crosses (Parsons et

al., 2020). The five selected QPP BC_2F_5 derived hybrids had relatively superior agronomics, elevated lysine levels in the kernels, and best maintained popcorn quality traits compared to the rest of the assessed hybrids.

Though the QPP hybrids were phenotypically indistinct from the original popcorn lines and held comparatively superior agronomics and adequate popping characteristics, previous studies have suggested that multiple rounds of backcrossing aid in restoring popping expansion volume (Babu et al., 2006). The five elite BC_2F_5 QPP hybrids chosen in 2019 had nonsignificant differences in popability (number of unpopped kernels/number of kernels tested), but slightly lower popping expansion volume compared to original popcorn germplasm (Parsons et al., 2020). To test the potential improvement of QPP popcorn quality traits, specifically popping expansion volume, by backcrossing, BC_2F_5 QPP inbreds were again backcrossed to elite popcorn parental lines, and *opaque-2* carrying, phenotypically vitreous BC_3F_4 QPP lines were produced in the fall of 2019. These BC_3F_4 inbreds were selectively crossed to produce the five pre-selected QPP hybrids from the 2019 analysis of BC_2F_5 crosses. In the summer of 2020, these ten QPP hybrids, five BC_2F_5 and five BC_3F_4 derived, and five chosen ConAgra Brands® original popcorn cultivars were grown in a generalized randomized block design at three locations to measure, compare, and rank QPP and ConAgra Brands® popcorn cultivars based on agronomic, popcorn quality, and protein quality traits. Overall, significant improvements in popcorn quality traits were observed in the BC_3 cultivars compared to their BC_2 counterparts, yield averages were significantly lower in BC_3 derived QPP hybrids compared to the BC_2 population, and protein quality traits were insignificantly different between QPP backcrossing populations and significantly superior to ConAgra elite varieties. Through

the use of a previously published ranking system and due to satisfactory agronomics, superior lysine content in the raw kernel and popped flakes, and most similar popcorn quality traits compared to ConAgra® Brands' elite hybrids, six QPP hybrids, three from the BC_2F_5 population and three from the BC_3F_4 population, were recommended to enter more robust testing for potential commercialization.

2. Materials and Methods

2.1 Plant Materials

2.1.1 BC_2F_5 inbred QPP lines

BC_2F_5 inbred QPP lines were produced by a Quality Protein Maize dent (QPM) by popcorn backcross breeding program as described in Ren et al. (Ren et al., 2018). Briefly, QPM lines CML154Q, Tx807, and K0326Y were crossed to ConAgra Brands® proprietary popcorn inbred lines labeled P1-P4 (proprietary names withheld) in 2013. Original ConAgra Brands® popcorn inbred lines were provided by ConAgra Brands®, K0326Y QPM dent maize was provided by Hans Gevers (Gevers and Lake, 1992), and CML154Q and Tx807 were provided from the North Central Regional Plant Introduction Station (Ren et al., 2018). To produce the BC_2F_5 inbred QPP lines, F_1 hybrids were backcrossed twice to the popcorn parent and self-pollinated five times with an expected level of heterozygosity at a given locus of 0.39% (Ren et al., 2018).

2.1.2 BC_3F_4 inbred QPP lines

BC_3 lines were produced by an additional cross of female ConAgra® popcorn lines with male BC_2F_5 QPP inbred lines during the summer of 2018. These BC_3F_1 QPP hybrids were self-pollinated in the winter of 2018 and the BC_3F_2 seed segregated for the QPM *opaque-2* allele. Homozygous recessive *opaque-2* kernels were selected through SDS-PAGE and

marker-assisted selection (as detailed below) and subsequently self-pollinated twice. BC_3F_4 seed was produced in the summer of 2019 concurrent with BC_2F_5 QPP hybrids analysis. Assuming a theoretical genetic contribution of popcorn to dent maize as 93.75% and 6.25%, respectively, and the homozygosity of an F_4 at 93.75%, the availability for heterozygosity in the BC_3F_4 inbred lines is synonymous to the BC_2F_5 lines at 0.39%. Comparatively, an F_8 inbred line has an available heterozygosity of 0.39% (Collard et al., 2005; Uptmoor et al., 2006; Gupta et al., 2010).

2.1.3 BC_2F_5, BC_3F_4, and ConAgra® Brands F_1 hybrid seed

After the 2019 summer field trials, five QPP BC_2F_5 hybrids were selected for further testing: Hybrid 20 (QPP BC_2F_5 Inbred 6 x QPP BC_2F_5 Inbred 10), Hybrid 25 (QPP BC_2F_5 Inbred 9 x QPP BC_2F_5 Inbred 3), Hybrid 28 (QPP BC_2F_5 Inbred 9 x QPP BC_2F_5 Inbred 6), Hybrid 38 (QPP BC_2F_5 Inbred 10 x QPP BC_2F_5 Inbred 5), and Hybrid 43 (QPP BC_2F_5 Inbred 10 x QPP BC_2F_5 Inbred 11) (Parsons et al., 2020). In the spring of 2020, BC_2F_5 and BC_3F_4 hybrids of the chosen crosses were produced and F_1 seed was harvested. These QPP cultivars were grown alongside five ConAgra check hybrids and varietals in the summer of 2020. {Popcorn parent 1 x Popcorn parent 2} seed and its reciprocal seed were produced in the spring of 2020 alongside QPP hybrids, and {Popcorn parent 1 x Popcorn parent 3} seed and two check ConAgra varietals were supplied by Dr. Oscar Rodriguez of ConAgra Brands® to compare both commercialized lines and respective non-QPM hybrids with QPP hybrids **(Table 1)**. In all, fifteen cultivars were planted in the summer of 2020 and numerically named 1-15 in order of BC_2F_5 hybrids, BC_3F_4 hybrids, and ConAgra test cultivars, respectively **(Table 1).**

2.2 2020 Field Design

The fifteen selected cultivars were grown in three locations over the summer of 2020. Seed was sown on April 30th in Lincoln, Nebraska (40°50'11.6"N 96°39'42.4"W DMS), May 1st in Mead, Nebraska (41°08'51.6"N 96°27'04.7"W DMS), and May 5th in Colby, Kansas (39°22'50.7"N 101°03'33.0"W DMS) in collaboration with Kansas State University's Northwest Research-Extension Center. Fields were designed in a Generalized Randomized Block Design (GRBD) with three replications of the treatment (genotype) per location. Experimental Units (EUs) were 17 foot (5.18 meters) by four row (10 feet or 3 meters) plots planted at ~34,500 population plants/acre (8.53 plants/m^2) and separated on all sides by 6-8 rows of dent border corn (45 EUs per location). The center two rows of EUs were machine harvested and random ears from the fourth row was hand-harvested for analysis.

2.3 Zein and non-Zein Protein Extraction and SDS-PAGE Profiling

F_1 hybrid seeds from all experimental crosses were subjected to zein and non-zein protein analysis as previously described (Wallace et al., 1990; Ren et al., 2018; Parsons et al., 2020). QPP F_1 and F_2 hybrid seed produced from BC_2F_5 and BC_3F_4 inbred lines were tested to verify a QPM-patterned proteome of high 27-kD γ-zein and low α-zeins. The specific procedures used for both zein and non-zein analysis are described in Parsons et al., 2020. Briefly, raw kernel powder was introduced to a borate extraction buffer and the protein supernatant was extracted. Zein and non-zein fractions were separated by adding 70% ethanol and incubating overnight. The soluble zein and non-soluble non-zein fractions were separated and proteins were profiled using acrylamide SDS-PAGE (Wallace et al., 1990).

2.4 Validating *o2o2* genotype in QPP inbreds

QPP BC_2F_5 and BC_3F_4 inbred lines utilized for hybrids, Inbreds 3, 5, 6, 9, 10, and 11, were genotyped for *o2o2* validation using *opaque-2* in-gene marker umc1066 and flanking marker bnlg1200 (Babu et al., 2005; Ren et al., 2018; Parsons et al., 2020). Inbreds 3, 9, 10, and 11 were genotyped by bnlg1200, while Inbreds 5 and 6 were genotyped by in-gene marker umc1066. Polymerase Chain Reaction (PCR) was carried out according to Ren et al. except TaKaRa Ex Taq DNA polymerase was used in the place of NEB Taq DNA polymerase (Ren et al., 2018). Annealing temperatures for umc1066 varied between 60-63° Celsius and held at approximately 55° C for bnlg1200. For DNA, two-week old leaf tissue was collected and DNA extracted according to a previously published procedure (Holding et al., 2008). Crude DNA was diluted 20-fold with double distilled or autoclaved water for an average concentration of 50 ng/µL.

2.5 Trait Analysis

Cultivars were harvested with a two-row plot combine capable of estimating test weight (lbs/bu), plot weight (lbs), and moisture content. Yield estimates were determined by Equations 1 and 2 and pounds of dry matter per bushel was measured for kernel size comparisons, as shown in Equation 3.

$$Yield\left(\frac{pounds\ of\ dry\ matter}{feet^2}\right) \quad (1)$$

$$= \frac{Plot\ weight\ (pounds) * (1 - moisture\ percentage)}{85\ feet^2}$$

$$Yield\left(\frac{56\ lb\ bushel\ at\ 15.5\%\ moisture}{acre}\right) = \quad (2)$$

$$\left(\frac{Plot\ weight\ (pounds) * (1 - \{moisture\ percentage - .155\} * .012)}{56}\right) * 512.5$$

$$\frac{pounds\ of\ dry\ matter}{volumetric\ bushel} = Test\ weight\ \left(\frac{pounds}{vol.\ bushel}\right) * (1 - moisture\ percentage) \quad (3)$$

Equation 1 estimated the amount of dry matter accumulated from each experimental unit while Equation 2 evaluated the yield of the plots on a 15.5% grain moisture bushel (the standard moisture value of a dry maize bushel) basis. 1.2% shrinkage due to expected water loss was incorporated into the equation (Hicks and Cloud, 1991). Equation 3 aided in estimating kernel size, kernel density, and packing efficiency. The yield estimate of Equation 1 was used in the 2020 Ranking System (detailed below).

Approximately two pound (~1000 gram) subsamples were obtained from the center two rows of each experimental unit to measure vitreousness, expansion volume, popability, and flake morphology. Approximately 50 kernels were assessed from each subsample for kernel vitreousness on a scale of 1-7 as previously described (Parsons et al., 2020). Five ears were randomly hand-harvested from the fourth row of every EU for one average ear length measurement per EU and amino acid profiling of the endosperm proteome. Three measurements of plant height were recorded and averaged for one height measurement per EU. Roughly 250 grams of machine-harvested seed from each EU (135 total samples) were placed in a conditioning room set at 14% moisture for six weeks for moisture equilibration prior to popping quality tests. After equilibration, the 250 gram samples were

popped and measured for expansion volume (cubic centimeters per grams), popability (({total number of kernels subjected to popping - number of unpopped kernels after popping}/total number of kernels subjected to popping) expressed as a percentage), and flake size index estimates. Flake size index (CFSI) was estimated using Equation 4:

$$OCFSI = (OE * 250)/(\left(\left(\frac{KC}{10}\right) * Weight\right) - UPK) \quad (4)$$

The OCFSI (Oil Crude Flake Size Index) is an estimate of an average individual kernel's flake expansion. '*OE'* is the expansion volume measured in a graduated cylinder (0-50 mL) of expansion volume per gram in cubic centimeters. The *'KC'* value is the number of kernels in a random sample of 10 grams. *Weight* is the sample weight (250 grams), and *UPK* represents the total number of unpopped kernels in the 250 gram sample. Measurement of expansion volume, popability, and OCFSI estimates were accomplished utilizing ConAgra Brands test oil popper and facilities in Brookston, Indiana. Categorical observation of flake morphology as either mushroom, butterfly, or mixed was ascertained. Free and protein bound amino acid profiles of all tested cultivars were analyzed at the University of Missouri according to previously published procedures (Angelovici et al., 2013; Yobi and Angelovici, 2018). Six samples from each cultivar, three in raw kernel powder and three in air-popped flake forms, were analyzed. Raw flour and air popped flake samples were prepared according to previously described procedures (Parsons et al., 2020). Briefly for popped samples, air-popped flakes were frozen in liquid nitrogen then ground in a mortar and pestle until a fine powder. B73 raw, ground flour was also submitted for reference.

2.6 Statistical Analysis

Cultivar trait estimates were analyzed by the statistical model given by Equation 5:

$$y_{ijk} = \mu + \beta_i + \tau_j + (\beta\tau_{ij}) + \epsilon_{ijk} \quad (5)$$

In which y_{ijk} is the cultivar's response, μ is the overall mean, β_i is the block, or locational, effect, τ_j is the treatment, or cultivar, effect, $(\beta\tau_{ij})$ is the location*treatment interaction, and ϵ_{ijk} is the experimental error (Addelman, 1969). Type II sums of squares was used to compute the Analysis of Variance and the treatment effect was fixed. The Central Limit Theorem was assumed for normality of the data. R Software was used to conduct all analysis including trait correlations and Tukey's Honest Significant Differences (R Core Team, 2018).

2.7 Cultivar Index Selection: 2020 Ranking System

As shown in Equation 6, the 2020 Ranking System described in previous study was utilized to rank the 15 tested cultivars (Parsons et al., 2020):

$$X_h = \sqrt{\sum_{i=1}^{m} \left(\frac{y_{i,h}}{y_{i,max}} - 1\right)^2 I_i \, {}^{(\sigma_{i,h}}/_{\sigma_{i,max}})} \quad (6)$$

The final rank of each cultivar, X_h, was determined by the summation of individually determined trait values calculated through trait performance relative to the tested population, $\left(\frac{y_{i,h}}{y_{i,max}} - 1\right)^2$, the trait's economic importance, I_i, and the cultivar's relative uniformity of trait values compared to the other tested lines, ${}^{(\sigma_{i,h}}/_{\sigma_{i,max}})$. Economic weights ($I_i$) were determined on an increasing 0-1 continuous scale paralleling consumer and producer concern for trait performance. Weights were determined to be '0.90', '0.90', '0.90', '0.85', '0.80', and '0.55' respectively for protein-bound lysine content (grams of

protein-bound lysine/100 grams total weight) and traits ‘Yield’, ‘Expansion Volume’, ‘OCFSI’, ‘Popability’, and ‘Vitreousness’. Plant height, number of ears per plant, ear length, and flake morphology were considered concurrently to the ranking system results for ultimate selection of best QPP hybrids.

3. Results

3.1 Breeding and Selection of BC_3F_4 QPP Inbred and Hybrid Cultivars

Vitreous BC_3F_4 QPP inbred lines were obtained by generational phenotypic and genotypic selection of vitreousness and the *opaque-2* allele, respectively **(Figures 1-2)**. Homozygous *o2o2* BC_3F_2 seedlings were selected in the spring of 2019 and self-pollinated until the BC_3F_4 generation. *o2* induced zein downregulation and non-zein upregulation in BC_3F_4 inbred seed was verified through protein extraction and SDS-PAGE, and homozygous allelic introgression of *opaque-2* was verified through marker-assisted selection (not shown). Since BC_3F_4 inbred lines were achieved through the F_1 cross of BC_2F_5 QPP with original popcorn parents, improved vitreousness of BC_3 cultivars compared to their BC_2 counterpart was not attainable **(Figure 2)**. Notably, QPP Inbred 3 differed in endosperm color between BC_2F_5 and BC_3F_4 lines, and QPP BC_3F_4 Inbred 3 gained cap opacity. All other QPP inbred lines maintained the same observable level of vitreousness between backcrossing generations **(Figure 2)**. Equation 3 estimates from test weight and moisture content revealed a decreased seed size in BC_3F_4 derived QPP hybrids compared to BC_2F_5 hybrids, and original popcorn parental hybrids had significantly smaller seeds than both QPP populations.

3.2 Phenotypic and quantitative assessment of *opaque-2* initiated proteomic rebalancing in Quality Protein Popcorn Hybrids

A random assortment of F_2 kernels from original popcorn parental crosses, QPP BC_2F_5 crosses, and QPP BC_3F_4 crosses was obtained for zein and non-zein protein extraction and free and protein-bound amino acid profiling **(Figure 3)**. The first two components in Principle Component Analysis of protein-bound amino acid profiles accounted for 95.47% of variation and clearly separated ConAgra hybrids from QPP hybrids **(Figure 3A)**. A general increase in lysine, arginine, and aspartate/asparagine in QPP hybrids markedly differentiated their cluster from leucine, glycine, and glutamate/glutamine-rich ConAgra hybrids. According to **Table 1** nomenclature, the BC_2 derived 'H2' displayed a unique protein-bound amino acid profile compared to the rest of the QPP hybrids, as shown by segregating with two CAG hybrids, H11 and H15 (outside both red and blue clusters) **(Figure 3A).** Alike to these profiling results, H2 held the least amount of protein-bound lysine compared to all QPP hybrids **(Figure 3B)**. Taken as an average, ConAgra hybrids held 0.189±0.02 g/100g of protein-bound lysine while QPP hybrids presented a 1.7 fold relative increase in protein-bound lysine and averaged 0.320±0.04 g/100g **(Tables 2-4)**. In concordance with these results, SDS-PAGE of extracted zein proteins from three ConAgra hybrids, three BC_2F_5 and three BC_3F_4 derived QPP hybrids exhibited expected profiles **(Figure 3C)**. ConAgra lines displayed the wild-type zein profile of abundant 22-kD α-zein, relatively downregulated 27-kD γ-zein, and variable 19-kD α-zein **(Figure 3C)**. All six QPP hybrids demonstrated the *opaque-2* triggered 22-kD α-zein negligibility, 19-kD α-zein variability, and significant 27-kD γ-zein upregulation characteristic of improved vitreousness **(Figure 3C)**. Interestingly, H1 and H4 displayed a semi-quantitative increase in 19-kD α-zein abundance that was visibly lost in the BC_3 counterparts H6 and H9, respectively.

3.3 Distinction in agronomic trait performance between QPP BC_2F_5, BC_3F_4, and popcorn parental hybrids

Yield Equation 2 offered a yield estimate in dry (15.5% moisture) bushels/acre, a common unit to evaluate maize yields. ConAgra Commercial Line 2 (H15) was grown as a high-yielding target with average popping traits while ConAgra Commercial Line 1 (H14) was grown and evaluated for its premier popping characteristics and average yield **(Table 1)**. H15 exhibited the maximum yield average at 89.53 bu/ac while H14 yielded 68.07 bu/ac **(Table 5)**. On average, BC_2 derived QPP hybrids yielded insignificantly different to ConAgra lines with ~62 bu/ac and ~67 bu/ac yields, respectively **(Table 5)**. BC_3 derived hybrids yielded an average of 53 bu/ac, significantly lower than the other two groups. Specifically comparing H15 to all QPP and ConAgra hybrids, only QPP H5 had an insignificantly different yield measure. Conversely, all QPP hybrids except H6, H7, and H8 conferred comparable yields to H14.

All QPP hybrids were insignificantly different in yield compared to their respective popcorn parental pedigrees except H2 and H7, two hybrids stemming out of the same, H13-equivalent, popcorn pedigree **(Table 1 and Table 5)**.

Plant height, ear length, and number of ears per plant were measured prior to combine harvesting but low, nonsignificant correlations were found between all hand measured traits and yield estimates except plant height and Yield Equation 2. The Pearson's correlation coefficient for this comparison was 0.215 (significant at $\alpha < 0.05$). Due to all other low and nonsignificant correlations, hand measured traits were not considered in the overall ranking of hybrids using the 2020 Ranking System.

3.4 Popping quality trait evaluation between ConAgra elite hybrids and differing QPP backcross-generated hybrids

Expansion volume, OCFSI, and popability measurements displayed ConAgra varietal advantage compared to all QPP hybrids **(Table 5)**. Percentage of grain moisture was ascertained prior to popping of each sample and had no significant effect on EV. Though the location effect was significant ($\alpha < 0.05$), no interactions were visually identified when analyzing the data through backcrossed groups. The Mead location experienced higher percentages of grain damage/mold, and a percentage of mold was noted per each experimental unit. H2 experienced 50% mold damage per sample, while all other QPP and ConAgra hybrids had insignificantly different levels of damage. After popping, ConAgra hybrids averaged an EV of 35.38±5.29 cubic centimeters/gram, BC_2- derived QPP hybrids averaged 22.8±4.6 cubic centimeters/gram, and BC_3- derived QPP hybrids averaged 25.28±4.63 cubic centimeters/gram, demonstrating significant differences between all groups and a significant improvement in EV after the third QPP backcross **(Table 5)**. Comparing QPP hybrids with commercial lines H14 and H15, H9 held the only insignificantly different EV measure compared to H15.

Concerning popability, H9 held insignificant differences compared to H11 (its corresponding ConAgra hybrid in pedigree), and H15. H6 and H8 also displayed insignificantly different popping values compared to their ConAgra-related hybrids (H12 and H11, respectively) and H15. Categorizing hybrids into backcross groups and ConAgra® controls rendered significant differences between all three groups **(Table 5)**. QPP BC_2-derived hybrids held the lowest popability percentage at 96%, while BC_3- hybrid

and ConAgra® hybrids were narrowly higher with averages of 97.1% and 98.4%, respectively **(Table 5)**.

OCFSI values displayed insignificant differences between backcrossing generations but ConAgra hybrids did hold a significantly higher flake size index compared to QPP hybrids **(Table 5)**. All OCFSI averages ranged from 2.56-5.52, with H2 holding the lowest value and H14 holding the highest **(Table 5)**.

An overview of these popping trait values identified trends between QPP hybrids, backcrossing groups, and ConAgra-respective hybrids. H2 and H5 held the lowest EV and OCFSI values out of all QPP hybrids, followed by their BC_3- counterparts H7 and H10. All four of these hybrids consistently held the lowest averages for all three popping traits compared to all other tested hybrids, though the BC_3 hybrids did have significantly higher popability values compared to the respective BC_2 varieties. These four QPP hybrids also were derived from the same PP1 x PP3 (H13) ConAgra pedigree, which did not hold correspondingly lower popping quality trait values compared to the other ConAgra varieties **(Table 5)**.

QPP hybrids that noticeably performed higher than average on popping quality traits were H1, H6, and H9 **(Table 5)**. These three hybrids held the highest EV measurements, H6 and H9 held the highest popability percentages, and the trio held the highest OCFSI measurements accompanied by H4 **(Table 5)**. H12, the corresponding ConAgra hybrid to H1 and H6, held the lowest OCFSI, lowest popability, and second lowest EV measurements compared to other ConAgra hybrids.

3.5 Flake morphology assessment of tested hybrids

Immediately after popping, flakes were assessed and each experimental unit was categorized into butterfly, mushroom, or mixed morphologies (blue, red, and white, respectively; **Figure 4**). QPP hybrids derived from corresponding backgrounds but different backcrosses showed mostly similar flake morphology patterns **(Figure 4)**. All ConAgra derived hybrids (H11-H13) and H14 were attributed unwavering 'butterfly' morphology. H1 and H6 overarchingly displayed a 'mixed' morphology with a single 'butterfly' distinction. H2 and H7 both had a majority of butterfly flake morphology assignments. H3, H7, and H9 held the most uniform butterfly morphology followed by H8. H4, H5, and H10 were assigned varying flake morphologies. H4 and H10 had a majority of mixed flakes while H5 held a majority 'butterfly'. All three of these QPP hybrids had at least one distinct 'mushroom' assignment. H15 was the only ConAgra line that had an assignment other than 'butterfly' in that three experimental units were categorized as 'mixed' morphology **(Figure 4)**.

3.6 Free and protein-bound lysine in QPP compared to parental popcorn hybrids in raw and air-popped forms

As previously stated, QPP hybrids held a 1.7 fold increase in protein-bound lysine compared to ConAgra hybrids in the raw flour form **(Tables 2-4)**. After popping, protein-bound lysine levels in popped flakes were 1.84 fold higher in QPP hybrids compared to ConAgra hybrids with 0.24±0.04 g/100g and 0.13±0.01 g/100g values, respectively **(Tables 2-4).** Lysine values between BC_2 and BC_3 backcrossed QPP populations were insignificantly different for both protein-bound and free lysine in both ground and air-popped forms ($\alpha < 0.05$). Air popping decreased protein-bound lysine levels by ~30.3% in all hybrids with a significant Pearson's correlation coefficient of

0.872 ($\alpha < 0.05$). However, H4 presented insignificant changes in protein-bound lysine content before and after popping likely due to sample preparation error. Excluding H4 data from the correlation test rendered a significant Pearson's correlation coefficient of 0.948 ($\alpha < 0.05$) between raw flour protein-bound lysine and air-popped protein bound lysine levels. Moreover, despite the 30% decrease in lysine, air-popped QPP hybrids still held higher protein-bound lysine levels than ConAgra lines in the raw flour form **(Tables 2-4)**.

An insignificant reduction differential after popping between ConAgra and QPP hybrids in both protein-bound and free lysine was found. Free lysine levels decreased after popping by roughly 20% in all cultivars though values held an comparatively inconsistent downward trend correlating with a significant 0.746 Pearson's coefficient **(Tables 6-8)**. Free lysine levels were minimal compared to protein-bound levels, rendering an average of 0.0014±0.0003 g/100g lysine in ConAgra hybrids and 0.0071±0.003 g/100g in QPP hybrids in the raw flour form **(Figure 5)**. These averages indicate QPP hybrids held a 4.95-fold relative increase in free lysine levels in raw flour and a 5.44-fold relative increase in free lysine retained after popping, with averages of 0.00519 and 0.00095 g/100g in QPP and ConAgra hybrids, respectively. Though these large fold-increases in free lysine were significant, free lysine in the air-popped samples only accounted for ~2% and ~0.7% of the total lysine in QPP and ConAgra hybrid popped flakes, respectively **(Tables 6-8)**.

Specifically comparing lysine levels between ConAgra commercial lines H14 and H15 and QPP hybrids, QPP H1, H4, H5, H6, H9, and H10 all held significantly higher protein-bound lysine levels in the raw form than H14, and H1, H5, and H6 held

significantly higher levels than H15 **(Figure 3B, Tables 2-4)**. In the popped form, all QPP hybrids except H2 held significantly higher protein-bound lysine levels than both H14 and H15, indicating a significantly higher lysine intake in the consumable form. Overall, QPP hybrids held higher levels of lysine in the ground kernels and popped flakes compared to ConAgra's currently commercialized popcorn cultivars.

3.7 2020 Ranking System: Evaluation and ranking of hybrids

Economic weights '0.90', '0.90', '0.90', '0.85', '0.80', and '0.55' respectively for protein-bound lysine content (g/100g), Yield (Eq.1), EV, OCFSI, Popability, and Vitreousness were utilized in the 2020 Ranking System **(Table 9; Figure 6)**. Consumer and producer interests were considered equally important (i.e. expansion volume and yield) along with protein-bound lysine content due to its pervasive goal in the QPP breeding program. OCFSI was considered less important to EV since it is an individual measure of kernel potential rather than a sample average, and popability was given a slightly lesser economic weight due to its more subjectively determined value of popping average. Finally, vitreousness was included in the model but given the least weight because of its indirect but significant positive correlations to popping traits. After computation, H13 held the best, lowest ranking due to its above average measurements in all traits except for protein-bound lysine content. H15 ranked second due to its relatively lower EV compared to other ConAgra hybrids. H11 ranked third in part to its poorer yield, and H12 ranked very low due to below average yield and popping traits. QPP BC_3 derived hybrid H10 ranked fourth overall despite its poor popping quality traits, followed by H14, H3, H4, H1, H6, and H9. H5 was ranked second lowest due to very poor popping traits, and H2 was ranked last due to low yields, popping traits, and relatively

lower lysine abundance **(Figure 6)**. Overall, most ConAgra® hybrids ranked higher than most QPP cultivars; however, H10, H3, H4, and H1 held close ranking values compared to commercial hybrids H15 and H14 **(Figure 6).**

4. Discussion

4.1 QPP backcross breeding and selection

The production of BC_2F_5 QPP inbred lines with highly vitreous endosperm, high lysine content, and restored popping characteristics offered scope for successful popcorn hybrid production utilizing dent maize germplasm. However, due to the temporary loss of popping capability in the early breeding stages of QPP and restoration in the final stage, popping traits such as expansion volume, popability, and OCFSI were not selected for during inbred production and final determination of elite QPP inbreds (Ren et al., 2018). Moreover, a preliminary popping test of selected inbreds identified overall reduced expansion volume with variability. After initial hybridization of inbred lines and selection of 44 BC_2F_5-derived QPP hybrids, popping traits were analyzed and found to be significantly, moderately lower than original popcorn parental lines (Parsons et al., 2020). Previous studies have postulated that popping expansion, the premier quality trait of popcorn, is predominantly a highly heritable additive trait regulated by three to five major genes (Dofing et al., 1991; Pereira and Amaral Junior, 2001; Ziegler, 2001; Li et al., 2003; Coan et al., 2019). A recent crossing study aimed at studying the mode of expansion volume inheritance found that one backcross to the original popcorn parental line recovered 75% of the popping expansion of the original parent in a flint (Zea mays var. indurata) by popcorn cross, and the BC_2- cross was not produced or tested (Coan et al., 2019). Indeed, previous inheritance-centered studies agree that a single popcorn parental backcross

following a dent by popcorn cross is sufficient for recovering a majority of popping capacity fit for genetic studies, but not enough to achieve synonymous popping trait measurements to the original popcorn parent (Li et al., 2003; 2007; 2008). Dating back to 1949, Crumbacker et al. postulated that two backcross generations to the original popcorn parent were sufficient for recovering popping expansion volume after a dent by popcorn cross, and limited but recent studies have validated this approach (Crumbacker et al., 1949; Li et al., 2004; Niu et al., 2008).

Though the theoretical genomic recovery of the recurrent parent in a BC_2 cross is 0.875, and 0.9375 for a BC_3 backcross, these proportions do not consider genomic or phenotypic selection measures employed throughout a breeding program (Collard et al., 2005; Uptmoor et al., 2006; Silva et al., 2007; Gupta et al., 2010; Ramos et al., 2011). One study converting two non-QPM dent lines into QPM found that the selected BC_2 lines recovered an average of 0.901-0.972 of the recurrent parental genome, and the BC_3 generation recovered 0.971-0.996 utilizing foreground selection (Thakur et al., 2014). This breeding program utilized two dent maize parents rather than a popcorn recurrent and dent maize donor parent, and solely the QPM *opaque-2* allele and required modifiers were selected. Considering the current study's aim to select for QPM-based amino acid and endosperm modifier genes and popcorn-based phenotypic traits such as seed size, kernel morphology, and popping traits – all of which have uncertain genetic locations – the genetic contribution of both parents in the QPP BC_2F_5 inbred lines could not be predicted as theoretically distributed nor necessarily favoring the recurrent parent to such an extent as found by the previous QPM-conversion study. Moreover, without knowledge of the location for necessary loci from both the recurrent and donor parents, sequencing the few QPP lines

available would have provided genetic contribution proportions but would do little to aid in identifying premier inbreds or popping or QPM trait Quantitative Trait Loci due to the limited number of lines available. Therefore, as previous studies have attributed popping trait improvement of dent by popcorn crosses to backcross-based breeding methods, and theoretical genetic contribution of the recurrent parent could be increased by 6.25% by an additional backcross, BC_2F_5 QPP inbred lines were crossed to the original popcorn parents and self-pollinated and selected to the F_4 generation. Given the availability for heterozygosity in the initial BC_3 cross was only ~6.25%, three generations of self-pollinating and selection rendered the availability for heterozygosity in the BC_3F_4 lines at 0.39%, or the equivalent to an F_8 generation without backcrossing (Semagn et al., 2006; Gupta et al., 2010).

Though the theoretical additional genetic contribution by the recurrent popcorn parental parent was 0.0625 between the BC_2 and BC_3 generations, empirical studies sequencing backcross population of various plants do indicate high variability between backcross populations and rather unpredictable genetic proportions (Uptmoor et al., 2006; Ramos et al., 2011; Thakur et al, 2014). Thus, without sequencing BC_2F_5 and BC_3F_4 inbred lines, the extent and location of selected QPM and popcorn loci, and the final genetic contributions of both parents, remains unknown. Future dent by popcorn breeding may benefit more profitably by backcrossing after genetic locations of popcorn traits and QPM endosperm-restorer and amino acid modifier genes have been identified. Previous and current work have suggested genetic whereabouts for both popping traits and *opaque-2* related genes, but the elucidation of exact locations coupled to available genetic markers remains unavailable (Holding et al., 2008; Li et al., 2009; Gutiérrez-Rojas et al., 2010; Wu

et al., 2010; Holding et al., 2011; Babu et al., 2015; Pandey et al., 2015; Senhorinho et al., 2019; Coan et al., 2019). The potential for verified markers in both suites of genes coupled to the declining cost of genomic sequencing offers scope for future dent by popcorn breeding systems that aim to improve agronomics within popcorn cultivars while maintaining synonymous popping characteristics.

4.2 Simultaneous comparisons between backcrossed generations and ConAgra elite lines

Rapid breeding of the BC_3F_4 QPP inbred lines enabled simultaneous comparison between the BC_2-and BC_3- derived hybrids and between all QPP lines and ConAgra elite cultivars. The kernel mold damage experienced at the Mead, NE location gave opportunity to test pest susceptibilities between BC_2-, BC_3-, and non-QPM popcorn lines. Initial introgression of *opaque-2* without necessary endosperm modifiers into various dent maize lines resulted in inferior agronomics and higher pest/rot susceptibility (Prasanna et al., 2001). Other than H2, a QPP hybrid inferior in all other evaluated traits, all QPP lines experienced insignificantly different mold susceptibility compared to ConAgra varieties. These results suggest the successful introgression of original dent allele *opaque-2* and essential endosperm modifiers into a popcorn background. Comparing QPP backcross populations, results indicated that an additional popcorn backcross improved QPP popping characteristics compared to BC_2- derived hybrids; however, average QPP popping traits were still significantly lower than ConAgra lines. Average BC_2 hybrid expansion volume measurements were roughly 64% of ConAgra volumes, while BC_3 hybrids held 71% of premier volume values. This significantly large improvement in EV suggests potential for improving popping traits by additional backcrossing. OCFSI values held similar ratios

between QPP and ConAgra lines, while popability measurements were similar between all hybrids. The discrepancy between previously published backcross-restored popping traits and QPP inbreds is likely due to the selection measures imposed during inbreeding (Coan et al., 2019). Without known locations and extent of required QPM dent maize loci introgression, and with known repulsion phase linkages between yield and expansion volume, and with inherent selection of agronomic characteristics throughout QPP inbred line production, unintentional selection against expansion volume could have been employed (Sprague and Dudley, 1988; Dofing et al., 1991; Ziegler and Ashman, 1994; Ren et al., 2018). Given these selection measures, it is probable that QPP inbred lines have higher than theoretical QPM genetic material after backcrossing and selection and suggests future scope in generally improving popping traits by further backcrossing.

Despite unattaining synonymous popping characteristics after an additional backcross to the original parents, BC_3- derived lines displayed significant improvements in these traits compared to BC_2- derived lines. However, the trade-off between popping and agronomic characteristics was apparent as BC_2- derived lines had significantly better yield averages. Therefore, utilization of the 2020 Ranking System proved helpful in holistically discriminating between BC_2- and BC_3- derived hybrids and comparing them individually to original popcorn lines (Parsons et al., 2020). Protein-bound endosperm lysine content, yield, and expansion volume were considered equally important in the final selection of QPP hybrids and were each given an economic weight of 0.90. Final ranking identified top QPP hybrids as H10, H3, H4, H1, H6, and H9, in respective order. Though the highest ranked hybrid was a BC_3- derived cross, BC_2- crosses H3, H4, and H1 were superior to BC_3- crosses H6 and H9. These results suggest that the third-backcrossed population did

not produce satisfactory popping results to warrant the time, assets, and effort allotted to producing it. However, the significant improvements in BC_3- derived hybrids H7 and H10 compared to their BC_2 counterparts H2 and H5, respectively, show specific potential in this breeding scheme if genetic selection could be conducted more specifically. Overall, the six most elite hybrids stemmed equally from the BC_2 population and the BC_3 population which rendered a diverse set of potentially marketable QPP varieties fit for consideration.

4.3 Flake morphology of selected QPP hybrids

All QPP hybrids exhibited varying mixtures of butterfly and mushroom flake morphologies. H3, H7, and H9 held the closest resemblance to their ConAgra respective hybrids, followed by H2 and H8. H1 and H6, hybrids from the same QPP cross but of differing backcrossed generations, exhibited the same morphological behavior in mostly a mixture of flakes. H4 and H9 differed most dramatically between backcrosses in this trait. H9 held a majority of butterfly flakes while H4 had a majority mixture, followed by some samples popping purely butterfly and one sample popping solely mushroom. This morphological profile was similarly mirrored by H10, though H10 had one more sample labeled ‘mushroom’ rather than a mixture. H5 interestingly only had one mixed sample; the rest popped either solely butterfly or solely mushroom. The location effect on these particular hybrid’s popping morphology was strikingly significant. Out of the nine samples analyzed, the three H5 samples taken from Lincoln, NE were considered ‘butterfly’, followed by the secondary location rendering two butterfly samples and one mushroom sample, and finally the Colby, KS location had two mushroom samples and one mixed sample. Similar to H5, H10 held three ‘mixed’ samples at Lincoln, NE, followed by two butterfly samples and one mixed sample at Mead, NE, and two mushroom and one mixed

sample taken from Colby, KS. Previous studies analyzing the environmental effect on popcorn flake morphology are limited, but one study in 2012 identified growing location as a significant factor in popcorn flake morphology though the extent of locational influence on morphology in comparison to other intrinsic and external factors remained elusive (Sweley et al., 2012). The narrow number of hybrids and samples tested per location limited these results' identification of particular flake morphological responses to certain environmental influences; however, like the 2012 study, the locational effect on flake morphology was found to be significant and warrants consideration when typifying future popcorn varietal flake morphologies.

4.4 QPP cultivars exhibit elevated lysine levels compared to ConAgra elite lines in raw flour and air-popped forms

Previous studies have shown that tryptophan and lysine levels within the same maize variety positively correlate in relative abundance in the zein fraction and thus in the entire endosperm (Hernandez and Bates, 1969; Krivanek et al., 2007; Olakojo et al., 2007; Ren et al., 2018; Parsons et al., 2020). Due to acidic hydrolysis' destruction of protein-bound tryptophan, lysine levels were recovered and used as a benchmark for *opaque-2* derived lysine and tryptophan increases compared to ConAgra varieties (Angelovici et al., 2013; Yobi and Angelovici, 2018). Protein-bound lysine levels in raw flour displayed a significant difference between ConAgra varieties and QPP cultivars, and no significant difference was found between BC_2 and BC_3 derived QPP cultivars. On average, QPP varieties held 0.320±0.039 and ConAgra cultivars held 0.189±0.019 grams protein-bound lysine/100 gram total weight in the raw flour, respectively. After popping, lysine levels decreased by ~30% to 0.235±0.042 grams of protein-bound lysine/100 grams total weight

and 0.128±0.006 g/100g in QPP and ConAgra cultivars, respectively. Even after air-popping, QPP cultivars held more lysine than non-QPM popcorn raw kernel flour.

Previous analysis of QPP and non-QPM popcorn lysine content revealed a slightly higher protein-bound lysine level than the current study indicates (Parsons et al., 2020). However, considering the ratio between non-QPM and QPM popcorn lysine levels is consistent between analyses, these results compositely suggest a stable and reliable increase in lysine content in air-popped QPP varieties compared to currently marketed popcorn. Contextually, a 68 kg (150 pound) individual is generally recommended to ingest 2.108 grams of lysine per day (Elango et al., 2009). These results suggest that the equivalent of one microwavable bag of QPP air-popped popcorn (~47 grams) would fulfill 5.2% of this daily lysine requirement as opposed to a 2.8% fulfillment available through currently commercialized popcorn varieties.

4.5 Conclusion: Final Selection of QPP hybrids

The holistic evaluation of Quality Protein Popcorn hybrids with ConAgra controls allowed for the simultaneous comparison of BC_2F_5 and BC_3F_4 genetic backgrounds with ConAgra elite lines to further select QPP best fit for potential commercialization. This evaluation found the BC_3 hybrids had significantly lower yields compared to both ConAgra and BC_2 groups, but the BC_3 cultivars had significantly improved popping traits compared to the BC_2 hybrids. In all popping traits evaluated, specifically expansion volume, OCFSI, and popability, all three groups had significantly different averages with ConAgra elite lines leading, followed by BC_3F_4 derived QPP hybrids, and lastly BC_2F_5 derived QPP hybrids. As only two BC_3- derived lines performed better than their BC_2- derived counterparts in the final ranking utilizing the 2020 Ranking System, it is uncertain whether the time and

resources spent introducing another backcross to this germplasm are justifiable. Thus, this study may evoke caution in further backcrossing for other dent by popcorn breeding programs aimed at improving agronomic and popping quality traits. However, the significant improvement in H7 and H10 compared to H2 and H5 demonstrates success, albeit rather indiscriminate, for this breeding plan. The significant increase in protein-bound lysine in all QPP hybrids except H2 compared to ConAgra elite lines in popped flakes validates the successful introgression of the QPM *opaque-2* allele and necessary endosperm modifier genes for restored popping. Additionally, the PCA of the protein-bound amino acid protein profile clusters all QPP separately from ConAgra lines except H2. H2 performed the worst out of all hybrids in multiple different analyses, holding the lowest protein-bound lysine content, expansion volume, OCFSI, second lowest popability, third lowest vitreousness, and eighth highest measurement in yield. Conversely, QPP hybrids H10, H3, H4, H1, H6, and H9 all held high lysine values and returned overall higher ranking values compared to the four other QPP hybrids. These selected hybrids' sufficient agronomic and popping quality trait evaluations and significantly higher lysine content compared to currently marketed varieties offer evidence for their consideration for commercialization.

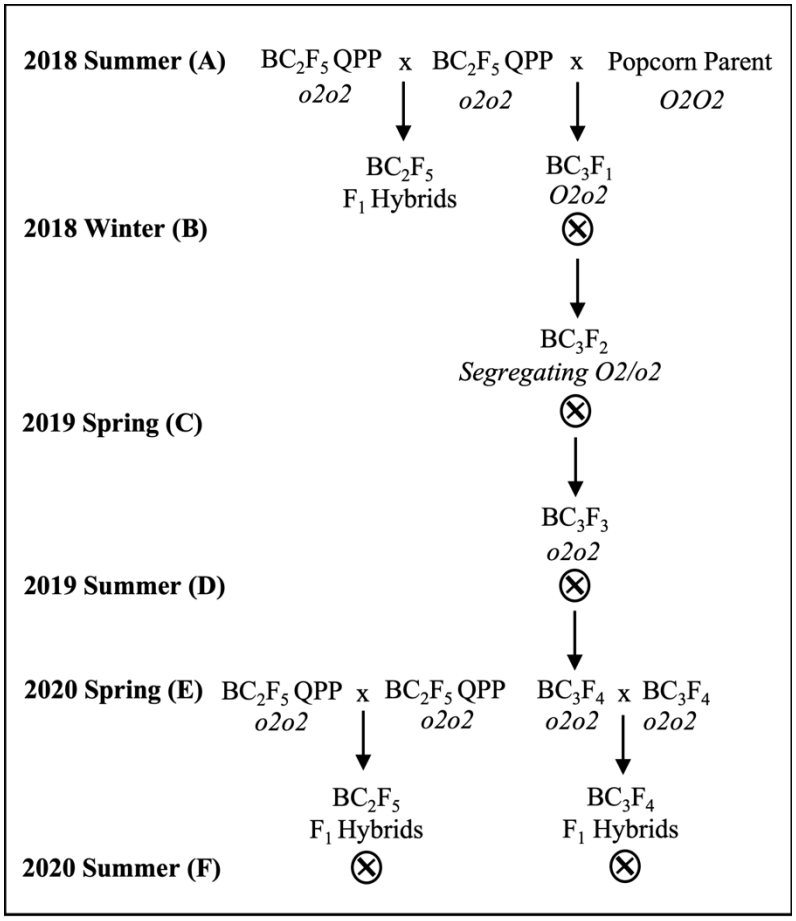


Figure 1 | Breeding Scheme to produce BC_2F_5 and BC_3F_4 QPP F_1 Hybrids. Overall breeding scheme from 2018-2020. **(A)** In the summer of 2018, BC_2F_5 QPP inbreds were crossed in full diallel to produce F_1 hybrids. BC_2F_5 inbreds were also selectively crossed to their respective original popcorn parents to produce heterozygous O_2o_2 BC_3F_1 offspring. **(B)** Heterozygotes were self-pollinated to produce segregating BC_3F_2 offspring which was selected at the seed based on *opaque-2* phenotyping of vitreousness, protein-profiling, and later marker-assisted selection. **(C)** Homozygous F_2 seed was grown and self-pollinated prior to 2019 summer. **(D)** Homozygous mutant o_2o_2 BC_3F_3 seed was harvested and grown to produce BC_3F_4 QPP seed in the summer of 2019. All inbred lines were identified as *o2*-carrying predominantly through protein-profiling. **(E)** BC_2F_5 and BC_3F_4 QPP inbred lines were grown in the spring of 2020 and selectively crossed to produce similar QPP hybrids of differing backcross generations. **(F)** BC_2F_5 and BC_3F_4 derived F_1 hybrids were grown in three locations and evaluated alongside ConAgra elite varieties for selection.

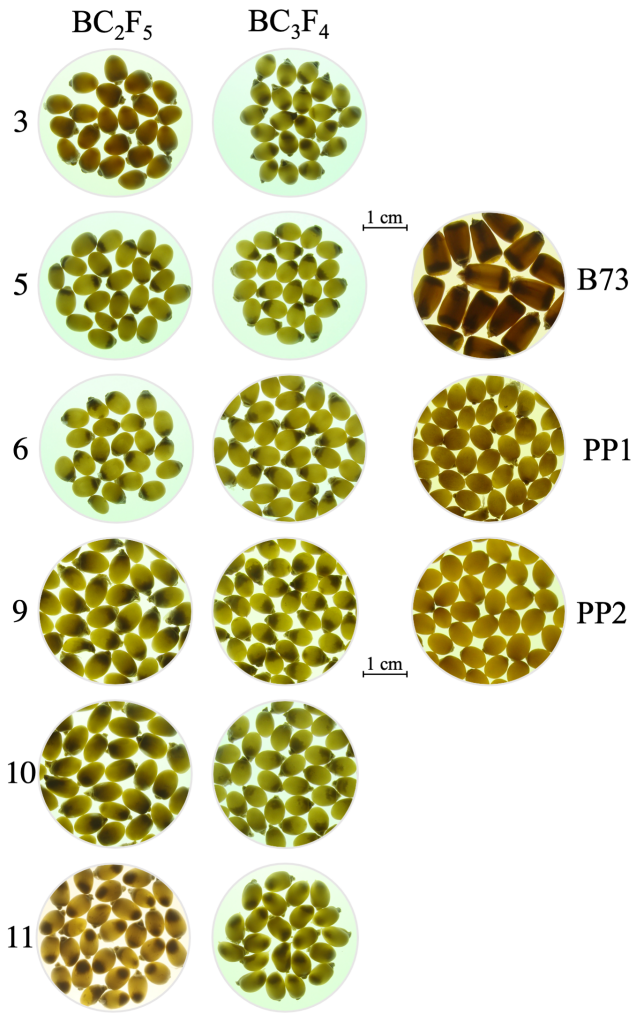


Figure 2 | Scaled comparison of BC_2F_5 and BC_3F_4 QPP hybrids and ConAgra Popcorn Parent 1, Popcorn Parent 2, and B73. Overall, BC_3- derived inbreds displayed smaller kernels which produced significantly smaller F_1 hybrid kernels compared to BC_2- derived hybrids, while popcorn parents produced the smallest seed size in non-QPM popcorn hybrids.

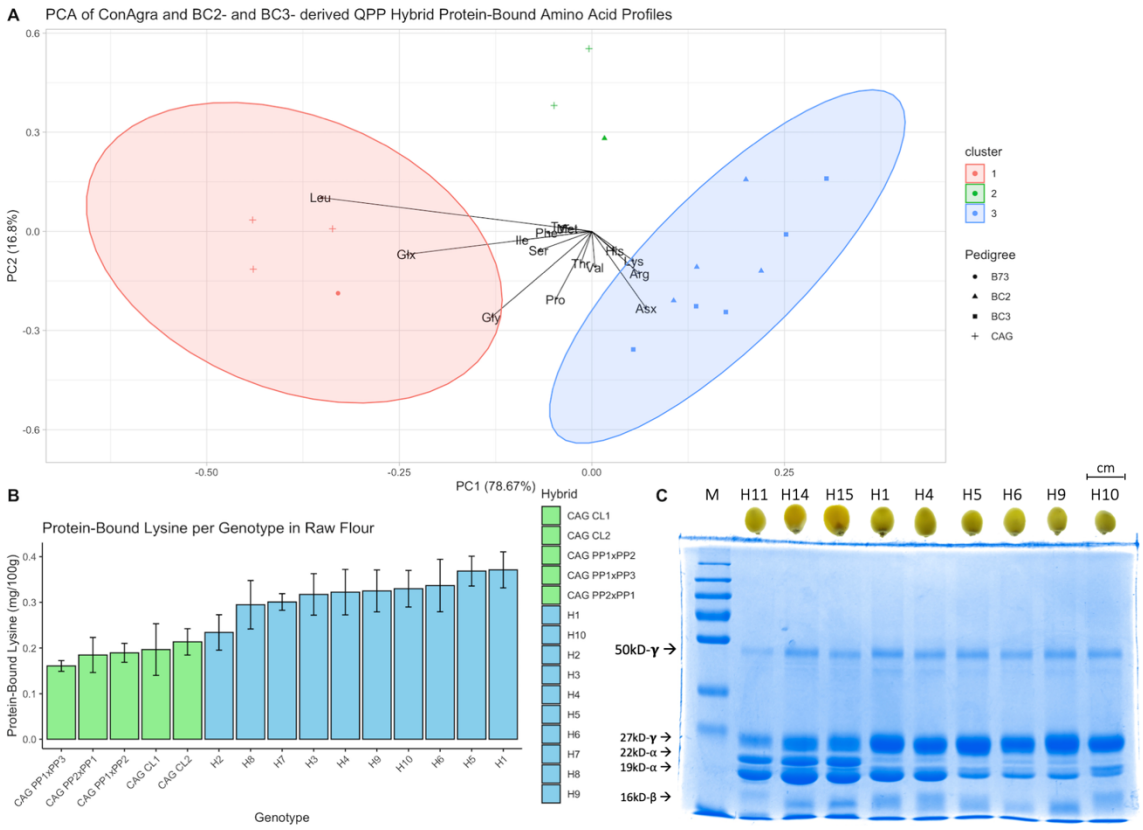


Figure 3 | Protein profiling of QPP and ConAgra elite lines. **(A)** Principle Component Analysis (PCA) of protein-bound amino acids in raw flour of ConAgra elite lines, B73 for reference, and QPP hybrids revealed a distinct segregation between QPP and original popcorn-derived cultivars. B73 grouped with ConAgra lines H12, H13, and H14, while H11 and H15 independently segregated with QPP H2. QPP H2 had a distinct proteome compared to all other QPP hybrids and held insignificantly different lysine levels compared to ConAgra lines. **(B)** Protein-bound lysine in raw flour of all genotypes revealed significantly higher lysine levels in all QPP lines except H2. **(C)** Zein extraction and SDS-PAGE analysis of randomly selected kernels revealed a significant reduction in 22kD-alpha zein, varying production of 19-kD-alpha zein, and increased expression of the 27-kD γ-zein in QPP lines compared to ConAgra lines, consistent with a homozygous *opaque-2* profile. Compositely, these results verifying the successful introgression and stabilization of the homozygous mutation in the BC_2F_5 and BC_3F_4 QPP populations.

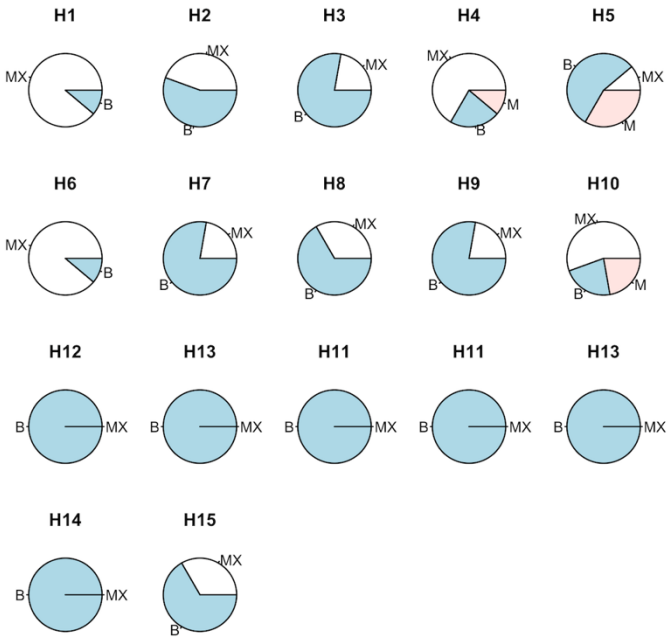


Figure 4 | Flake morphology assessment of QPP and ConAgra elite lines. Random samples of QPP and ConAgra lines from each experimental plot were given a description of butterfly (B - blue), mushroom (M - red), or mixed flake morphology (MX - white). Each cultivar was assigned a total of nine descriptions (three from each of the three locations). All ConAgra varieties were assigned butterfly morphology except H15, which was assigned three MX morphologies. QPP BC_2- derived hybrids are displayed on the first row, respective QPP BC_3-derived hybrids are on the second row, and original popcorn hybrids from the respective pedigrees are arranged on the third row to enable column comparison between similar QPP and ConAgra pedigrees. Commercial lines H14 and H15 are positioned on the fourth row.

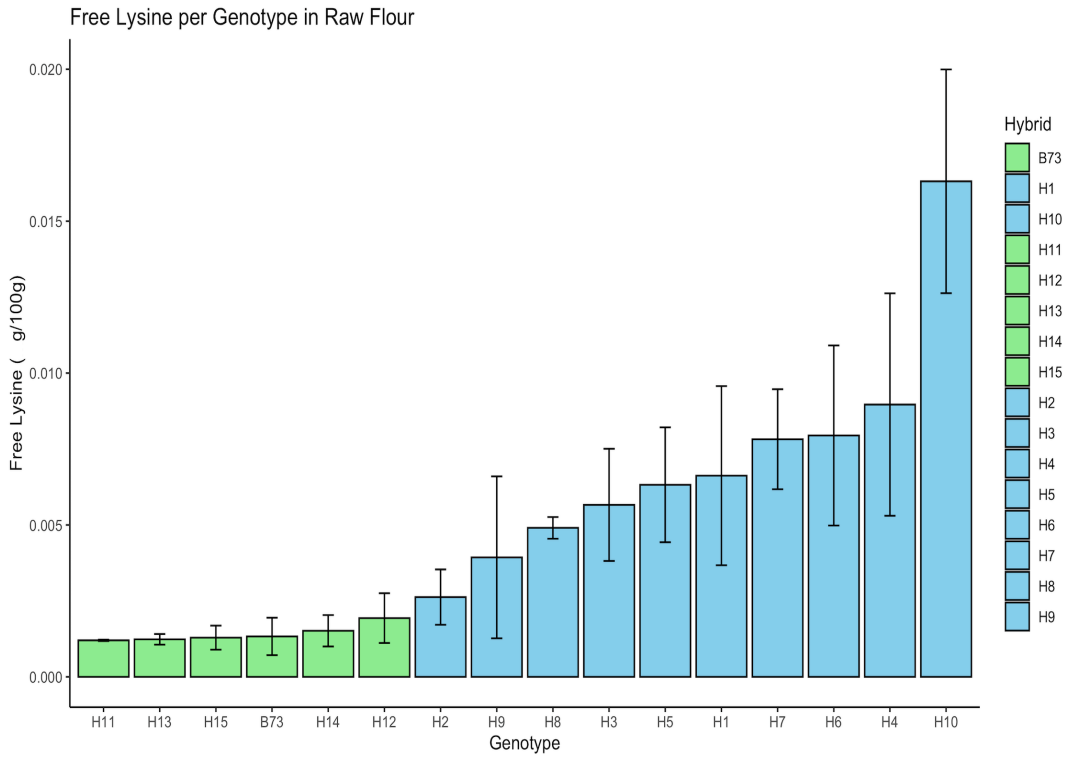


Figure 5 | Free lysine content per genotype in raw flour (g/100g). ConAgra derived lines (green columns) had significantly lesser free lysine in raw flour compared to QPP hybrids. Hybrids differed more in free lysine content compared to protein-bound lysine, but g/100g measurements were significantly lesser in free lysine than protein-bound (<3% compared to protein-bound).

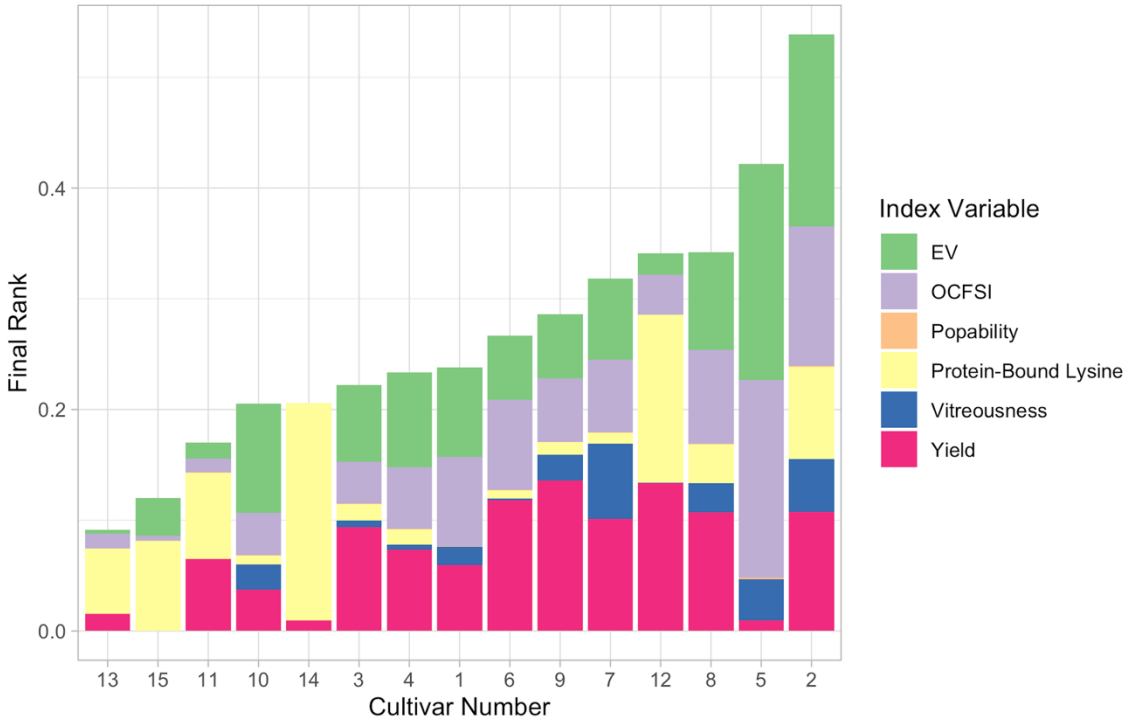


Figure 6 | 2020 Ranking System Selection Index Results. Utilization of the 2020 Ranking System enabled a visual display of overall cultivar ranking from best to worst, left to right, respectively. Color by variable identified individual hybrid pitfalls (the longer the stacked column, the farther from the best hybrid) and high trait values. H13, PP1 x PP3, ranked highest out of all hybrids, scoring relatively lower only because of its lack of protein-bound lysine content. QPP H10 ranked the best compared to all other QPP lines and ranked higher than H14, or Commercial Line 1. QPP BC_2- derived hybrids H3, H4, and H1 ranked respectively higher than the rest, while BC_3- derived hybrids H6, H9, and H7 all ranked higher than original popcorn hybrid line H12. H8, H5, and H2 ranked lowest out of all hybrids.

Cultivar	Previous Nomenclature	Ref. No.	Pedigree
BC_2F_5 F_1 Hybrid	Hybrid 20	H1	(PP2 x (K0326Y x PP2))F_5 x (PP1 x (CML154Q x PP1))F_5
	Hybrid 25	H2	(PP1 x (CML154Q x PP1))F_5 x (PP3 x (CML154Q x PP3))F_5
	Hybrid 28	H3	(PP1 x (CML154Q x PP1))F_5 x (PP2 x (K0326Y x PP2))F_5
	Hybrid 38	H4	(PP1 x (CML154Q x PP1))F_5 x (PP2 x (K0326Y x PP2))F_5
	Hybrid 43	H5	(PP1 x (CML154Q x PP1))F_5 x (PP3 x (Tx807 x PP3))F_5
BC_3F_4 F_1 Hybrid	Hybrid 20	H6	(PP2 x ((PP2 x (K0326Y x PP2))F_5)F_4 x (PP1 x ((PP1 x (CML154Q x PP1))F_5)F_4
	Hybrid 25	H7	(PP1 x ((PP1 x (CML154Q x PP1))F_5)F_4 x (PP3 x ((PP3 x (CML154Q x PP3))F_5)F_4
	Hybrid 28	H8	(PP1 x ((PP1 x (CML154Q x PP1))F_5)F_4 x (PP2 x ((PP2 x (K0326Y x PP2))F_5)F_4
	Hybrid 38	H9	(PP1 x ((PP1 x (CML154Q x PP1))F_5)F_4 x (PP2 x ((PP2 x (K0326Y x PP2))F_5)F_4
	Hybrid 43	H10	(PP1 x ((PP1 x (CML154Q x PP1))F_5)F_4 x (PP3 x ((PP3 x (Tx807 x PP3))F_5)F_4
ConAgra® Brands Popcorn	PP1 x PP2	H11	PP1 x PP2
	PP2 x PP1	H12	PP2 x PP1
	PP1 x PP3	H13	PP1 x PP3
	Cultivar 1	H14	Commercial Line (CL) 1
	Cultivar 2	H15	Commercial Line (CL) 2

Table 1 | Description of cultivars tested in 2020 summer trials. 15 total cultivars were grown and evaluated in the summer of 2020. 'Previous Nomenclature' as described in Parsons et al., 2020 is in reference to QPP pedigree. For simplicity, Reference Numbers H1-H15 were utilized for identification of hybrids in this analysis.

Backcross	Hybrid	Type	Arg	Asx	Glx	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
BC2	H1	*Raw*	0.520	0.807	1.562	1.062	0.367	0.340	0.872	0.329	0.101	0.400	0.914	0.414	0.443	0.286	0.442
			0.567	0.947	1.550	0.880	0.372	0.345	0.810	0.376	0.102	0.396	0.848	0.396	0.449	0.300	0.462
			0.697	1.005	1.876	1.285	0.425	0.435	1.077	0.408	0.126	0.487	1.145	0.553	0.602	0.362	0.555
		Air	0.382	0.751	1.897	1.150	0.394	0.386	1.114	0.211	0.112	0.448	1.084	0.461	0.516	0.324	0.453
			0.378	0.703	1.733	0.959	0.354	0.355	0.987	0.217	0.126	0.403	1.012	0.412	0.473	0.278	0.440
			0.484	0.716	1.688	0.978	0.345	0.338	0.907	0.236	0.113	0.409	0.947	0.408	0.439	0.284	0.443
	H2	*Raw*	0.329	0.647	1.946	1.036	0.261	0.392	1.232	0.215	0.164	0.442	0.845	0.474	0.475	0.352	0.387
			0.434	0.801	1.849	1.170	0.345	0.410	1.098	0.279	0.134	0.438	1.037	0.462	0.530	0.346	0.459
			0.326	0.656	1.661	0.908	0.273	0.404	1.262	0.209	0.172	0.481	0.848	0.474	0.439	0.369	0.380
		Air	0.327	0.695	1.996	1.351	0.327	0.381	1.203	0.175	0.153	0.442	0.970	0.467	0.448	0.330	0.427
			0.305	0.654	1.776	0.988	0.332	0.331	1.028	0.176	0.131	0.398	0.959	0.426	0.470	0.298	0.411
			0.275	0.590	1.661	1.058	0.315	0.347	1.079	0.140	0.118	0.419	0.934	0.373	0.389	0.286	0.389
	H3	*Raw*	0.397	0.725	1.520	0.958	0.345	0.351	1.046	0.266	0.102	0.433	1.042	0.455	0.461	0.330	0.433
			0.566	0.876	1.832	1.078	0.360	0.413	1.111	0.332	0.131	0.452	1.063	0.494	0.559	0.337	0.484
			0.582	0.893	1.722	1.336	0.393	0.355	0.868	0.353	0.129	0.418	0.987	0.448	0.528	0.302	0.479
		Air	0.473	0.756	1.640	1.093	0.369	0.370	1.031	0.243	0.116	0.421	1.134	0.448	0.495	0.301	0.460
			0.458	0.750	1.785	1.360	0.390	0.367	1.072	0.234	0.119	0.434	1.204	0.480	0.544	0.311	0.502
			0.521	0.779	1.931	1.189	0.397	0.407	1.092	0.247	0.122	0.450	1.153	0.515	0.557	0.325	0.523
	H4	*Raw*	0.668	0.869	1.972	1.197	0.379	0.510	1.283	0.357	0.149	0.523	1.093	0.578	0.602	0.372	0.546
			0.588	0.732	1.657	1.075	0.349	0.382	0.919	0.344	0.114	0.431	0.900	0.446	0.451	0.306	0.473
			0.425	0.602	1.157	0.784	0.317	0.298	0.767	0.265	0.093	0.347	0.784	0.370	0.385	0.257	0.385
		Air	0.611	0.855	2.222	1.185	0.386	0.452	1.157	0.314	0.152	0.495	1.152	0.523	0.586	0.348	0.543
			0.605	0.886	2.038	1.071	0.376	0.476	1.195	0.323	0.133	0.504	1.157	0.503	0.545	0.372	0.561
			0.651	0.900	2.277	1.340	0.384	0.518	1.312	0.339	0.149	0.522	1.284	0.570	0.602	0.362	0.579
	H5	*Raw*	0.469	0.736	1.406	0.878	0.301	0.325	0.827	0.331	0.113	0.409	0.856	0.442	0.481	0.283	0.423
			0.614	0.964	2.215	1.185	0.382	0.462	1.138	0.384	0.150	0.499	1.204	0.523	0.566	0.365	0.531
			0.660	1.068	1.838	1.170	0.372	0.469	1.120	0.390	0.175	0.476	1.172	0.516	0.584	0.338	0.562
		Air	0.578	0.872	1.760	1.138	0.359	0.428	1.077	0.300	0.149	0.461	1.006	0.514	0.539	0.315	0.499
			0.458	0.747	1.899	1.158	0.351	0.387	1.057	0.231	0.126	0.435	1.042	0.415	0.476	0.305	0.462
			0.507	0.785	1.706	1.069	0.339	0.382	0.975	0.266	0.132	0.412	0.999	0.431	0.476	0.292	0.468

Table 2. Protein-bound amino acid profiles of Quality Protein Popcorn BC_2F_5-derived hybrids. Three replicates of raw flour and air-popped flakes were submitted for analysis (g/100g).

Backcross	Hybrid	Type	Arg	Asx	Glx	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
BC3	H6	*Raw*	0.539	0.876	2.074	1.081	0.352	0.433	1.131	0.272	0.131	0.452	1.224	0.456	0.572	0.318	0.514
			0.643	0.951	1.958	1.174	0.363	0.429	0.995	0.380	0.140	0.438	1.142	0.530	0.594	0.311	0.535
			0.602	1.042	1.970	1.105	0.363	0.373	0.914	0.359	0.125	0.424	1.033	0.480	0.518	0.293	0.490
		Air	0.468	0.846	2.166	1.126	0.356	0.396	1.006	0.245	0.125	0.425	1.069	0.429	0.513	0.314	0.485
			0.529	0.929	2.007	1.139	0.360	0.434	1.100	0.266	0.132	0.465	1.188	0.464	0.559	0.320	0.523
			0.444	0.838	2.010	1.152	0.340	0.428	1.103	0.238	0.122	0.446	1.167	0.434	0.510	0.314	0.513
	H7	*Raw*	0.443	0.836	1.531	0.931	0.304	0.306	0.813	0.287	0.126	0.368	0.903	0.406	0.461	0.278	0.397
			0.474	0.852	1.740	0.936	0.329	0.364	0.893	0.294	0.138	0.396	0.983	0.406	0.480	0.306	0.452
			0.569	0.882	1.487	1.028	0.324	0.318	0.813	0.321	0.126	0.382	0.895	0.444	0.476	0.265	0.442
		Air	0.538	0.856	1.919	1.154	0.364	0.405	1.027	0.259	0.136	0.444	1.059	0.462	0.510	0.300	0.488
			0.397	0.818	1.801	0.939	0.336	0.343	0.911	0.227	0.133	0.385	1.010	0.385	0.470	0.262	0.449
			0.397	0.714	1.587	0.902	0.321	0.319	0.842	0.204	0.116	0.359	0.971	0.392	0.426	0.235	0.406
	H8	*Raw*	0.536	0.864	1.544	1.022	0.332	0.342	0.841	0.290	0.132	0.396	1.026	0.433	0.447	0.274	0.446
			0.645	0.916	1.788	1.234	0.346	0.393	0.907	0.350	0.146	0.441	1.046	0.503	0.499	0.296	0.503
			0.466	0.821	1.619	0.846	0.329	0.322	0.834	0.245	0.108	0.368	0.930	0.393	0.412	0.263	0.431
		Air	0.510	0.797	1.940	1.048	0.349	0.357	0.899	0.252	0.117	0.371	1.101	0.401	0.498	0.265	0.478
			0.370	0.728	1.769	1.054	0.321	0.326	0.849	0.197	0.116	0.359	1.019	0.381	0.438	0.253	0.437
			0.419	0.615	1.562	0.980	0.339	0.344	0.903	0.200	0.106	0.370	1.075	0.407	0.475	0.247	0.454
	H9	*Raw*	0.577	0.936	1.861	1.173	0.325	0.391	0.955	0.315	0.140	0.410	1.130	0.475	0.545	0.280	0.491
			0.524	0.843	1.570	0.929	0.329	0.351	0.878	0.285	0.133	0.396	0.924	0.445	0.452	0.283	0.450
			0.684	0.907	1.852	1.266	0.328	0.449	1.089	0.375	0.180	0.457	1.141	0.545	0.593	0.328	0.542
		Air	0.497	0.727	1.969	0.912	0.330	0.374	0.932	0.237	0.125	0.407	1.082	0.423	0.486	0.264	0.478
			0.435	0.707	1.697	0.950	0.335	0.357	0.938	0.216	0.119	0.394	1.104	0.423	0.460	0.267	0.452
			0.412	0.605	1.500	0.789	0.324	0.332	0.873	0.193	0.115	0.381	1.000	0.410	0.465	0.259	0.425
	H10	*Raw*	0.512	0.938	1.740	1.193	0.321	0.364	0.885	0.310	0.154	0.405	1.034	0.435	0.486	0.296	0.459
			0.485	0.956	1.554	0.878	0.288	0.358	0.810	0.303	0.130	0.397	0.861	0.407	0.462	0.277	0.428
			0.640	1.064	2.069	1.304	0.326	0.427	1.005	0.376	0.180	0.433	1.135	0.535	0.606	0.323	0.528
		Air	0.492	0.940	2.046	1.006	0.344	0.392	0.966	0.267	0.165	0.430	1.032	0.431	0.488	0.291	0.480
			0.370	0.783	1.800	1.115	0.338	0.324	0.835	0.193	0.120	0.379	0.931	0.392	0.423	0.260	0.423
			0.385	0.768	1.891	1.036	0.310	0.331	0.842	0.208	0.133	0.374	0.969	0.387	0.458	0.257	0.438

Table 3. Protein-bound amino acid profiles of Quality Protein Popcorn BC_3F_4-derived hybrids. Three replicates of raw flour and air-popped flakes were submitted for analysis (g/100g).

Backcross	Hybrid	Type	Arg	Asx	Glx	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
ConAgra Hybrids	H11	*Raw*	0.320	0.591	1.741	0.963	0.216	0.395	1.265	0.171	0.166	0.460	0.815	0.416	0.385	0.343	0.353
			0.327	0.644	2.201	0.999	0.248	0.442	1.383	0.186	0.198	0.483	0.959	0.487	0.453	0.400	0.394
			0.355	0.591	1.756	0.907	0.225	0.359	1.064	0.212	0.160	0.417	0.735	0.395	0.384	0.321	0.352
		Air	0.318	0.615	2.255	0.986	0.243	0.506	1.580	0.114	0.187	0.508	1.020	0.509	0.441	0.368	0.415
			0.321	0.640	2.124	1.173	0.253	0.498	1.551	0.119	0.218	0.493	0.986	0.547	0.497	0.373	0.413
			0.342	0.628	1.999	1.176	0.242	0.455	1.440	0.127	0.172	0.515	0.937	0.510	0.440	0.356	0.386
	H12	*Raw*	0.347	0.736	2.244	1.173	0.259	0.622	1.859	0.165	0.225	0.605	1.178	0.623	0.565	0.449	0.479
			0.419	0.712	1.944	1.277	0.269	0.492	1.439	0.229	0.200	0.536	0.921	0.547	0.493	0.365	0.432
			0.363	0.748	2.411	1.362	0.264	0.604	1.848	0.160	0.245	0.606	1.154	0.645	0.574	0.457	0.456
		Air	0.348	0.705	2.385	1.332	0.270	0.608	1.795	0.146	0.220	0.583	1.193	0.597	0.527	0.439	0.456
			0.308	0.680	2.387	1.210	0.265	0.572	1.810	0.113	0.214	0.592	1.168	0.568	0.486	0.430	0.439
			0.319	0.652	2.592	1.186	0.252	0.563	1.729	0.115	0.211	0.570	1.089	0.550	0.511	0.407	0.435
	H13	*Raw*	0.379	0.732	2.690	1.294	0.303	0.578	1.763	0.163	0.200	0.580	1.251	0.568	0.564	0.455	0.516
			0.284	0.601	2.010	1.003	0.245	0.468	1.411	0.171	0.160	0.497	0.938	0.471	0.467	0.388	0.388
			0.344	0.679	2.417	1.210	0.265	0.616	1.868	0.148	0.207	0.634	1.123	0.561	0.546	0.464	0.456
		Air	0.333	0.707	2.317	1.198	0.258	0.581	1.756	0.140	0.225	0.582	1.083	0.529	0.485	0.428	0.460
			0.356	0.752	2.391	1.198	0.262	0.617	1.791	0.135	0.221	0.591	1.153	0.607	0.517	0.426	0.467
			0.298	0.594	2.051	1.149	0.250	0.488	1.525	0.125	0.195	0.507	0.967	0.497	0.449	0.356	0.401
	H14	*Raw*	0.318	0.653	2.273	1.143	0.266	0.559	1.714	0.154	0.198	0.576	1.083	0.524	0.475	0.421	0.441
			0.409	0.724	2.258	1.230	0.271	0.523	1.619	0.176	0.187	0.541	1.050	0.545	0.475	0.386	0.441
			0.484	0.821	1.954	1.170	0.269	0.537	1.480	0.261	0.211	0.546	1.033	0.579	0.537	0.399	0.467
		Air	0.303	0.618	2.206	0.974	0.244	0.479	1.539	0.107	0.193	0.538	0.982	0.531	0.441	0.356	0.394
			0.361	0.742	2.174	1.172	0.278	0.573	1.732	0.131	0.192	0.582	1.140	0.553	0.480	0.405	0.465
			0.345	0.712	2.305	1.138	0.272	0.541	1.713	0.141	0.211	0.560	1.080	0.594	0.498	0.420	0.457
	H15	*Raw*	0.343	0.670	2.050	1.074	0.256	0.474	1.458	0.187	0.190	0.509	0.997	0.553	0.500	0.395	0.423
			0.328	0.653	1.900	0.990	0.249	0.429	1.315	0.210	0.182	0.476	0.943	0.465	0.475	0.371	0.382
			0.409	0.691	1.620	0.947	0.243	0.410	1.169	0.244	0.156	0.456	0.810	0.444	0.403	0.331	0.378
		Air	0.331	0.534	1.612	0.860	0.241	0.370	1.193	0.126	0.172	0.447	0.841	0.405	0.363	0.302	0.350
			0.269	0.559	1.833	1.043	0.232	0.398	1.269	0.113	0.161	0.450	0.835	0.454	0.386	0.308	0.355
			0.372	0.667	1.899	1.087	0.252	0.440	1.280	0.163	0.189	0.470	0.889	0.491	0.442	0.331	0.404
B73 Reference			0.535	0.779	2.204	1.262	0.276	0.544	1.498	0.268	0.209	0.543	1.029	0.606	0.526	0.390	0.492
			0.575	0.838	2.266	1.193	0.290	0.628	1.731	0.257	0.213	0.599	1.171	0.595	0.530	0.418	0.515
			0.203	0.813	2.107	1.228	0.290	0.521	1.425	0.316	0.192	0.540	0.995	0.577	0.508	0.402	0.492

Table 4. Protein-bound amino acid profiles of ConAgra derived hybrids and B73 for reference. Three replicates of raw flour and air-popped flakes were submitted for analysis (g/100g).

Cultivar	Ref. No.	Yield 1		Yield 2		PB Lysine (Raw Flour)		Expansion Volume		OCFSI		Popability		Vitreousness	
		Grams dry matter/m² (lbs/ft²)	*sd*	*15.5% moisture kg/m² (bushel/ac)*	*sd*	*g/100 g*	*sd*	*cc per gram*	*sd*	*unit*	*sd*	*%*	*sd*	*1-7 scale*	*sd*
BC_2F_5 F_1 Hybrid	**H1**	312.48^{cd} (0.064)	*63.47 (0.013)*	0.3934^{cde} (62.54)	*0.106 (16.82)*	0.371^{a}	*0.039*	26.11^{efg}	*3.98*	3.53^{d}	*0.62*	96.37^{fgh}	*1.00*	5.56^{bc}	*0.92*
	H2	273.42^{cd} (0.056)	*73.24 (0.015)*	0.3560^{de} (56.59)	*0.085 (13.43)*	0.234^{bcdef}	*0.039*	19.11^{j}	*3.76*	2.56^{f}	*0.44*	95.49^{hi}	*1.29*	4.75^{cde}	*1.10*
	H3	273.42^{cd} (0.056)	*58.59 (0.012)*	0.3388^{de} (53.86)	*0.092 (14.65)*	0.317^{abcd}	*0.045*	24.22^{ghi}	*2.64*	3.14^{de}	*0.20*	96.64^{efg}	*1.31*	5.33^{bcd}	*0.25*
	H4	273.42^{cd} (0.056)	*48.83 (0.010)*	0.3532^{de} (56.15)	*0.073 (11.56)*	0.322^{abc}	*0.050*	25.00^{fghi}	*3.61*	3.42^{d}	*0.38*	96.49^{efgh}	*1.25*	6.22^{ab}	*0.83*
	H5	400.37^{ab} (0.082)	*63.47 (0.013)*	0.5038^{ab} (81.10)	*0.109 (17.30)*	0.368^{a}	*0.033*	19.56^{j}	*4.42*	2.68^{ef}	*0.67*	94.84^{i}	*1.23*	4.64^{de}	*0.78*
average		306.24^{b} (0.063)	*77.124 (0.016)*	0.389^{a} (61.848)	*0.108 (17.192)*	0.323^{a}	*0.062*	22.8^{c}	*4.61*	3.06^{b}	*0.614*	95.97^{c}	*1.36*	5.30^{b}	*0.98*
BC_3F_4 F_1 Hybrid	**H6**	244.13^{d} (0.050)	*58.59 (0.012)*	0.3091^{e} (49.14)	*0.081 (12.93)*	0.337^{ab}	*0.057*	27.78^{ef}	*3.70*	3.36^{d}	*0.54*	97.57^{bcde}	*1.23*	6.47^{a}	*0.54*
	H7	253.89^{d} (0.052)	*53.71 (0.011)*	0.3105^{e} (49.37)	*0.073 (11.62)*	0.300^{abcde}	*0.018*	21.67^{ij}	*2.06*	2.74^{ef}	*0.26*	96.89^{defg}	*0.98*	3.94^{e}	*0.85*
	H8	244.13^{d} (0.050)	*53.71 (0.011)*	0.3072^{e} (48.84)	*0.085 (13.53)*	0.295^{abcde}	*0.053*	25.33^{fgh}	*3.87*	3.15^{de}	*0.46*	97.22^{cdef}	*0.93*	5.06^{cd}	*0.81*
	H9	273.42^{cd} (0.056)	*87.89 (0.018)*	0.3391^{de} (53.92)	*0.130 (20.66)*	0.325^{abc}	*0.046*	29.44^{de}	*4.98*	3.38^{d}	*0.38*	97.95^{bcd}	*0.97*	5.56^{bc}	*1.31*
	H10	317.36^{cd} (0.065)	*39.06 (0.008)*	0.3901^{cde} (62.01)	*0.065 (10.33)*	0.330^{abc}	*0.040*	22.22^{hij}	*2.95*	2.81^{ef}	*0.16*	96.03^{gh}	*1.18*	5.44^{bcd}	*1.10*
average		265.56^{c} (0.054)	*63.37 (0.013)*	0.331^{b} (52.657)	*0.091 (14.533)*	0.317^{a}	*0.042*	25.29^{b}	*4.63*	3.09^{b}	*0.457*	97.13^{b}	*1.21*	5.29^{b}	*1.23*
ConAgra ® Brands Popcorn	**H11**	283.19^{cd} (0.058)	*48.83 (0.010)*	0.3459^{de} (54.99)	*0.106 (16.81)*	0.190^{ef}	*0.021*	34.44^{bc}	*4.67*	4.63^{bc}	*0.48*	98.12^{abc}	*1.19*	7.00^{a}	*0.00*
	H12	253.89^{d} (0.052)	*68.36 (0.014)*	0.3107^{e} (49.39)	*0.076 (12.07)*	0.185^{ef}	*0.038*	33.22^{c}	*4.09*	4.32^{c}	*0.77*	97.93^{bcd}	*1.00*	6.72^{a}	*0.83*
	H13	390.60^{ab} (0.080)	*78.12 (0.016)*	0.4693^{bc} (74.61)	*0.103 (16.31)*	0.161^{f}	*0.012*	37.33^{ab}	*5.29*	4.77^{bc}	*0.71*	98.64^{ab}	*0.89*	7.00^{a}	*0.00*
	H14	346.66^{bc} (0.071)	*19.53 (0.004)*	0.4282^{bcd} (68.07)	*0.044 (7.05)*	0.197^{def}	*0.057*	39.89^{a}	*3.79*	5.52^{a}	*0.85*	99.16^{a}	*0.23*	7.00^{a}	*0.00*
	H15	458.96^{a} (0.094)	*97.65 (0.020)*	0.5632^{a} (89.53)	*0.156 (24.73)*	0.214^{cdef}	*0.029*	32.00^{cd}	*5.17*	4.93^{b}	*0.37*	98.22^{abc}	*1.24*	7.00^{a}	*0.00*
average		346.17^{a} (0.071)	*98.67 (0.020)*	0.423^{a} (67.32)	*0.134 (21.338)*	0.189^{b}	*0.034*	35.38^{a}	*5.29*	4.83^{a}	*0.748*	98.41^{a}	*1.03*	6.94^{a}	*0.37*

Table 5 | Select trait measurements of cultivars tested in 2020 summer trials. Trait values and standard deviations that were utilized for the 2020 Ranking System (except for Yield 2) are shown.

Backcross	Hybrid	Type	Ala	Arg	Asn	Asp	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Trp	Thr	Tyr	Val	Cys
BC2	H1	Raw	0.0114	0.0148	0.1276	0.0810	0.0205	0.1123	0.0708	0.0019	0.0010	0.0012	0.0038	0.0006	0.0021	0.1458	0.0059	0.0037	0.0234	0.0128	0.0047	0.0011
			0.0091	0.0364	0.3190	0.0831	0.0325	0.1237	0.0723	0.0030	0.0010	0.0012	0.0064	0.0004	0.0024	0.1846	0.0064	0.0046	0.0302	0.0162	0.0048	0.0014
			0.0103	0.0488	0.3212	0.0758	0.0108	0.0794	0.0764	0.0033	0.0008	0.0009	0.0097	0.0002	0.0014	0.2608	0.0033	0.0022	0.0360	0.0102	0.0050	0.0017
		Air	0.0170	0.0126	0.1589	0.0849	0.0020	0.0918	0.0709	0.0015	0.0030	0.0018	0.0041	0.0010	0.0026	0.0889	0.0077	0.0036	0.0206	0.0137	0.0062	0.0008
			0.0129	0.0100	0.1002	0.1029	0.0032	0.0889	0.0677	0.0016	0.0029	0.0021	0.0049	0.0014	0.0030	0.0778	0.0081	0.0042	0.0173	0.0166	0.0072	0.0006
			0.0100	0.0108	0.0873	0.0828	0.0002	0.0661	0.0632	0.0014	0.0018	0.0017	0.0032	0.0011	0.0023	0.1123	0.0067	0.0043	0.0184	0.0150	0.0054	0.0006
	H2	Raw	0.0115	0.0120	0.0422	0.0281	0.0023	0.0242	0.0471	0.0010	0.0006	0.0007	0.0030	0.0004	0.0008	0.1230	0.0048	0.0102	0.0176	0.0063	0.0027	0.0009
			0.0094	0.0090	0.1426	0.1061	0.0450	0.1522	0.0742	0.0015	0.0008	0.0019	0.0033	0.0008	0.0024	0.0982	0.0067	0.0032	0.0192	0.0167	0.0045	0.0009
			0.0111	0.0054	0.0372	0.0320	0.0080	0.0328	0.0347	0.0009	0.0004	0.0009	0.0016	0.0006	0.0008	0.0654	0.0056	0.0028	0.0102	0.0049	0.0029	0.0005
		Air	0.0153	0.0063	0.0721	0.0886	0.0060	0.0717	0.0576	0.0012	0.0024	0.0033	0.0040	0.0008	0.0029	0.0635	0.0087	0.0078	0.0130	0.0155	0.0068	0.0005
			0.0099	0.0058	0.0587	0.1114	0.0056	0.0945	0.0642	0.0014	0.0016	0.0026	0.0044	0.0010	0.0034	0.0601	0.0073	0.0040	0.0131	0.0206	0.0060	0.0006
			0.0129	0.0043	0.0542	0.0939	0.0052	0.0843	0.0614	0.0012	0.0024	0.0033	0.0041	0.0010	0.0031	0.0503	0.0085	0.0043	0.0123	0.0163	0.0074	0.0005
	H3	Raw	0.0058	0.0134	0.1201	0.0865	0.0147	0.0830	0.0705	0.0019	0.0006	0.0009	0.0038	0.0002	0.0016	0.1229	0.0025	0.0030	0.0196	0.0096	0.0028	0.0009
			0.0065	0.0239	0.2047	0.0855	0.0251	0.1017	0.0662	0.0020	0.0008	0.0012	0.0058	0.0004	0.0021	0.1495	0.0048	0.0020	0.0228	0.0124	0.0040	0.0011
			0.0114	0.0265	0.1967	0.0993	0.0316	0.1397	0.0726	0.0027	0.0026	0.0020	0.0074	0.0011	0.0027	0.1650	0.0080	0.0065	0.0309	0.0142	0.0072	0.0015
		Air	0.0081	0.0174	0.1245	0.1006	0.0038	0.1078	0.0736	0.0019	0.0011	0.0016	0.0043	0.0008	0.0027	0.1258	0.0060	0.0040	0.0216	0.0149	0.0050	0.0010
			0.0085	0.0140	0.1158	0.1098	0.0011	0.0856	0.0647	0.0020	0.0010	0.0014	0.0045	0.0006	0.0032	0.1328	0.0049	0.0043	0.0211	0.0156	0.0041	0.0009
			0.0075	0.0137	0.1087	0.0868	0.0008	0.0674	0.0676	0.0016	0.0011	0.0014	0.0037	0.0008	0.0018	0.1205	0.0042	0.0019	0.0199	0.0100	0.0047	0.0008
	H4	Raw	0.0107	0.0225	0.0641	0.0177	0.0041	0.0182	0.0511	0.0027	0.0025	0.0055	0.0131	0.0024	0.0020	0.1237	0.0071	0.0011	0.0195	0.0093	0.0063	0.0008
			0.0046	0.0119	0.0497	0.0353	0.0023	0.0312	0.0360	0.0017	0.0008	0.0023	0.0060	0.0010	0.0008	0.0669	0.0032	0.0010	0.0101	0.0055	0.0027	0.0005
			0.0065	0.0181	0.0636	0.0303	0.0028	0.0466	0.0509	0.0021	0.0012	0.0024	0.0078	0.0011	0.0010	0.0755	0.0045	0.0012	0.0139	0.0066	0.0033	0.0006
		Air	0.0030	0.0085	0.0690	0.0526	0.0003	0.0561	0.0588	0.0010	0.0002	0.0005	0.0029	0.0004	0.0010	0.0912	0.0022	0.0013	0.0136	0.0072	0.0022	0.0005
			0.0049	0.0119	0.1013	0.0653	0.0005	0.0613	0.0688	0.0015	0.0000	0.0003	0.0035	0.0002	0.0008	0.1670	0.0026	0.0014	0.0226	0.0062	0.0021	0.0009
			0.0040	0.0092	0.1275	0.0474	0.0002	0.0500	0.0548	0.0011	0.0000	0.0004	0.0032	0.0002	0.0004	0.1270	0.0025	0.0009	0.0182	0.0044	0.0017	0.0006
	H5	Raw	0.0146	0.0242	0.2335	0.1131	0.0175	0.1100	0.0755	0.0029	0.0010	0.0012	0.0061	0.0004	0.0025	0.2282	0.0039	0.0048	0.0349	0.0186	0.0054	0.0018
			0.0145	0.0104	0.1155	0.0842	0.0113	0.1040	0.0743	0.0021	0.0019	0.0014	0.0045	0.0004	0.0018	0.1644	0.0046	0.0024	0.0239	0.0157	0.0050	0.0014
			0.0174	0.0256	0.3523	0.1178	0.0157	0.1078	0.0790	0.0033	0.0017	0.0020	0.0083	0.0006	0.0027	0.2296	0.0055	0.0023	0.0360	0.0166	0.0062	0.0018
		Air	0.0185	0.0216	0.1768	0.1034	0.0028	0.1032	0.0765	0.0027	0.0011	0.0014	0.0064	0.0004	0.0020	0.2319	0.0049	0.0017	0.0334	0.0142	0.0054	0.0013
			0.0130	0.0102	0.0740	0.0913	0.0014	0.0914	0.0682	0.0024	0.0012	0.0014	0.0049	0.0006	0.0020	0.1372	0.0058	0.0038	0.0216	0.0154	0.0046	0.0008
			0.0113	0.0211	0.0815	0.0969	0.0015	0.1029	0.0727	0.0028	0.0006	0.0009	0.0055	0.0002	0.0020	0.1537	0.0034	0.0036	0.0254	0.0144	0.0039	0.0010

Table 6. Free amino acid profiles of Quality Protein Popcorn BC_2F_5-derived hybrids. Three replicates of raw flour and air-popped flakes were submitted for analysis (g/100g).

Backcross	Hybrid	Type	Ala	Arg	Asn	Asp	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Trp	Thr	Tyr	Val	Cys
BC3	H6	Raw	0.0112	0.0117	0.1350	0.1041	0.0459	0.1434	0.0782	0.0021	0.0021	0.0025	0.0053	0.0014	0.0022	0.1157	0.0088	0.0016	0.0239	0.0118	0.0066	0.0013
			0.0122	0.0224	0.2299	0.1080	0.0747	0.2250	0.0770	0.0030	0.0022	0.0026	0.0074	0.0021	0.0023	0.1778	0.0101	0.0022	0.0363	0.0171	0.0083	0.0017
			0.0168	0.0364	0.3369	0.1340	0.0971	0.2402	0.0778	0.0030	0.0032	0.0038	0.0111	0.0023	0.0043	0.1689	0.0150	0.0022	0.0434	0.0198	0.0111	0.0021
		Air	0.0219	0.0137	0.1694	0.1441	0.0026	0.1147	0.0766	0.0025	0.0043	0.0035	0.0073	0.0021	0.0036	0.1542	0.0146	0.0017	0.0313	0.0150	0.0111	0.0012
			0.0126	0.0083	0.1368	0.1285	0.0010	0.1011	0.0750	0.0021	0.0021	0.0026	0.0052	0.0012	0.0036	0.0946	0.0072	0.0021	0.0202	0.0146	0.0065	0.0008
			0.0064	0.0065	0.1133	0.1263	0.0028	0.1027	0.0772	0.0021	0.0004	0.0014	0.0039	0.0004	0.0028	0.0835	0.0045	0.0018	0.0164	0.0140	0.0029	0.0008
	H7	Raw	0.0164	0.0154	0.2389	0.1369	0.0472	0.1368	0.0743	0.0023	0.0027	0.0032	0.0060	0.0012	0.0032	0.0866	0.0082	0.0037	0.0190	0.0172	0.0082	0.0011
			0.0189	0.0216	0.2117	0.1363	0.0478	0.1617	0.0765	0.0025	0.0048	0.0042	0.0091	0.0016	0.0042	0.1408	0.0115	0.0028	0.0272	0.0166	0.0114	0.0014
			0.0223	0.0269	0.2465	0.1527	0.0752	0.1919	0.0798	0.0029	0.0064	0.0049	0.0084	0.0021	0.0042	0.1546	0.0172	0.0025	0.0325	0.0198	0.0140	0.0016
		Air	0.0147	0.0106	0.1190	0.1411	0.0050	0.1054	0.0729	0.0017	0.0047	0.0042	0.0052	0.0014	0.0034	0.0663	0.0087	0.0036	0.0158	0.0144	0.0089	0.0008
			0.0373	0.0084	0.1765	0.1483	0.0237	0.1598	0.0776	0.0022	0.0108	0.0081	0.0121	0.0033	0.0060	0.0703	0.0191	0.0055	0.0257	0.0213	0.0179	0.0013
			0.0241	0.0106	0.1183	0.1680	0.0122	0.1207	0.0788	0.0023	0.0083	0.0067	0.0094	0.0022	0.0049	0.0823	0.0151	0.0054	0.0215	0.0189	0.0135	0.0010
	H8	Raw	0.0072	0.0180	0.2155	0.0931	0.0190	0.0956	0.0703	0.0020	0.0014	0.0010	0.0053	0.0004	0.0023	0.0756	0.0051	0.0037	0.0159	0.0115	0.0038	0.0008
			0.0085	0.0180	0.2147	0.0818	0.0151	0.0721	0.0622	0.0020	0.0010	0.0009	0.0047	0.0004	0.0012	0.0812	0.0043	0.0026	0.0145	0.0084	0.0036	0.0008
			0.0093	0.0161	0.2247	0.1100	0.0359	0.1240	0.0722	0.0017	0.0024	0.0018	0.0047	0.0010	0.0023	0.0624	0.0071	0.0049	0.0154	0.0135	0.0052	0.0008
		Air	0.0107	0.0101	0.1340	0.1051	0.0034	0.0841	0.0709	0.0016	0.0026	0.0020	0.0046	0.0009	0.0035	0.0970	0.0062	0.0035	0.0187	0.0140	0.0056	0.0008
			0.0200	0.0093	0.1540	0.1419	0.0070	0.1225	0.0742	0.0022	0.0073	0.0048	0.0070	0.0027	0.0045	0.0813	0.0132	0.0041	0.0222	0.0165	0.0125	0.0011
			0.0060	0.0053	0.0662	0.0976	0.0003	0.0684	0.0558	0.0015	0.0009	0.0009	0.0024	0.0004	0.0021	0.0828	0.0027	0.0022	0.0157	0.0124	0.0028	0.0005
	H9	Raw	0.0091	0.0178	0.2495	0.1091	0.0401	0.1315	0.0740	0.0024	0.0010	0.0014	0.0059	0.0008	0.0018	0.1044	0.0043	0.0040	0.0207	0.0097	0.0043	0.0011
			0.0070	0.0017	0.0142	0.0148	0.0012	0.0172	0.0169	0.0005	0.0004	0.0007	0.0009	0.0002	0.0004	0.0366	0.0025	0.0006	0.0051	0.0022	0.0020	0.0002
			0.0051	0.0153	0.1639	0.0578	0.0067	0.0579	0.0633	0.0018	0.0006	0.0014	0.0050	0.0008	0.0010	0.1090	0.0044	0.0017	0.0169	0.0078	0.0029	0.0008
		Air	0.0130	0.0090	0.1019	0.0989	0.0016	0.0846	0.0718	0.0015	0.0008	0.0016	0.0043	0.0010	0.0018	0.1297	0.0056	0.0014	0.0202	0.0102	0.0044	0.0008
			0.0189	0.0110	0.1036	0.1077	0.0021	0.0768	0.0682	0.0018	0.0025	0.0026	0.0066	0.0019	0.0020	0.0862	0.0082	0.0019	0.0168	0.0097	0.0067	0.0006
			0.0061	0.0085	0.0711	0.0884	0.0003	0.0517	0.0522	0.0014	0.0002	0.0007	0.0029	0.0002	0.0014	0.0789	0.0017	0.0029	0.0118	0.0081	0.0019	0.0005
	H10	Raw	0.0347	0.0328	0.2910	0.1363	0.0989	0.2013	0.0793	0.0034	0.0067	0.0055	0.0202	0.0017	0.0053	0.1161	0.0168	0.0068	0.0317	0.0306	0.0157	0.0018
			0.0242	0.0430	0.3838	0.1203	0.1093	0.2285	0.0813	0.0034	0.0074	0.0048	0.0129	0.0018	0.0051	0.1427	0.0160	0.0046	0.0365	0.0273	0.0156	0.0021
			0.0434	0.0431	0.2422	0.1473	0.1299	0.2398	0.0796	0.0046	0.0061	0.0046	0.0159	0.0018	0.0057	0.1802	0.0184	0.0040	0.0398	0.0313	0.0153	0.0021
		Air	0.0185	0.0189	0.1876	0.1441	0.0024	0.1070	0.0738	0.0024	0.0010	0.0021	0.0069	0.0008	0.0039	0.0898	0.0082	0.0083	0.0183	0.0246	0.0053	0.0008
			0.0275	0.0067	0.1387	0.1419	0.0061	0.1155	0.0771	0.0022	0.0060	0.0049	0.0069	0.0025	0.0051	0.0789	0.0187	0.0057	0.0227	0.0321	0.0122	0.0011
			0.0260	0.0105	0.1294	0.1455	0.0065	0.1187	0.0765	0.0026	0.0054	0.0044	0.0074	0.0014	0.0048	0.0727	0.0154	0.0084	0.0214	0.0352	0.0126	0.0009

Table 7. Free amino acid profiles of Quality Protein Popcorn BC_3F_4-derived hybrids. Three replicates of raw flour and air-popped flakes were submitted for analysis (g/100g).

Backcross	Hybrid	Type	Ala	Arg	Asn	Asp	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Trp	Thr	Tyr	Val	Cys
ConAgra Hybrids	H11	Raw	0.0070	0.0026	0.0238	0.0212	0.0015	0.0236	0.0182	0.0007	0.0006	0.0009	0.0012	0.0004	0.0008	0.0342	0.0028	0.0025	0.0057	0.0041	0.0024	0.0003
			0.0115	0.0027	0.0243	0.0242	0.0020	0.0279	0.0322	0.0008	0.0008	0.0012	0.0012	0.0006	0.0008	0.0583	0.0044	0.0009	0.0104	0.0039	0.0030	0.0005
			0.0062	0.0032	0.0209	0.0166	0.0009	0.0218	0.0251	0.0008	0.0004	0.0007	0.0012	0.0002	0.0006	0.0491	0.0024	0.0016	0.0077	0.0040	0.0019	0.0003
		Air	0.0089	0.0016	0.0108	0.0212	0.0003	0.0253	0.0153	0.0006	0.0004	0.0008	0.0012	0.0006	0.0008	0.0257	0.0036	0.0018	0.0049	0.0037	0.0026	0.0002
			0.0063	0.0019	0.0113	0.0125	0.0000	0.0126	0.0127	0.0005	0.0002	0.0005	0.0009	0.0002	0.0004	0.0333	0.0019	0.0016	0.0049	0.0025	0.0015	0.0002
			0.0054	0.0023	0.0120	0.0201	0.0000	0.0159	0.0209	0.0005	0.0004	0.0007	0.0011	0.0002	0.0006	0.0432	0.0020	0.0014	0.0059	0.0027	0.0019	0.0002
	H12	Raw	0.0039	0.0060	0.0202	0.0138	0.0018	0.0207	0.0179	0.0009	0.0004	0.0007	0.0016	0.0004	0.0006	0.0430	0.0018	0.0046	0.0060	0.0038	0.0019	0.0003
			0.0048	0.0083	0.0220	0.0093	0.0008	0.0129	0.0182	0.0006	0.0002	0.0004	0.0029	0.0002	0.0004	0.0472	0.0022	0.0059	0.0068	0.0036	0.0015	0.0003
			0.0069	0.0045	0.0176	0.0140	0.0014	0.0153	0.0174	0.0006	0.0002	0.0005	0.0014	0.0002	0.0004	0.0473	0.0031	0.0049	0.0068	0.0034	0.0016	0.0003
		Air	0.0029	0.0027	0.0070	0.0101	0.0002	0.0110	0.0075	0.0004	0.0002	0.0003	0.0008	0.0002	0.0004	0.0215	0.0012	0.0022	0.0025	0.0026	0.0012	0.0002
			0.0039	0.0017	0.0049	0.0151	0.0002	0.0138	0.0044	0.0004	0.0002	0.0005	0.0007	0.0004	0.0004	0.0134	0.0024	0.0022	0.0018	0.0023	0.0012	0.0002
			0.0033	0.0014	0.0035	0.0100	0.0000	0.0086	0.0052	0.0003	0.0000	0.0003	0.0007	0.0002	0.0004	0.0131	0.0015	0.0018	0.0016	0.0024	0.0010	0.0002
	H13	Raw	0.0037	0.0029	0.0141	0.0175	0.0017	0.0196	0.0140	0.0007	0.0002	0.0005	0.0013	0.0002	0.0006	0.0304	0.0017	0.0045	0.0038	0.0034	0.0015	0.0003
			0.0034	0.0035	0.0130	0.0071	0.0013	0.0159	0.0109	0.0005	0.0002	0.0003	0.0010	0.0002	0.0004	0.0222	0.0015	0.0015	0.0027	0.0019	0.0012	0.0002
			0.0049	0.0039	0.0132	0.0101	0.0011	0.0168	0.0119	0.0005	0.0002	0.0003	0.0014	0.0004	0.0004	0.0303	0.0023	0.0049	0.0036	0.0033	0.0012	0.0003
		Air	0.0053	0.0022	0.0073	0.0113	0.0000	0.0108	0.0100	0.0004	0.0002	0.0003	0.0008	0.0002	0.0004	0.0347	0.0021	0.0027	0.0041	0.0026	0.0014	0.0002
			0.0043	0.0019	0.0054	0.0138	0.0002	0.0135	0.0097	0.0004	0.0002	0.0005	0.0009	0.0004	0.0006	0.0217	0.0023	0.0030	0.0027	0.0025	0.0015	0.0002
			0.0048	0.0018	0.0082	0.0118	0.0002	0.0101	0.0087	0.0004	0.0000	0.0003	0.0007	0.0002	0.0004	0.0247	0.0021	0.0011	0.0030	0.0024	0.0012	0.0002
	H14	Raw	0.0029	0.0036	0.0274	0.0144	0.0018	0.0211	0.0122	0.0006	0.0004	0.0005	0.0011	0.0002	0.0004	0.0217	0.0014	0.0019	0.0037	0.0025	0.0015	0.0002
			0.0035	0.0037	0.0373	0.0259	0.0021	0.0253	0.0184	0.0010	0.0006	0.0007	0.0014	0.0004	0.0008	0.0313	0.0018	0.0029	0.0052	0.0035	0.0022	0.0003
			0.0041	0.0081	0.0761	0.0229	0.0021	0.0242	0.0254	0.0015	0.0006	0.0005	0.0021	0.0002	0.0008	0.0532	0.0014	0.0052	0.0079	0.0041	0.0022	0.0005
		Air	0.0024	0.0026	0.0175	0.0173	0.0000	0.0153	0.0084	0.0005	0.0002	0.0005	0.0009	0.0002	0.0006	0.0172	0.0009	0.0033	0.0023	0.0025	0.0015	0.0002
			0.0028	0.0021	0.0149	0.0175	0.0000	0.0126	0.0083	0.0004	0.0002	0.0005	0.0009	0.0002	0.0004	0.0249	0.0011	0.0026	0.0034	0.0028	0.0015	0.0002
			0.0031	0.0021	0.0160	0.0172	0.0000	0.0121	0.0103	0.0005	0.0004	0.0005	0.0010	0.0002	0.0006	0.0272	0.0014	0.0022	0.0036	0.0030	0.0017	0.0002
	H15	Raw	0.0060	0.0020	0.0297	0.0196	0.0014	0.0219	0.0158	0.0008	0.0006	0.0009	0.0009	0.0004	0.0006	0.0267	0.0030	0.0012	0.0051	0.0035	0.0019	0.0003
			0.0062	0.0025	0.0437	0.0317	0.0022	0.0282	0.0249	0.0009	0.0012	0.0012	0.0014	0.0006	0.0008	0.0301	0.0033	0.0018	0.0068	0.0049	0.0031	0.0003
			0.0085	0.0043	0.0499	0.0400	0.0038	0.0282	0.0365	0.0009	0.0013	0.0013	0.0016	0.0008	0.0010	0.0524	0.0051	0.0012	0.0103	0.0054	0.0035	0.0005
		Air	0.0039	0.0018	0.0142	0.0331	0.0002	0.0208	0.0093	0.0007	0.0010	0.0009	0.0010	0.0006	0.0008	0.0255	0.0025	0.0015	0.0043	0.0042	0.0021	0.0002
			0.0067	0.0026	0.0197	0.0333	0.0005	0.0227	0.0189	0.0007	0.0015	0.0012	0.0012	0.0006	0.0008	0.0369	0.0036	0.0021	0.0067	0.0049	0.0032	0.0002
			0.0076	0.0028	0.0358	0.0396	0.0003	0.0286	0.0232	0.0009	0.0019	0.0016	0.0014	0.0006	0.0010	0.0355	0.0043	0.0014	0.0078	0.0060	0.0041	0.0003
B73 Reference			0.0086	0.0031	0.0137	0.0076	0.0002	0.0070	0.0249	0.0007	0.0002	0.0004	0.0011	0.0000	0.0006	0.0580	0.0014	0.0003	0.0066	0.0022	0.0017	0.0005
			0.0092	0.0026	0.0129	0.0103	0.0002	0.0098	0.0267	0.0007	0.0002	0.0004	0.0009	0.0000	0.0006	0.0637	0.0009	0.0003	0.0090	0.0028	0.0017	0.0006
			0.0211	0.0070	0.0379	0.0182	0.0002	0.0156	0.0489	0.0016	0.0006	0.0007	0.0020	0.0002	0.0013	0.1283	0.0025	0.0005	0.0188	0.0048	0.0035	0.0014

Table 8. Free amino acid profiles of ConAgra derived hybrids and B73 for reference. Three replicates of raw flour and air-popped flakes were submitted for analysis (g/100g).

Trait	Selection Index Economic Value (I_v)
Protein-Bound Lysine (g/100g)	0.90
Yield *(lbs/ft²)*	0.90
Expansion Volume *(cc/g)*	0.90
OCFSI	0.85
Popability *(%)*	0.80
Vitreousness	0.55

Table 9 | Economic Values assigned for traits in 2020 Ranking System. Economic weighting values were determined. Protein-bound lysine, yield, and expansion volume were each considered equally important traits during selection, while OCFSI, popability, and vitreousness were respectively given lesser importance for overall selection.

References

Addelman, S. (1969). The Generalized Randomized Block Design. The American Statistician. 23, 35-36.

Angelovici R., Lipka A. E., Deason N., Gonzalez-Jorge S., Lin H., Cepela J. (2013). Genome-wide analysis of branched-chain amino acid levels in arabidopsis seeds. *Plant Cell.* 25, 4827–4843. doi:10.1105/tpc.113.119370

Babu, B.K., Agrawal, P.K., Saha, S., Gupta, H.S. (2015). Mapping QTLs for opaque2 modifiers influencing the tryptophan content in quality protein maize using genomic and candidate gene-based SSRs of lysine and tryptophan metabolic pathway. *Plant Cell Rep.* 34, 37-45.

Babu R., Nair S. K., Kumar A., Venkatesh S., Sekhar J. C., Singh N. N. (2005). Two-generation marker-aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). *Theor. Appl. Genet.* 111, 888–897. doi: 10.1007/s00122-005-0011-6

Babu, R., Nair, S.K., Kumar, A., Rao, H.S., Verma, P., Gahalain, A., Sigh, I.S., and Gupta, H.S. (2006). Mapping QTLs for popping ability in a popcorn x flint corn cross. *Theor Appl Genet.* 112, 7: 1392-1399.

Coan, M. M. D., Pinto, R. J. B., Kuki, M. C., Amaral, A. T. Jr., Figueiredo, A. S. T., Scapim, C. A., et al. (2019). Inheritance study for popping expansion in popcorn vs. flint corn genotypes. *Agron. J.* 111, 2174–2183. doi: 10.1007/s00122-006-0242-1

Collard, B., Zahufer, M., Brouwer, J., Pang, E. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts, *Euphytica.* 142, 169-196. https://doi.org/10.1007/s10681-005-1681-5

Crumbaker, D. E., Johnson, I., and Eldredge, J. (1949). Inheritance of popping volume and associated characters in crosses between popcorn and dent corn. *Agron. J.* 41, 207–212. doi: 10.2134/agronj1949.00021962004100050009x

Dofing, S. M., ĎCroz-Mason N., Thomas-Compton, M. A. (1991). Inheritance of Expansion Volume and Yield in Two Popcorn × Dent Corn Crosses. *Crop Sci.* 31,715-718. doi:10.2135/cropsci1991.0011183X003100030035x

Elango, R., Ball, R. O., and Pencharz, P. B. (2009). Amino acid requirements in humans: with a special emphasis on the metabolic availability of amino acids. *Amino Acids* 37:19. doi: 10.1007/s00726-009-0234-y

Gevers H. O., Lake J. K. (1992). "Development of modified opaque-2 maize in South Africa," in Quality Protein Maize, ed. E. T. Mertz (Saint Paul, MN: AACC), 49–78.

Gupta, P. K., Kumar, J., Mir, R. R., and Kumar, A. (2010). Marker-assisted selection as a component of conventional plant breeding. *Plant Breed. Rev.* 33, 145–217. doi: 10.1002/9780470535486.ch4

Gutiérrez-Rojas, A., Betrán, J., Paul Scott, M., Atta, H., Menz, M. (2010). Quantitative Trait Loci for Endosperm Modification and Amino Acid Contents in Quality Protein Maize. *Crop Sci.* 50, 870-879.

Hernandez, H. H., and Bates, L. S. (1969). A Modified Method for Rapid Tryptophan Analysis in Maize. *CIMMYT Research Bulletin no. 13*. Mexico: CIMMYT.

Hicks, D.R. and Cloud, H.A. (1991). Calculating Grain Weight Shrinkage in Corn Due to Mechanical Drying. National Corn Handbook Publication NCH-61. National Corn Handbook – 61. Accessible at: https://www.extension.purdue.edu/extmedia/nch/nch-61.html

Holding, D. R. (2014). Recent advances in the study of prolamin storage protein organization and function. *Front. Plant Sci.* 5:276. doi: 10.3389/fpls.2014.00276

Holding, D. R., Hunter, B. G., Chung, T., Gibbon, B.C., Ford, C.F., Bharti, A.K. (2008). Genetic analysis of opaque2 modifier loci in quality protein maize. *Theor. Appl. Genet*. 117, 157–170. doi: 10.1007/s00122-008-0762-y

Holding, D. R., Hunter, B. G., Klingler, J. P., Wu, S., Guo, X., and Gibbon, B. C. (2011). Characterization of opaque2 modifier QTLs and candidate genes in recombinant inbred lines derived from the K0326Y quality protein maize inbred. *Theor. Appl. Genet.* 122, 783–794.

Hoseney R., Zeleznak K., Abdelrahman, A. (1983). Mechanism of popcorn popping. *J. Cereal Sci*. 1, 43–52. doi: 10.1002/anie.201402040

Krivanek, A. F., De Groote, H., Gunaratna, N. S., Diallo, A. O., and Friesen, D. (2007). Breeding and disseminating quality protein maize (QPM) for Africa. Afr. J. Biotechnol. 6, 312–324.

Li, Y., Wu, S., Dong, Y., Zhang, Y., Li, Z. (2004). Study on the Popping Characteristics of Different Populations from Normal Corn× Popcorn Crosses. *Journal of Maize Sciences,* 2.

Li, Y., Wu, S., Zhang, Y., Wang, Y., Du, Z., Wang, C. (2003). Study on the Combining Ability of Advanced Pedigree Selected Lines from Popcorn x Normal Corn Crosses and Their Utilization. *Acta Agri. Boreali – Sinica,* 4.

Li, Y., Dong, Y., Niu, S., and Cui, D. (2007). QTL for popping characteristics in popcorn. Plant Breed. 126, 509–514. doi: 10.1111/j.1439-0523.2007.01372.x

Li, Y. L., Dong, Y., Niu, S., Cui, D., Wang, Y., Liu, Y., et al. (2008). Identification of agronomically favorable quantitative trait loci alleles from a dent corn inbred Dan232 using advanced backcross QTL analysis and comparison with the F2:3 population in popcorn. *Mol. Breed.* 21, 1–14. doi: 10.1007/s11032-007-9104-z

Li, Y. L., Dong, Y. B., Niu, S. Z., and Cui, D. Q. (2009). Identification of QTL for popping characteristics using a BC2F2 population and comparison with its F2:3 population in popcorn. Agric. Sci. China 8, 137–143. doi: 10.1016/S1671-2927(09)60020-1

Liu, H., Shi J., Sun C., Gong H., Fan X., Qiu F. (2016). Gene duplication confers enhanced expression of 27-kDa γ-zein for endosperm modification in quality protein maize. *Proc. Natl. Acad. Sci.* U.S.A. 113, 4964–4969. doi: 10.1073/pnas.1601352113

Matz S.A. (1984). Snacks Based on Popcorn. In: Snack Food Technology. pg. 138-149. Springer, Dordrecht

Mordor Intelligence. (2018). POPCORN MARKET – GROWTH, TRENDS AND FORECAST (2020-2025). Hyderabad, India: Mordor Intelligence.

Niu, A., Li, Y., Chen, H. (2008). Popping Characteristics of BC_2S_1 Lines Derived from A Normal Corn×Popcorn Cross and Their Relationships with Ear-kernel Characters. *Journ. of Henan Ag. Sci.* 1.

Olakojo, S. A., Omueti, O., Ajomale, K., and Ogunbodede, B. A. (2007). Development of quality protein maize: biochemical and agronomic evaluation. Trop. Subtrop. Agroecosyst. 7, 97–104.

Pandey, N., Hossain, F., Kumar, K., Vishwakarma, A.K., Muthusamy, V., Saha, S., Agrawal, P.K., Guleria, S.K., Reddy, S.S., Thirunavukkarasu, N., Gupta, H.S. (2015). *Plant*

Breeding. Molecular characterization of endosperm and amino acids modifications among quality protein maize inbreds. 135:1, 47-54.

Parsons, L., Ren, Y., Yobi, A., Hurst, P., Angelovici, R., Rodriguez, O., & Holding, D. (2020). Production and Selection of Quality Protein Popcorn Hybrids Using a Novel Ranking System and Combining Ability Estimates. *Frontiers In Plant Science, 11.* https://doi.org/10.3389/fpls.2020.00698

Pereira, M. G., and Amaral Júnior, A. T. (2001). Estimation of genetic components in popcorn based on the nested design. *Crop Breed. Appl. Biotechnol*. 1, 3–10. doi: 10.13082/1984-7033.v01n01a01

Prasanna B. M., Vasal S. K., Kassahun B., Singh N. N. (2001). Quality protein maize. *Curr. Sci.* 81, 1308–1319.

R Core Team (2018). R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing.

Ramos, H., Pereira, M., Silva, F., Gonçalves, L., Pinto, F., de Souza Filho, G., Pereira, T. (2011). Genetic characterization of papaya plants (*Carica papaya* L.) derived from the first backcross generation. *Genet. Mol. Res.* 10(1), 393-403.

Ren, Y., Yobi, A., Marshall, L., Angelovici, R., Rodriguez, O., Holding, D. (2018). Generation and Evaluation of Modified *Opaque-2* Popcorn Suggests a Route to Quality Protein Popcorn. *Front. Plant Sci.* 9, 1803. doi: 10.3389/fpls.2018.01803

Robbins, W. A., and Ashman, R.B. (1984). Parent-offspring popping expansion correlations in progeny of dent corn x popcorn and flint corn x popcorn crosses. *Crop Sci.* 24, 119-121. doi: 10.2135/cropsci1984.0011183X002400010027x

Semagn, K., Bjørnstad, A., Ndjiondjop, M. (2006). An overview of molecular marker methods for plants. *Afric. Journ. of Biotech.* 5(25), 2540-2568.

Senhorinho, H. J. C., Coan, M. M. D., Marino, T. P., Kuki, M. C., Pinto, R. J. B., Scapim, C. A., et al. (2019). Genomic-wide association study of popping expansion in tropical popcorn and field corn germplasm. *Crop Sci.* 59, 2007–2019. doi: 10.2135/cropsci2019.02.0101

Silva, F, Pereira, M, Campos, W, Damasceno-Júnior P, Daher, R., Pereira, T., Souza Filho, G., Viana, A., Ferreguetti, G. (2007). Monitoring of the genetic variability in papaya parent 'Formosa' of 'UENF/CALIMAN 01' hybrid via RAPD. *Crop Breed. Appl. Biotechnol.* 7(1), 36-42.

da Silva, W., Vidal, B., Martins, M., Vargas, H., Pereira, C., Zerbetto, M., and Miranda, L. (1993) What makes popcorn pop. *Nature.* 362**,** 417. doi: https://doi.org/10.1038/362417a0

Smith, C. Wayne, J.B., and Runge, E.C.A. (2004). Corn. Hoboken, New Jersey: John Wiley.

Sprague, G.F. and Dudley, J.W. (1988). Corn and Corn Improvement. Madison: America Society of Agronomy.

Sweley, J., Meyer, M., Rose, D., & Jackson, D. (2012). Effects of hybrid, environment, oil addition, and microwave wattage on popped popcorn morphology. *Journal Of Cereal Science,* 56(2), 276-281. https://doi.org/10.1016/j.jcs.2012.05.009

Thakur, V. Tiwari, S., Tripathi, N. (2014). Studies on Genome Recovery Percentage for Opaque2 Introgressed in Backcross Population for Marker Assisted Selection of Quality Protein Maize. *Trends in Biosciences,* 7, 1897-1901.

Topping, R. (2011). Just Call Me Orville: The Story of Orville Redenbacher. West Lafayette, Indiana: Purdue University Press.

Uptmoor, R., Wenzel, W., Ayisi, K., Donaldson, G., Gehringer, A., Friedt, W., Ordon, F. (2006). Variation of the genomic proportion of the recurrent parent in BC1 and its relation to yield performance in sorghum (Sorghum bicolor) breeding for low-input conditions. *Plant Breeding,* 125(5), 532-534. https://doi.org/10.1111/j.1439-0523.2006.01270.x

Wallace J. C., Lopes M. A., Paiva E., Larkins B. A. (1990). New methods for extraction and quantitation of zeins reveal a high content of gamma-zein in modified opaque-2 maize. *Plant Physiol.* 92, 191–196. doi: 10.1104/pp.92.1.191

Wu Y., Holding D. R., Messing J. (2010). γ-zeins are essential for endosperm modification in quality protein maize. *Proc. Natl. Acad. Sci.* U.S.A. 107, 12810–12815. doi: 10.1073/pnas.1004721107

Yobi, A., and Angelovici, R. (2018). A High-Throughput Absolute-Level Quantification of Protein-Bound Amino Acids in Seeds. *Curr Protoc Plant Biol.* 3:4 doi: 10.1002/cppb.20084.

Ziegler, K. E. (2001). "Popcorn," in Specialty Corn, 2nd edn, ed. A. R. Hallauer, Boca Raton FL: CRC Press, 199–234.

Ziegler, K.E., and Ashman, B. (1994). "Popcorn", in Specialty corns, ed. A.R. Hallauer (New York, NY: CRC Press), 189-223.

CHAPTER 4: SENSORY EVALUATION OF NOVEL QUALITY PROTEIN POPCORN REVEALS IMPROVED DIVERSITY IN TASTE AND TEXTURE COMPARED TO CONVENTIONAL VARIETIES

1. Introduction

Popcorn is considered a specialty maize crop grown primarily for human consumption. It is characterized by its spherical and highly vitreous kernel morphology, and unique ability to pop into light flakes after applying heat. Popcorn has been sold and enjoyed as a snack product since the beginning of the 20th century, and sales and market diversification significantly increased after 2012 (Dawson and Telford, 1912; Dawande, 2018). In correlation with rises in consumer health-awareness, disposable income, and consumption of Ready-To-Eat (RTE) products, the popcorn industry enjoyed a 32% increase in retail popcorn sales from 2012 to 2018. Moreover, the market is projected to rise from a 2016 estimate of $9.06 billion to more than $15 billion by 2023 (Dawande, 2018, Mordor Intelligence, 2018).

Diversification of marketable popcorn products has relied on exterior supplements such as coatings, RTE additives, and blending with other food products rather than the breeding and production of novel popcorn cultivars (Matz, 1984; Lusas and Rooney, 2001; Tandjung, 2003). In fact, genetic influence on the sensory attributes of popcorn has been argued as an unimportant factor as the popcorn should be considered a neutral receptacle for diverse, exterior additives (Matz, 1984). In comparison with flavor additions, breeding of quality traits is not surprisingly a less favorable option for diversifying the popcorn market since breeding requires time and expense without ensured success. Given that the popcorn gene pool is significantly limited, within-pool breeding for productivity traits has

been unsuccessful, and attempts to diversify popcorn germplasm involving dent by popcorn crosses have resulted in a loss or serious reduction of characteristic popcorn quality traits. Popcorn breeding has the potential for improvement in agronomic traits such as pest/rot susceptibility, standability, and yield by dent germplasm introgression. However, a significantly negative correlation between yield and expansion volume has hindered producers from breeding high yielding, high expansion volume popcorn lines (Brunson, 1937; Robbins and Ashman, 1984; Dofing et al., 1991; Pereira and Amaral Júnior, 2001; Daros et al., 2002; Ziegler and Ashman, 1994; Li et al., 2002; Lu et al., 2003; Li et al., 2006; 2007, 2008, 2009; Dhliwayo, 2008; Ren et al., 2018; Parsons et al., 2020). Additionally, funding for popcorn breeding is limited compared to conventional corn grown on 99% of all maize-sown U.S. acres, and limited resources have restricted the number of popcorn breeding programs (Nebraska Corn Board, 2019). Nevertheless, some privately funded breeding programs remain (*National Plant Breeding Study-1*, 1996; Paula et al., 2010; Guimarães et al., 2018; Ren et al., 2018; Parsons et al., 2020). In 2018, Ren et al. described a subspecies breeding program which crossed Quality Protein Maize (QPM), highly vitreous and elevated lysine maize varieties, to popcorn (Ren et al., 2018). A four-year backcross recurrent breeding scheme utilized marker assisted selection for the *opaque-2* mutant allele, a characteristic allele introgressed into QPM conferring higher lysine and tryptophan in the maize kernel (Mertz et al., 1964; Babu et al., 2005), and phenotypic selection for endosperm and amino acid modifier genes (Vasal et al., 2002). Inbred Quality Protein Popcorn (QPP) lines culminated in 2017 that were highly vitreous, had popcorn-like kernel morphology, high popability, and a QPM-equaling elevated lysine (Ren et al., 2018). These inbred QPP lines were hybridized and evaluated, and select

hybrids with superior agronomic, protein quality, and popcorn quality traits were chosen for continued evaluation in 2020 (Parsons et al., 2020).

Popcorn sensory traits such as texture and taste have been associated with multiple popcorn characteristics, such as flake morphology, kernel morphology, pericarp color, and increasing genetic diversity of the popcorn cultivar (Sweley et al., 2011; Sweley et al., 2013; Paraginski et al., 2016; unpublished observations). To compare QPP hybrids with currently marketed, conventional popcorn cultivars and test for correlations between certain physical and sensory traits, a popcorn tasting evaluation utilizing 112 participants and eight popcorn cultivars was conducted. Overall, one QPP variety ranked higher than a popcorn control in taste, all QPP varieties ranked higher than a control in texture, and two QPP varieties ranked higher than a conventional popcorn control in overall likability. Additionally, taste and texture sensory trait rank were found to be highly correlated to overall likability while aroma and appearance were weakly correlated. These results, in concert with previous agronomic, popcorn quality, and protein quality trait comparisons with conventional popcorn cultivars, reveal a significant potential for QPP marketability.

2. Materials and Methods

2.1 Production and Selection of Plant Materials

Ten Quality Protein Popcorn (QPP) hybrids were grown in Lincoln, NE (40°50'11.6"N 96°39'42.4"W DMS) in the summer of 2020 alongside five ConAgra Brands® popcorn hybrid cultivars for comparative evaluation of agronomic, popcorn quality, and endosperm protein quality traits. After popcorn was harvested and evaluated, six QPP hybrids, herein labeled QPP Hybrids H1, H3, H4, H6, H8, and H9, were chosen for sensory comparison to two ConAgra® Brands conventional cultivars, labeled CL1 and CL2.

2.2 Sample Preparation

Directly after harvest, a random subsample of 400 grams (~0.88 lbs) of QPP and conventional hybrid kernels were placed and held in a conditioning room set at 14% moisture for six weeks. After the required duration for equilibration, all popcorn kernels were transferred to labeled, sealed plastic jars for long-term maintenance of moisture content. Immediately prior to participant sampling, 15-20 kernels of two varieties (measured in a half teaspoon measure) were simultaneously popped in Orville Redenbacher's® Hot Air Poppers. Participants were given two samples at a time, approximately in three minute intervals while the samples popped, and delivered immediately after popping for a total of six cultivars to evaluate. All popcorn kernels were popped by air without additives. After informed consent was obtained, popped samples were presented to panelists in five ounce multipurpose paper cups accompanied with six copies of the sensory evaluation form and an optional bottle of water.

2.3 Recruitment and Sensory Evaluation

Recruitment of individuals for the taste-testing panel took place at Colby Community College in Colby, Kansas from October 27th through November 9th of 2020. No data relating to the demographics of the panelists was asked for or recorded. Sole requirements for participation included being older than the age of 18 years, having no known allergic or negative reaction to popcorn, and experiencing no illness symptoms during both recruitment and taste-testing. Participant evaluations were scheduled over a two-week timeframe between November 2nd, 2020 and November 13th, 2020 in 30 minute increments (with walk-ins accepted) to individually taste and evaluate six popcorn samples. Participants were asked to specify popcorn appearance, aroma, taste, and texture on a 1-6

scale and overall likability on a 1-10 scale by completing individualized evaluation forms for each popcorn sample given (Figure 1). Evaluation forms also included two questions concerning taste and texture asking participants to designate one or two descriptors out of originative word banks for both sensory traits. Nutty, pungent, rancid, sweet, umami, and bland were chosen as descriptors for taste, while airy, adhesive, crispy, crunchy, doughy, and tender were selected to describe texture (Figure 1). Definitions for taste and texture terms were available on the evaluation sheets. Participants were also given the opportunity to write general comments at the end of the evaluation sheet. Overall, 112 participants individually ranked six popcorn cultivars and 84 evaluations for each of the six QPP cultivars and two ConAgra Brands® commercial cultivars were recorded.

2.4 Experimental Design

Two ConAgra® Brands and six QPP cultivars were randomly assigned to 112 participants in a Balanced Incomplete Block Design (BIBD). Twenty-eight subgroups of treatment combinations were randomized and treatments were randomized by block position and block labeling (i.e. Variety '1-6' and Blocks 'A-F', respectively) using R® Software.

2.5 Statistical Analysis

All sensory trait evaluations were analyzed using R® Software and the Balanced Incomplete Block Design model as shown in Equation 1:

$$y_{ij} = \mu + \tau_i + \beta_j + \epsilon_{ij} \quad (1)$$

Where y_{ij} is the y^{th} evaluation, μ is the overall mean, τ_i is the effect of the 'i^{th}' treatment, β_j is the block effect, and ϵ_{ij} is experimental error. In the BIBD, eight popcorn

treatments (*t*) in 112 blocks (*b)* of six elements (*k*) each were replicated (*r*) 84 times, and treatment pairs in the same block (λ) were tested 60 times. All analysis was performed in R® Studio using packages 'crossdes', 'ibd', 'GGally', 'ggplot2', 'cowplot', 'dplyr', 'readxl', 'xlsx', 'doBy', 'car', 'lsmeans', 'lme4', 'gridExtra', 'forcats', and 'RColorBrewer', with references listed respectively (Sailer, 2013; Mandal, 2019; Schloerke et al., 2020; Wickham, 2016; Wilke, 2019; Wickham et al., 2020; Wickham and Bryan, 2019; Dragulescu and Arendt, 2020; Højsgaard and Halekoh, 2020; Fox and Weisberg, 2019; Lenth, 2016; Bates et al., 2015; Augie, 2017; Wickham, 2020; Neuwirth, 2014; R Core Team, 2020).

3. Results

3.1 Overall likability ranking suggests top QPP hybrids

Commercial Line 1 (CL1) scored the highest Overall Likability (OL) mark with a mean rank of 6.75±2.34 (Figure 1). QPP Hybrids 4 (H4) and 8 (H8) ranked second and third highest with average values of 6.46±2.11 and 6.32±2.11, respectively. Analysis of variance indicated a significant effect due to the hybrid variable, and Tukey's Honest Significance Difference (HSD) test only identified CL1 higher than H3 and H6 at the 0.05 level of significance. All other OL comparisons were insignificantly different. Individualizing rank and variety, H4 was ranked most frequently as '10', followed by CL1, H6 and H8 (Figure 2). Combining OL ranks 7-10, CL1 was ranked within a range of 7-10 the most times followed by H4, at 50 and 41 marks, respectively. H1 and H6 noticeably ranked in the lower OL range; both holding the most '2' and '3' rankings. CL2 maintained a mediocre ranking throughout the '5-7' range (Figure 2). H3 held the highest '5-6' ranking, though numbers dropped significantly above '7'. Like CL2, H8

did not have a standout OL ranking though it had the third highest average. H1 and H9 had the lowest rankings at an average of 6.07 and 6.10 respectively, though H1 was more strongly disliked by certain participants than H9, since H9 received only 7 counts under a rank of '4' in comparison to 14 counts for H1. Overall, OL averages and comparative individualized rankings identified H1 and H3 as less desirable popcorn varieties compared to CL1, and QPP hybrids H4 and H8 as insignificantly different to CL1 and frequently ranked within in the 7-10 range (Figure 2).

3.2 High correlations found between 'overall likability' and taste and texture ranking

Spearman's correlation coefficients were calculated between numerically ranked variables and all ten correlations were significant (Figure 3). OL and Taste held the highest correlation coefficient at 0.777, followed by OL and Texture at 0.656. Taste and Texture were moderately correlated (0.562), as well as Smell and OL (0.51). Appearance held a weak association to OL (0.397), Texture (0.42), Taste (0.359), and Smell (0.391). These correlations displayed the high influence quality traits Taste and Texture imposed on participant decision for OL ranking, followed by Smell and lastly the weakly influential trait, Appearance (Figure 3).

3.3 Conventional popcorn appearance ranked highest compared to QPP hybrids

Analysis of Variance on appearance ranking held the treatment effect (variety) as significant. Tukey's HSD revealed conventional popcorn variety CL1 held a significantly higher rank compared to all QPP hybrids, and conventional popcorn variety CL2 was significantly higher than H3. Multiple comments positively related CL1 and CL2 yellow flakes with a buttery appearance despite the lack of additives and

complimented the relatively larger popped flakes compared to the QPP hybrids (Figure 5). H4 was the highest ranking QPP in appearance, averaging a 5.04 rank compared to CL1's 5.40 mean rating (out of 6) (Figure 4). H9 held the lowest average score of 4.83 with a standard deviation of 1.11. Comparing the OL preference to appearance ranking, CL1 maintained the highest ranking in both categories, while CL2 dropped to fourth preference in OL compared to second in appearance (Figure 2 and Figure 4). H4 maintained the third highest appearance ranking, similar to its overall secondary OL ranking. H8 noticeably had a very low appearance ranking, sixth out of the eight varieties, compared to its third preference in OL. H6, the least preferred overall to participants, was the fourth most appealing popcorn in appearance. Compared to all other traits, appearance held the highest average across popcorn cultivars (5.00 out of 6) and held a very small range of 0.57. Especially given the small range of values available from appearance scores, it was of no surprise that the orders of preference were dissimilar between appearance and OL rankings and that the relationship between these two traits held the lowest correlation coefficient. These results suggested that other sensory traits exerted greater influences on a participant's overall likability of the popcorn.

3.4 Ranking of popcorn aroma suggests desirability of minimal scent and aversion to a 'burnt' aroma

Like appearance, participants were asked to rank each popcorn's aroma using a desirability scale of 1-6. Analysis of variance identified the variety effect as significant, with Tukey's HSD comparisons between CL1 and H3, H4, and H9, and CL2 and H9, as significant. H9 had a considerably lower aroma rank compared with all other popcorn cultivars, holding an average rank of 3.88 out of 6 (Figure 6). Overall, the average aroma

ranking was 4.28 with a range of 3.88-4.58, which is lower and more broad compared to appearance ratings. Like appearance, CL1 and CL2 ranked first and second above all QPP hybrids and H9 ranked last. However, almost opposite to appearance ratings, H8 was third highest and H4 was second to last in aroma ratings (Figure 6). No specific comments were mentioned concerning H8's aroma, however participants noted H4 having little to no aroma. H4's considerably lower aroma ranking suggests that participants desire a popcorn-like smell. However, multiple comments concerning CL1 and CL2 aroma also described no/minimal aroma detected. H9, the lowest ranked, had some comments describing a burnt/smoky taste and smell. Overall, comparative aroma rankings were similar to appearance for commercial lines CL1 and CL2, both ranking highest compared to QPP, but within QPP lines, the order was substantially different between aroma, appearance, and overall likability.

3.5 Taste rank and associated descriptors suggest 'Nutty' and 'Sweet' as consumer preferences

Along with indicating a numeric rank of taste on a 1-6 scale, participants were asked to circle 1-2 descriptors of taste from a word bank of six terms. Numeric ranking of taste was significant at the treatment effect when the analysis of variance was tested, but Tukey's HSD only identified one comparison, H9 with CL1, as significant. Taste averages ranged from 3.65-4.33, slightly lower than aroma rankings with approximately the same range. Like appearance and smell, CL1 ranked highest at 4.33 out of 6. H4 ranked second in taste, akin to OL rank, followed by CL2 and H3. H1 and H9 were the lowest ranking hybrids. Comparing OL scores with taste descriptors revealed multiple relationships. The 'Bland' descriptor was most often used, followed by 'Nutty' and

‘Sweet’ (Figure 7A). As OL scores increased from 1 to 5, the number of ‘Bland’ counts increased to its peak and decreased to its lowest value at an OL score of 10 (Figure 7A). The ‘Nutty’ descriptor was nonexistent in varieties scored ‘1’, and it slowly climbed with increasing OL until it overtook ‘Bland’ at OL rank ‘7’ and continued to be the most abundant descriptor for all high OL rankings (Figure 7A). More subtly, the ‘sweet’ descriptor was not used for any popcorn cultivar ranked under an OL of 4, and its count slowly increased until rank ‘7’, after which the counts decreased at a slow rate. The descriptor ‘Rancid’ was used for a few cultivars ranging in OL ranks from 1-6, but was rarely used for hybrids ranked with an OL higher than 7. ‘Umami’ and ‘Pungent’ descriptors followed this trend to a lesser extent and were utilized by a few participants to describe cultivars with an OL of 10. Overall, ‘Nutty’ and ‘Sweet’ descriptors displayed trends suggesting they were the most appealing taste terms, ‘Bland’ was average and acceptable, and ‘Rancid’, ‘Umami’ (a savory, meaty flavor), and ‘Pungent’ were least appealing (Figure 7A).

Counts of descriptors specific to cultivar revealed a high proportion of the ‘Sweet’ term utilized to describe CL1, followed more distantly by H4, H8 and H9 (Figure 7B). Notably, both commercial lines and H9 were very low in counts for the ‘Nutty’ taste followed by H4. Hybrids H1, H3, H6, and H8 were particularly high for ‘Nutty’, however H1 also had higher rankings for ‘Pungent’, and ‘Rancid’ which may explain its overall low numeric taste ranking. H3 had the highest count for the selection of ‘Rancid’ and was also high in the ‘Umami’ flavor. However, H3 also had a very high ‘Nutty’ ranking which likely promoted its overall rank in taste to fourth. CL2 and H9 were remarkably higher than the other hybrids with the ‘Bland’ classification, although H9

also gained a relatively higher number of ‘Pungent’ and ‘Sweet’ marks (Figure 7B). Since H9 was the lowest ranking cultivar in the numeric ranking, it is plausible that its taste was vaguely unpleasant. Some participants negatively associated H9’s flavor with a nutty, smoky, burnt, and meat-like taste, though a few participants indicated they enjoyed H9’s specifically nutty flavor. Though ‘Nutty’ had the clearest trend as a positive indicator of participant taste preference, the highest numeric rankings for taste in CL1 and H4 was driven by other factors since they ranked relatively lower in that category. H4 held no noticeably high descriptors, though it ranked highest in ‘Pungent’, lowest in ‘Rancid’ and ‘Umami’, and moderately higher in ‘Sweet’. CL1’s high ‘Sweet’ rating likely explained its enjoyability. Overall, CL1, H4, CL2, and H3 were the top numerically ranked cultivars according to taste and were each described differently, as ‘Sweet’, ‘Pungent’, ‘Bland’, and ‘Nutty’, respectively (Figure 7A-B).

3.6 Hybrids with high texture ranking primarily associated with four texture descriptors

No significant differences were found between hybrids for the numeric texture ranking, and the values were in a narrow range from 4.27-4.62. CL1 held the highest average ranking while CL2 held the lowest. Similar to the taste rankings, H4 had the second highest texture ranking and H6 and H9 held lower ranks. Participants were also asked to circle 1-2 descriptors of texture from a word bank of six terms, and descriptors ‘Airy’, ‘Crispy’, and ‘Adhesive’ were most commonly utilized. Comparing descriptor trends with OL rankings, ‘Adhesive’ rankings trended similarly to the ‘Bland’ taste rankings by increasing until an OL of 5 and then decreasing to a minimal number by an OL of 10 (Figure 8A). The ‘Airy’ descriptor was substantially the highest descriptor at an OL of 7,

though it subtly dropped to similar counts with ‘Crispy’ and ‘Crunchy’ by an OL ranking of 10. Both ‘Doughy’ and ‘Tender’ descriptors generally increased from an OL ranking of 1 to 8, however both descriptors were negligibly used for any popcorn rated 9 or above. Taken together, the ‘Airy’ descriptor seemed most utilized for popcorn cultivars with above average and superior texture, while ‘Crispy’ and ‘Crunchy’ descriptors were more specifically utilized for cultivars with highest OL. ‘Adhesive’ was a slightly negative descriptor, and ‘Doughy’, and Tender’ supported a slight trend toward above average hybrids but decreased in use as the OL rating increased (Figure 8A). Comparing frequency of descriptor use per cultivar with texture numeric ranking, CL2, H3, and H8 had the highest counts of ‘Adhesive’ texture, likely demoting CL2’s texture ranking (Figure 8B). However, H8 had a substantially high number of ‘Crunchy’ descriptor marks, the probably causing its third highest texture ranking. CL1 held the highest number of ‘Airy’ descriptions, while H4 held the highest number of ‘Crispy’ (Figure 8B). Taken together, these descriptions clearly depict the overall texture ranking of CL1, H4, and H8 as superior with ‘Airy’, ‘Crispy’, and ‘Crunchy’ textures respectively, and CL2 was deemed inferior due to its relatively higher counts of ‘Adhesive’ texture.

3.7 Cumulative evaluation of sensory data and comparison of conventional and QPP popcorn hybrids

Overall, CL1 had the highest rankings in appearance, aroma, taste, texture, and overall likability. The cumulative superiority of CL1 suggests it as the top popcorn variety chosen by participants though CL1’s OL was only significantly higher than OL values for H3 and H6. Hybrid 4 ranked directly below CL1 in taste, texture, and OL, giving

credence to the significant, moderately high correlations identified between these two traits and overall likability. CL2 ranked second in appearance and smell; however, a severely low texture score and lesser taste rank pushed its OL ranking to fourth behind H8. H8 appearance and taste were both lower than average, but its smell and texture appealed participants enough for it to earn the third highest OL ranking. Hybrids H1, H3, and H6 ranked relatively lower in all categories. Despite H3's fourth ranking in texture, taste, and smell, it dropped to sixth in OL. H6 ranked last in OL despite fourth, fifth, and sixth (twice) rankings in appearance, smell, taste, and texture, respectively. Interestingly, H9 ranked fifth, above H3, H1, and H6, in overall likability despite having the lowest rank for appearance, smell, and taste, and second lowest rank in texture.

Without informing participants of Quality Protein Popcorn's nutritional improvement of increased lysine and tryptophan in the popped flake, all QPP hybrids ranked insignificantly different than CL2 in OL and H1, H4, H8, and H9 were insignificantly different than CL1 in Overall Likability. H4 ranked higher than CL2 in taste, while all QPP hybrids ranked higher than CL2 in texture rankings.

4. Discussion

4.1 Intentional withholding of QPP nutritional characteristics

This taste-test was employed to identify consumer likability and preference of six Quality Protein Popcorn hybrids compared to two currently marketed popcorn varieties supplied by ConAgra Brands®. The breeding and selection of QPP inbred and hybrid lines commenced at the University of Nebraska-Lincoln in 2013, and six optimal QPP hybrids were selected in the fall of 2020. To separate a potential confounding factor of prior familiarity and identification of QPP compared to commercialized varieties, the taste test

was held at Colby Community College in Colby, Kansas and participants were asked to rate six popcorn varieties based solely on sensory factors without prior knowledge of QPP's higher levels of essential amino acids lysine and tryptophan in the popped flake compared to commercialized varieties (Parsons et al., 2020). This specific increase in these two deficient amino acids in maize allows QPP to be considered a complete protein source. Moreover, due to the novel inclusion of these amino acids to popcorn and the proven health benefits of increased lysine and tryptophan intake, QPM popcorn may also be considered a 'Functional Food', or a food with an inclusion of certain substances with proven health benefits (Murphey et al., 2006; Ghosh et al., 2008; 2010; Ritze et al., 2013; Tahergorabi et al., 2015; Payne et al., 2018). This innovative product can also be considered within the 'superfood' category, or a relatively more nutrient dense and healthy product (Curll et al., 2016; MacGregor et al., 2018; Meyerding et al., 2018). Furthermore, unlike dent maize and due to consumer preferences, breeders have refrained from genetically modifying popcorn germplasm, and QPP was conventionally bred through crossing and genetic selection (Fernandez-Cornejo et al., 2014; Jones et al., 2015; Ren et al., 2018, Barnes, 2019). Thus, QPP and marketed popcorn remains under the 'non-GMO' label (Parsons et al., 2020). QPP, like conventional popcorn, is ideal for production under organic conditions.

Collectively, the above specialty food niches have experienced individual increases in consumer demand over the past decade. Previous studies have shown that consumers have a growing, positive attitude towards 'superfoods' and 'Functional Foods', are increasing in awareness and willingness to adjust their eating habits toward a more health-oriented diet. Furthermore, they are willing to pay more for both 'Functional

Foods' and organically grown products (Niva and Mäkelä, 2007; Siró et al., 2008; Traill et al., 2008; Chen, 2011; Falguera et al., 2012; Annunziata and Vecchio, 2013; Weitkamp & Eidsvaag, 2014; Curll et al., 2016; MacGregor et al., 2018; Meyerding et al., 2018; Graeff-Hönninger & Khajehei, 2019; Kuesten and Hu, 2020). Unsurprisingly, sales in these categories has significantly increased over the past decade, with the U.S. having the largest consumer base and interest in the world for these products, outpacing the growth of the overall U.S. food and beverage market (Siró et al, 2008; Kapsak et al., 2011; Hartmann, 2020).

Though interest and sales in specialty products have clearly increased, studies have shown that consumer acceptance and consumption of them are not unconditional. Findings have observed consumers are more receptive toward innovations in traditional food products that strengthen the product's original, raw, or traditional character (i.e. a guarantee of raw material, 'all-natural', or 'non-GMO'), or reduce a traditionally negative side-effect with the product (i.e. a reduction of fat, calorie, or salt content) (Vanhonacker et al., 2013). Moreover, studies have also found that consumers are not willing to pay for these labels without certain satisfactory food characteristics, the most important of which is taste (Annunziata and Vecchio, 2013). One of the fastest growing niches in the specialty food sector is 'increased protein content' in products by supplementation or alternative sources (Banovic et al., 2018). To test consumer interest, acceptability, and appeal of added protein supplementation in protein beverages, Oltman et al. studied consumer reaction toward certain label claims, protein types, amount of protein and carbohydrates added, and sweeteners. They found that despite advertising larger protein supplementation, consumers preferred a lesser protein, more appealing tasting beverage

(Oltman et al., 2015). Similarly, a study in 2020 testing consumer acceptability of seaweed incorporated into wheat bread found very low acceptability of this blend due to its dry, dense, and seaweed-tasting flavor (Lamont and McSweeney, 2020).

While current markets for novel, plant-based protein sources are widening and becoming more acceptable and desirous for US consumers, sensory appeal, brand, price, convenience, and trustworthiness of health claims have all shown to be important factors that influence ultimate purchase and consumption of these new products (Siró et al., 2008; Kuesten and Hu, 2020). Therefore, to better gauge consumer opinion solely on QPP's sensory appeal, QPP was tested against two currently marketed varieties without participant knowledge of higher quality protein content or potential health benefits. Given results from previous consumer acceptance studies and this taste-testing, it is reasonable to conclude that certain QPP hybrids are not significantly different in sensory appeal than current popcorn varieties. Given prior knowledge of QPP's superior nutritional profile relative to commercialized lines, participants may have had an even higher inclination toward QPP than conventional varieties if they were informed of QPP's protein quality using creative marketing tools.

4.2 Sensory effects of dent maize introgression into QPP

The main purpose of dent maize introgression into popcorn germplasm for this breeding program was to achieve higher levels of protein-bound and free lysine and tryptophan in the kernel and popped flake of QPP cultivars (Ren et al., 2018; Parsons et al., 2020). Quality Protein Maize dent germplasm carrying the *opaque-2* allele was utilized in the initial crossing and the *opaque-2* allele was selected throughout breeding using marker-assisted selection. Unknown dent modifier genes were also selected phenotypically to

restore maize endosperm vitreousness, an absolute requirement for popcorn popping. Due to this introgression and selection of a suite of dent loci during the production of QPP, it is of no surprise that certain popcorn-like characteristics appeared inferior in QPP compared to ConAgra® controls (Ren et al, 2018; Parsons et al., 2019). The most obvious difference between QPP and controls was appearance. CL1 held the only significant advantage in the 'Appearance' trait compared to all QPP popcorn cultivars likely due to larger expansion volume of the popped kernel and the appearance of yellow flakes (Figure 5). Previous studies have shown that yellow popcorn is more desirable than white popcorn in both color and aroma (Eldredge and Thomas, 1959; Park and Maga, 2000). However, studies have also suggested that dent introgression into popcorn enhances more influential sensory traits such as taste and texture despite lowering certain popcorn traits such as popability and expansion volume (Crumbaker et al., 1949; Johnson and Eldredge, 1953; Robbins and Ashman, 1984; Parsons et al., 2020). All of these findings agreed well with the current results ranking both CL1 and CL2 higher than white QPP in both appearance and smell, certain QPP ranking higher than CL2 in taste, all QPP ranking higher than CL2 in texture, and two QPP – H4 and H8 – ranking higher than CL2 in Overall Likability (OL).

The increase in lysine accumulation in QPP popped flakes adds an additional aspect to possibly enhancing the flavor profile. The Maillard Reaction is a well-known reaction that induces browning, enhances flavor and aroma, and can produce antioxidative compounds in heated foods (van Boekel et al., 2006). This reaction is nonenzymatic, initiates between a carbonyl compound and an amine under heated conditions, and ends with a diverse array of products dependent on starting materials and conditions (Nursten

et al., 2005; Parker, 2016). The innate process of popping popcorn, whether by oil, air, or microwave, inevitably offers adequate conditions for the Maillard Reaction to occur (Byrd and Perona, 2005; Bocharova et al., 2017). Studies have shown that lysine and arginine are the most effective amines in initiating the Maillard Reaction, and lysine, tryptophan, and histidine are most effective in pushing the reaction forward with xylose to produce antioxidants (Parker, 2016). In fact, a 2013 study specifically biofortified biscuits with lysine and found that the glucose-lysine reaction produced high amounts of antioxidants through the Maillard reaction (Virág et al., 2013). Along with enhancing the rate of the Maillard reaction and pushing the reaction forward toward antioxidant products, lysine has also been found to produce high amounts of flavor compounds pyrazines and pyrroles (Hwang et al, 1994; 1995a; 1995b). One recent study analyzing low-molecular weight pigments produced by the Maillard reaction identified an upsurge of a certain cysteine-glucose initiated compound after the addition of lysine. Further characterization identified this compound as pyrrolothiazolate, an antioxidative pigment found in soy sauce and miso (Noda et al., 2015; 2016; Murata, 2020). Previous protein-bound amino acid analysis of QPP hybrids identified H1 as holding high abundance of lysine, and therefore it is interesting that taste-testing participants generally commented about the hybrid's burnt, odd taste. In fact, one participant specifically wrote that the hybrid tasted like 'miso soup'. Other studies have found that the addition of lysine to certain processed or cured meats, such as sausage, salted meat, and Jinhua ham, enhanced the unique flavor profiles of the respective products (dos Santos Alves et al, 2017; Zhu et al., 2017; Vidal et al., 2020). Overall, increases in lysine and tryptophan due to the introgression of the *opaque-2* allele in QPP likely played a role in producing unique

flavor profiles through the Maillard reaction. Reviewing commonly used taste descriptors, all QPP except H9 held more 'Nutty' descriptors than the commercialized lines. Interestingly, both CL1 and CL2 held higher marks in 'Umami', a commonly savory and meat-associated taste that was associated more with lower OL scores, in all QPP except H3 and H6. However, all QPP except H8 held higher 'Pungent' marks, and all QPP but H4 and H9 had more 'Rancid' counts than the control varieties. The higher 'Umami' marks for the control lines may be due to the fact that the more sharp descriptors 'Pungent' and 'Rancid' were utilized instead for H1, H4, H8, and H9 to describe QPP's more potent taste.

4.3 Conclusions: QPP potential for commercialization based on sensory evaluation

Overall, this analysis identified main sensatory contrasts and themes between QPP and conventional commercial cultivars. Broadly, QPP had more distinct flavor profiles and adequate to superior texture. Participants indicated that 'Nutty' and 'Sweet' descriptors were most positive for taste, and 'Crunchy', 'Airy', and 'Crispy' were all positive descriptors for texture. Out of the two commercialized lines, CL1 performed superior to CL2 in all rankings, almost all QPP performed insignificantly different to CL2 in all categories, and CL1 only performed significantly better than H3 and H6 in Overall Likability. Participants indicated that the appearance of CL1 and CL2 was superior to QPP varieties, though this trait was least influential to participant's OL decision. Only one significant difference was identified in taste, the highest correlated trait to OL, between H9 and CL1.

Due to its unique germplasm and proteome, QPP can be considered a 'Functional', all-natural, higher quality protein, non-GMO superfood that will very likely be organically

grown. Commercialized popcorn already fits well into the niches of the growing health-aware U.S. market and popcorn products, specifically within the Ready-To-Eat (RTE) sector, have already experienced increases in sales over the past decade (Dawande, 2018, Mordor Intelligence, 2018). Due to QPP's suitability for the RTE market in which packets are inflated with nitrogen gas to prevent damage and preserve the product, its nutritional, texture and taste improvements may outweigh the slight reduction in expansion volume compared to commercialized lines sold by weight to retailers (i.e. movie theaters) and by volume to consumers. With participants blind to QPP's higher lysine and tryptophan content and the potential health benefits associated with quality protein, the stand-alone, satisfactory and in some cases improved sensory results of this study indicate that Quality Protein Popcorn varieties, especially H4 and H8, have a promising future in commercialization and marketability.

Evaluation Form: **VARIETY X**

Preface: Popcorn samples include <u>no additives</u> (salt, butter, oil, etc.) and have been air-popped.
Please *relatively* rank all samples.

Please circle the appropriate ranking	☹			😐		☺
<u>Appearance</u>: How appealing does this popcorn look?	**1**	**2**	**3**	**4**	**5**	**6**
<u>Smell</u>: How appealing is this popcorn's aroma?	**1**	**2**	**3**	**4**	**5**	**6**
<u>Taste</u>: How appealing is this popcorn's taste?	**1**	**2**	**3**	**4**	**5**	**6**
<u>Taste:</u> What are the best descriptors of this popcorn's taste? **Please circle 1-2 descriptors* or add in specific comments below!**	**Nutty Pungent Rancid Sweet Umami Bland**					
<u>Texture</u>: How appealing is this popcorn's texture?	**1**	**2**	**3**	**4**	**5**	**6**
<u>Texture:</u> What are the best descriptors of this popcorn's texture? **Please circle 1-2 descriptors* or add in specific comments below!**	**Airy Adhesive Crispy Crunchy Doughy Tender**					
<u>Overall Likability:</u> How enjoyable is this popcorn, overall?	**1**	**2**	**3**	**4**	**5**	**6** **7** **8** **9** **10**
<u>General Comments (optional)</u>						

*Definitions for terminology *(reminder - taste and texture are solely derived from the essential product without additives)*:

Airy: a light pillow-like texture
Adhesive: description for foods that stick to tongue, teeth, or upper-palate, gum-like texture
Bland: of little taste or flavor
Crispy: a light texture with a slight crunch
Crunchy: a firm texture associated with the sharp, audible noise of being chewed
Doughy: a soft and heavy texture
Nutty: a flavor reminiscent of nuts
Pungent: a sharp and strong taste
Rancid: a stale and unpleasant aroma or flavor
Sweet: a pleasing taste that exhibits sugar characteristics
Tender: soft texture that is easy to break down
Umami: a savory flavor in meats and broths

Figure 1 | <u>Taste-Testing Evaluation Form.</u> Each participant was given six pages with the 'X' replaced with the number 1-6, respectively.

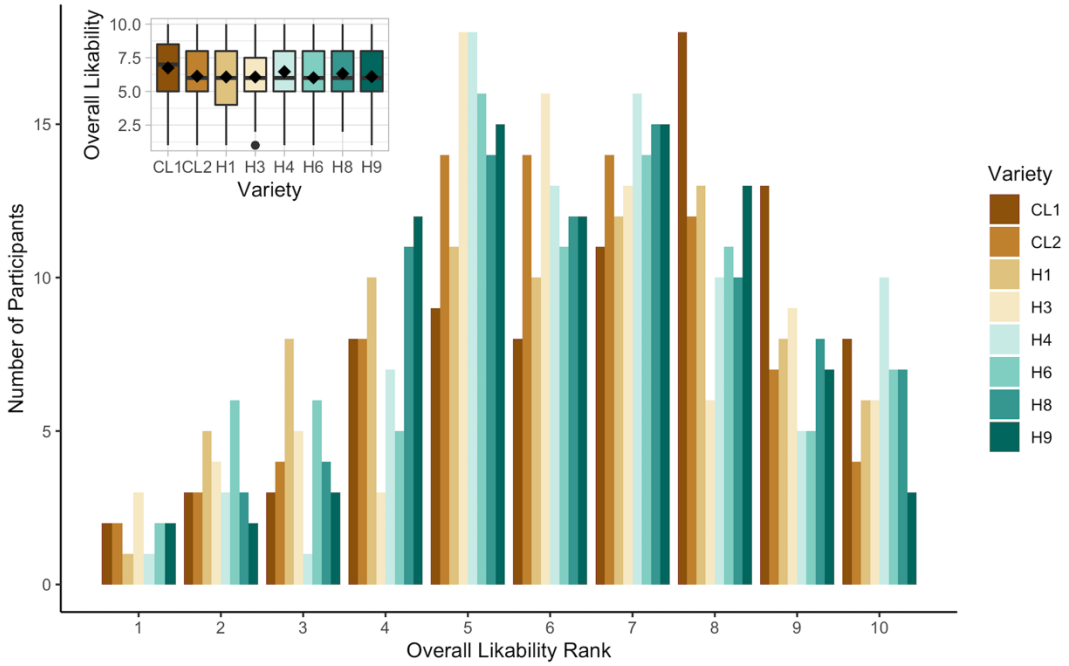


Figure 2 | Average and Individual Overall Likability scores per Variety. Participants offered an overall likability (OL) score for each popcorn variety sampled. OL scores 1-10 (1 as worst, 10 as best) were utilized for all varieties in different frequencies, except H8 did not receive an OL rank of '1'.
Top left inset graph: Range, median, and mean of OL scores for each variety. Black diamond within box-and-whiskers plots represents mean, horizontal line within box represents median, and vertical extent of black line represents range.

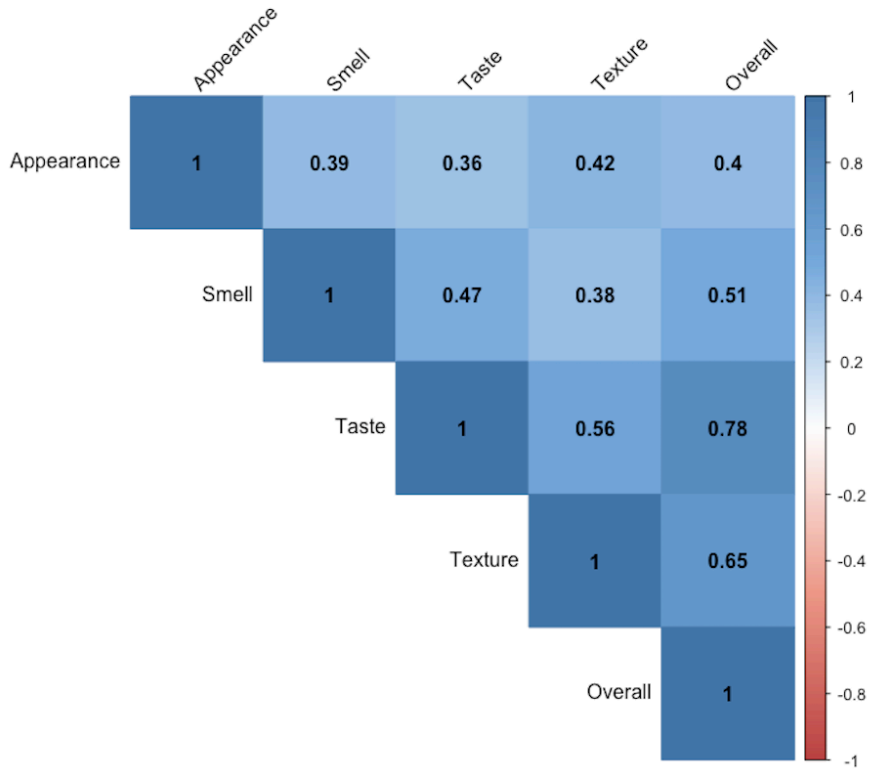


Figure 3 | Spearman's Correlations between Sensory Traits and Overall Likability. Numeric ranking values for Appearance (1-6), Smell (1-6), Taste (1-6), Texture (1-6), and Overall Likability (1-10) were tested for significant correlations. All ten correlations were found to be significant with $\alpha \leq 0.05$.

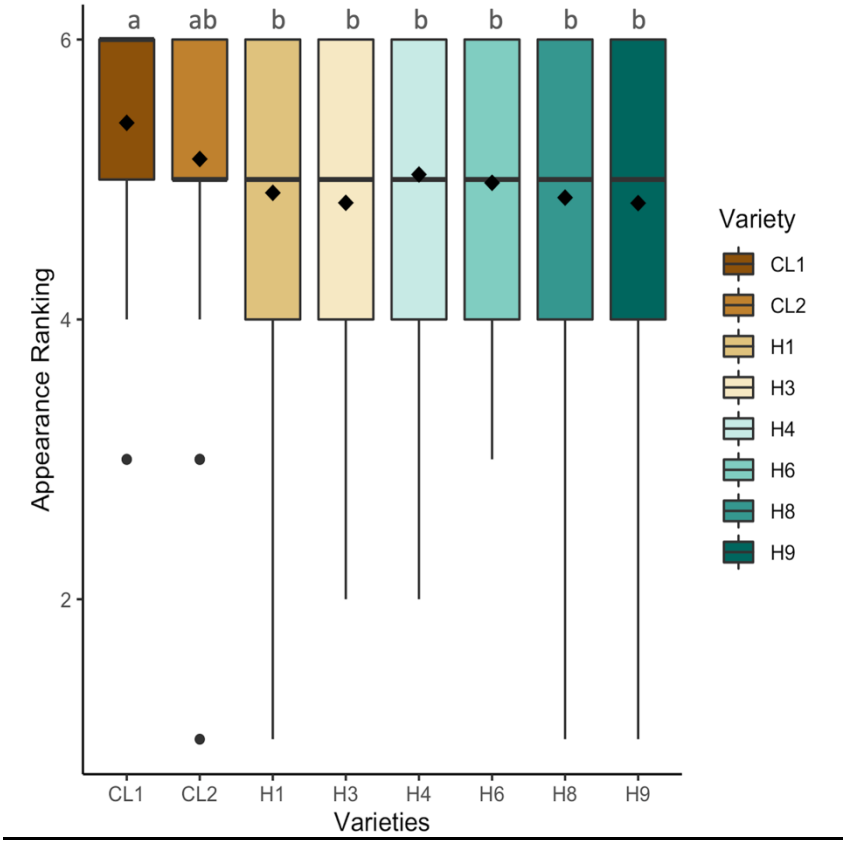


Figure 4 | Average Appearance Ranking of Individual Cultivars. Ranking was based on a scale of 1-6, with 1 being least ideal and 6 being most ideal. Significance differences between values are indicated above each column.

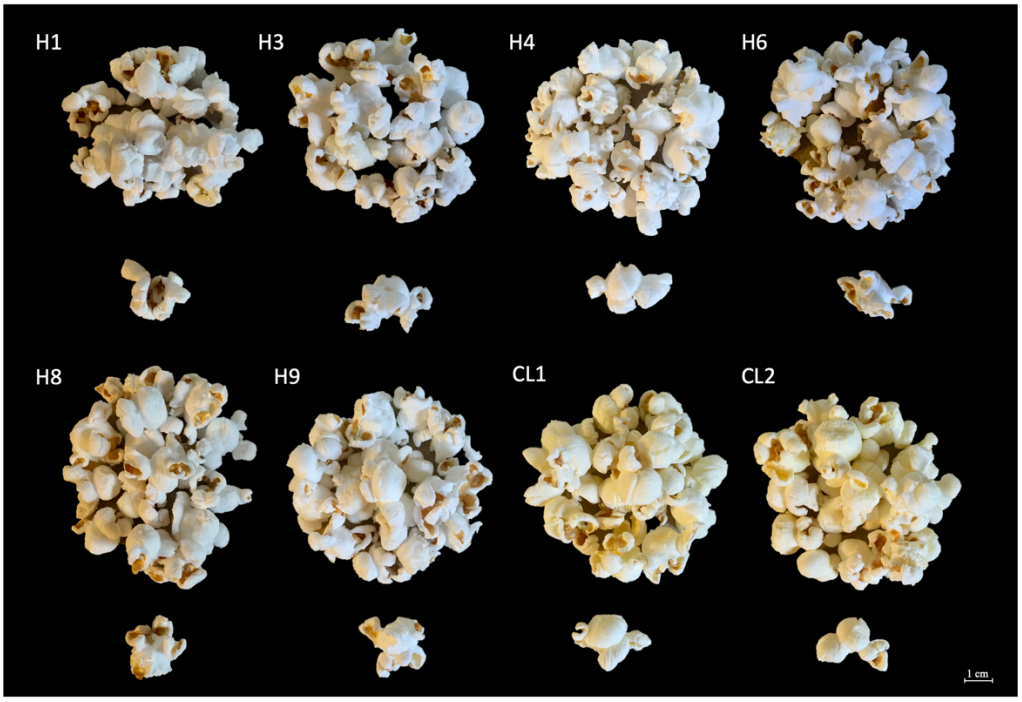


Figure 5. | Appearance of QPP and Commercial Varieties. Popped flakes held individual differences that participants identified. Specifically, few participants positively commented on the yellow appearance and larger flake size of the commercialized lines (CL1 and CL2).

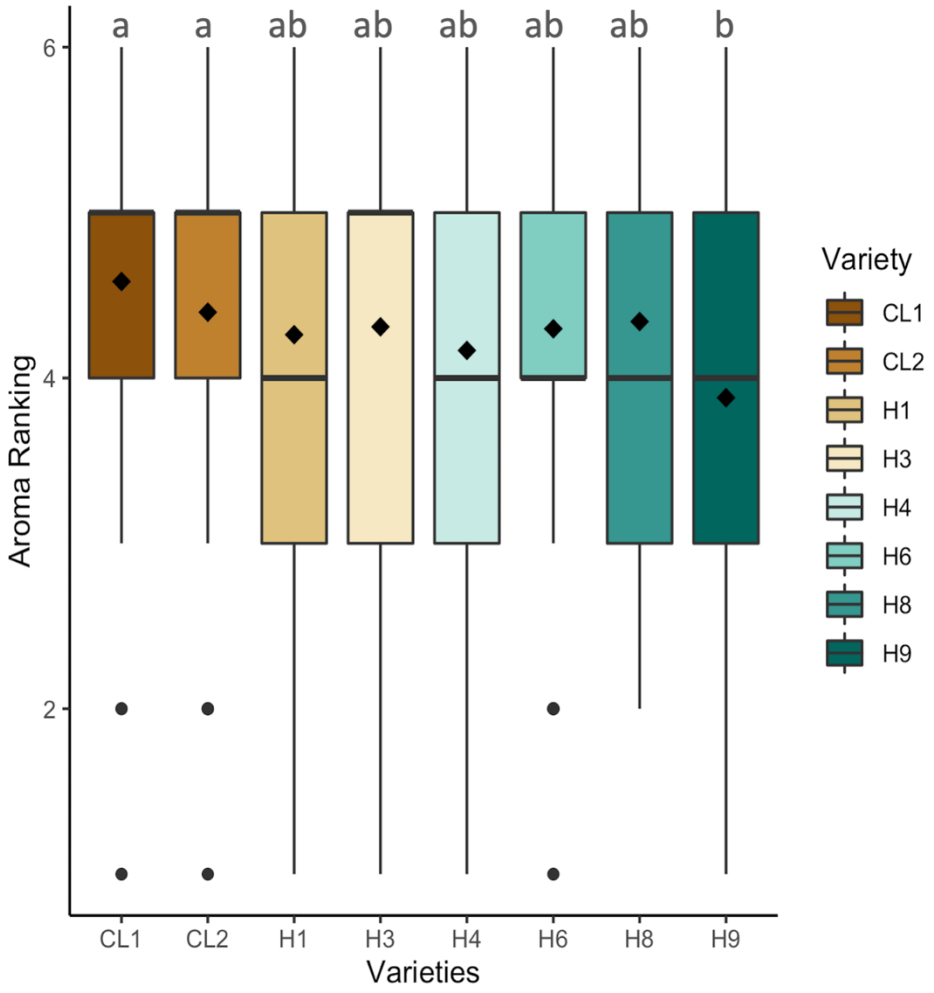


Figure 6 | Average Aroma Ranking of Individual Cultivars. Ranking was based on a scale of 1-6, with 1 being least ideal and 6 being most ideal. Significance differences between values are indicated above each column.

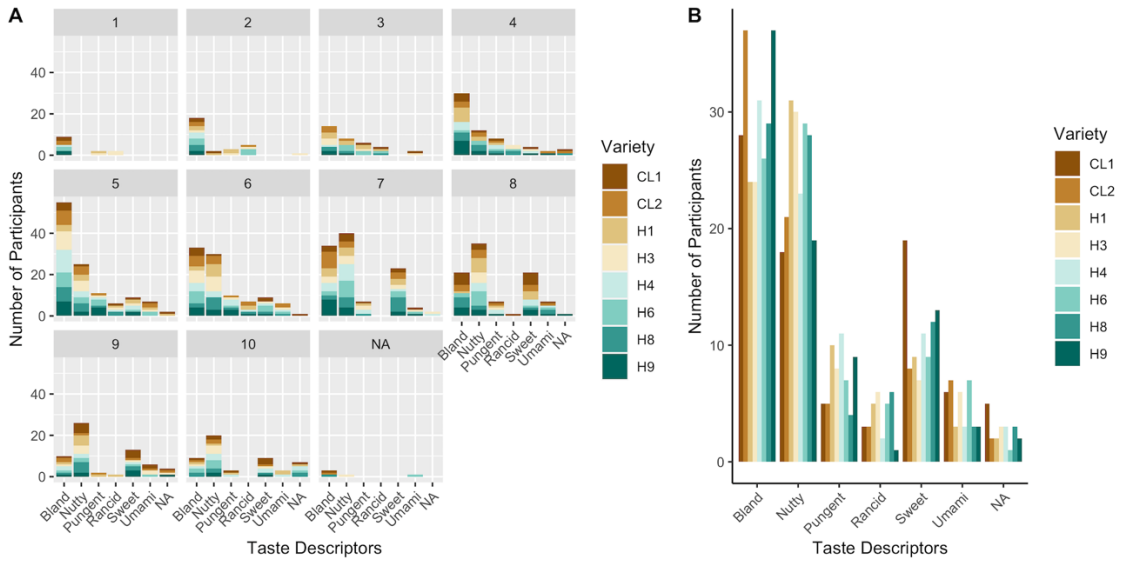


Figure 7 | Rank and Description of Taste. (A) Taste descriptors 'Bland', 'Nutty', 'Pungent', 'Rancid', 'Sweet', and 'Umami' were utilized in different frequencies when categorized by Overall Likability (OL) scores. OL scores are specified above each individual graph, starting at an OL score of '1' (least likable) in the top left corner. 'NA' represents taste descriptors identified by a participant unaccompanied by an OL score. (B) Frequency of taste descriptors associated with individual varieties across OL ranking. 'Bland' was most used, followed by 'Nutty' and 'Sweet'. NA represents participant lack of indicating taste descriptors.

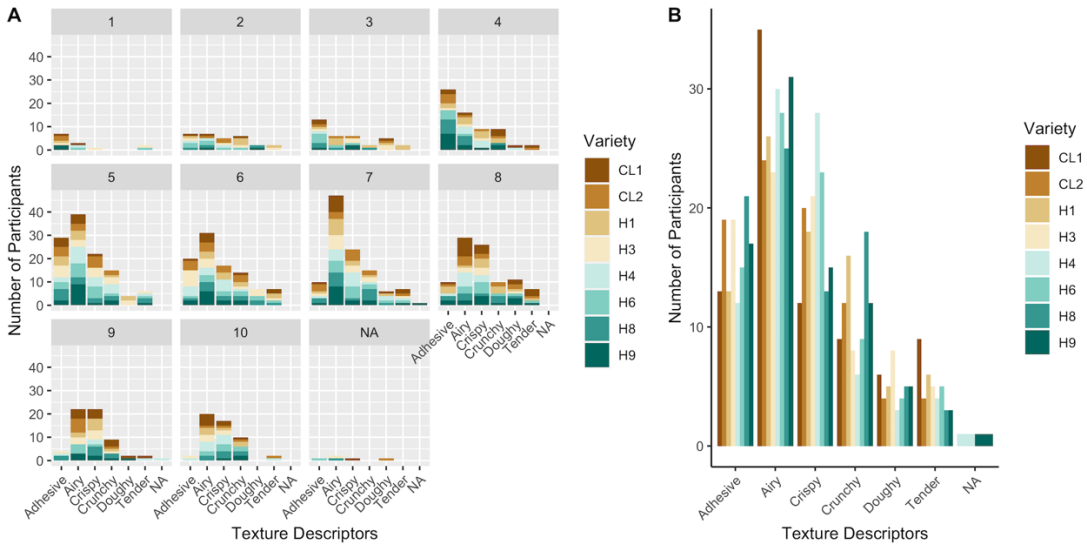


Figure 8. | Rank and Description of Texture. (A) Texture descriptors 'Adhesive', 'Airy', 'Crispy', 'Crunchy', 'Doughy', and 'Tender' were utilized in different frequencies for different cultivars when categorized by Overall Likability (OL) scores. OL scores are specified above each individual graph, starting at an OL score of '1' (least likable) in the top left corner. 'NA' represents texture descriptors identified by participants but unaccompanied by an OL score. (B) Frequency of texture descriptors associated with specific varieties. 'Airy' was most commonly used, followed by 'Crispy'. NA represents participant lack of indicating a description of texture.

References

Annunziata, A., & Vecchio, R. (2013). Consumer perception of functional foods: A conjoint analysis with probiotics. *Food Quality And Preference*, *28*(1), 348-355. https://doi.org/10.1016/j.foodqual.2012.10.009

Auguie, B. (2017). gridExtra: Miscellaneous Functions for "Grid" Graphics. R package version 2.3. https://CRAN.R-project.org/package=gridExtra

Babu, R., Nair S. K., Kumar A., Venkatesh S., Sekhar J. C., Singh N. N. (2005). Two-generation marker-aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). Theor. Appl. Genet. 111, 888–897. doi: 10.1007/s00122-005-0011-6

Banovic, M., Arvola, A., Pennanen, K., Duta, D., Brückner-Gühmann, M., Lähteenmäki, L., & Grunert, K. (2018). Foods with increased protein content: A qualitative study on European consumer preferences and perceptions. *Appetite*, *125*, 233-243. https://doi.org/10.1016/j.appet.2018.01.034

Barnes, E. (2019). *Challenges and Opportunities for Weed Control in Nebraska Popcorn* (Ph.D. Dissertation). University of Nebraska-Lincoln.

Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software, 67(1), 1-48. doi:10.18637/jss.v067.i01.

Bocharova, O., Reshta, S., & Bocharova, M. (2017). Investigation of the chemical safety of microwaved popcorn in respect of acrylamide formation. *International Food Research Journal*, *24*(5), 2274-2277. Retrieved 21 November 2020, from.

van Boekel, M. (2006). Formation of flavour compounds in the Maillard reaction. *Biotechnology Advances*, *24*(2), 230-233. https://doi.org/10.1016/j.biotechadv.2005.11.004

Brunson, A.M. (1937). “Popcorn Breeding,” in Specialty corns, ed. A.R. Hallauer (New York, NY: CRC Press), 189–223.

Byrd, J., & Perona, M. (2005). Kinetics of Popping of Popcorn. *Cereal Chemistry Journal*, *82*(1), 53-59. https://doi.org/10.1094/cc-82-0053

Chen, M. (2011). The mediating role of subjective health complaints on willingness to use selected functional foods. *Food Quality And Preference*, *22*(1), 110-118. https://doi.org/10.1016/j.foodqual.2010.08.006

Crumbaker, D. E., Johnson, I., and Eldredge, J. (1949). Inheritance of popping volume and associated characters in crosses between popcorn and dent corn. *Agron. J.* 41, 207–212. doi: 10.2134/agronj1949.00021962004100050009x

Curll, J., Parker, C., MacGregor, C., & Petersen, A. (2016). Unlocking the Energy of the Amazon? the Need for a Food Fraud Policy Approach to the Regulation of Anti-Ageing Health Claims on Superfood Labelling. *Federal Law Review*, *44*(3), 419-449. https://doi.org/10.1177/0067205x1604400304

Daros, M., Amaral Júnior., A.T., Pereira, M.G., (2002). Genetic gain for grain yield and popping expansion in full-sib recurrent selection in popcorn. Crop Breeding and Applied Biotechnology. 2, 339-344.

Dawande, R. (2018). Popcorn Market by Type (Microwave Popcorn and Ready-to-eat Popcorn) and End User (Household and Commercial) - Global Opportunity Analysis and Industry Forecast, 2017-2023. Portland: Allied Market Research.

Dawson, M., and Telford, E.P. (1912). The Book of Parties and Pastimes. Philadelphia, PA: David McKay.

Dhliwayo T. (2008) *Genetic Mapping and Analysis of Traits Related to Improvement of Popcorn. [*Ph.D. thesis]. [Ames, (IA)]: Iowa State University

Dofing, S. M., ĎCroz-Mason N., Thomas-Compton, M. A. (1991). Inheritance of Expansion Volume and Yield in Two Popcorn × Dent Corn Crosses. Crop Sci. 31,715-718. doi:10.2135/cropsci1991.0011183X003100030035x

Dragulescu, A. & Arendt, C. (2020). xlsx: Read, Write, Format Excel 2007 and Excel 97/2000/XP/2003. Files. R package version 0.6.5. https://CRAN.R-project.org/package=xlsx

Falguera, V., Aliguer, N., & Falguera, M. (2012). An integrated approach to current trends in food consumption: Moving toward functional and organic products?. *Food Control*, *26*(2), 274-281. https://doi.org/10.1016/j.foodcont.2012.01.051

Fernandez-Cornejo, J., Wechsler, S., Livingston, M., Mitchell, L. (2014) Genetically Engineered Crops in the United States, *ERR-162 U.S.* Department of Agriculture, Economic Research Service. https://www.fda.gov/food/ingredientspackaginglabeling/geplants/ucm461805.htm

Fox, J. & Weisberg, S. (2019). An {R} Companion to Applied Regression, Third Edition. Thousand Oaks CA: Sage. URL: https://socialsciences.mcmaster.ca/jfox/Books/Companion/

Ghosh, S., Pellett, P., Aw-Hassan, A., Mouneime, Y., Smriga, M., & Scrimshaw, N. (2008). Impact of Lysine-Fortified Wheat Flour on Morbidity and Immunologic Variables among Members of Rural Families in Northwest Syria. *Food And Nutrition Bulletin*, *29*(3), 163-171. https://doi.org/10.1177/156482650802900302

Ghosh, S., Smriga, M., Vuvor, F., Suri, D., Mohammed, H., Armah, S., & Scrimshaw, N. (2010). Effect of lysine supplementation on health and morbidity in subjects belonging to poor peri-urban households in Accra, Ghana. *The American Journal Of Clinical Nutrition*, *92*(4), 928-939. https://doi.org/10.3945/ajcn.2009.28834

Graeff-Hönninger, S., & Khajehei, F. (2019). The Demand for Superfoods: Consumers' Desire, Production Viability and Bio-intelligent Transition. *Food Tech Transitions*, 81-94. Retrieved 20 November 2020, from.

Guimarães, A G., Amaral Júnior, A.T., Almeida Filho, J.E., Pena, G.F., Vittorazzi, C., and Pereira, M.G. (2018). Population structure and impact of recurrent selection on popcorn using EST-SSR markers. *Acta Scientiarum. Agronomy*, *40*, e35218. https://doi.org/10.4025/actasciagron.v40i1.35218

Hartmann, P. (2020). *The Hype of Superfoods A Marketing Success Story to Deceive Consumers?* (Master's Thesis). Radboud Universitiet.

Højsgaard, S. & Halekoh U. (2020). doBy: Groupwise Statistics, LSmeans, Linear Contrasts, Utilities. R package version 4.6.5. https://CRAN.R-project.org/package=doBy

Hwang, H., Hartman, T., & Ho, C. (1995). Relative Reactivities of Amino Acids in Pyrazine Formation. *Journal Of Agricultural And Food Chemistry*, *43*(1), 179-184. https://doi.org/10.1021/jf00049a033

Hwang, H., Hartman, T., & Ho, C. (1995). Relative Reactivities of Amino Acids in the Formation of Pyridines, Pyrroles, and Oxazoles. *Journal Of Agricultural And Food Chemistry*, *43*(11), 2917-2921. https://doi.org/10.1021/jf00059a027

Hwang, H., Hartman, T., Rosen, R., Lech, J., & Ho, C. (1994). Formation of Pyrazines from the Maillard Reaction of Glucose and Lysine-.alpha.-amine-15N. *Journal Of Agricultural And Food Chemistry*, *42*(4), 1000-1004. https://doi.org/10.1021/jf00040a031

Iowa State University. (1996). *National Plant Breeding Study-I.*

Jones, Z., Goodman, M., & Krakowsky, M. (2015). Identification of resistance to the Ga1-m gametophyte factor in maize. *Euphytica*, *206*(3), 785-791. https://doi.org/10.1007/s10681-015-1518-9

Johnson, I., and Eldredge, J. (1953). Performance of recovered popcorn inbred lines derived from outcrossed to dent corn. *Agron. J.* 45, 105–110. doi: 10.2134/agronj1953.00021962004500030005x

Kapsak, W., Rahavi, E., Childs, N., & White, C. (2011). Functional Foods: Consumer Attitudes, Perceptions, and Behaviors in a Growing Market. *Journal Of The American Dietetic Association*, *111*(6), 804-810. https://doi.org/10.1016/j.jada.2011.04.003

Kuesten C., Hu C. (2020) Functional Foods and Protein Supplementation. *Meiselman H. (eds) Handbook of Eating and Drinking.* Springer, Cham. https://doi.org/10.1007/978-3-030-14504-0_175

Lamont, T., & McSweeney, M. (2020). Consumer acceptability and chemical composition of whole-wheat breads incorporated with brown seaweed (Ascophyllum nodosum) or red seaweed (Chondrus crispus). *Journal Of The Science Of Food And Agriculture*. https://doi.org/10.1002/jsfa.10765

Lenth, R.V. (2016). Least-Squares Means: The R Package lsmeans. Journal of Statistical Software, 69(1), 1-33. doi:10.18637/jss.v069.i01

Li, Y.L., Lu, F.Y., Du, Z.W., Wu, S.W., Han, C.P. (2002). Preliminary report on improvement result to popcorn using normal corn germplasm and backcross. Acta Agriculturae Boreali-Sinica. 17, 37–43.

Li, Y. L., Dong, Y.B., and Niu, S. Z. (2006). QTL analysis of popping fold and the consistency of QTLs under two environments in popcorn. Acta Genet. Sin. 33, 724–732. doi: 10.1016/S0379-4172(06)60105-4

Li, Y., Dong, Y., Niu, S., and Cui, D. (2007). QTL for popping characteristics in popcorn. Plant Breed. 126, 509–514. doi: 10.1111/j.1439-0523.2007.01 372.x

Li, Y. L., Dong Y., Niu, S., Cui, D., Wang, Y., Liu, Y., Wei, M., Li, X. (2008). Identification Of Agronomically Favorable Quantitative Trait Loci Alleles From A Dent Corn Inbred Dan232 Using Advanced Backcross QTL Analysis And Comparison With The F2:3 Population In Popcorn. Molecular Breeding. 21,1-14. doi:10.1007/s11032-007-9104-z

Li, Y. L., Dong, Y. B., Niu, S. Z., and Cui, D. Q. (2009). Identification of QTL for popping characteristics using a BC2F2 population and comparison with its F2:3 population in popcorn. Agric. Sci. China 8, 137–143. doi: 10.1016/S1671- 2927(09)60020- 1

Li, C., Qiao, Z., Qi, W., Wang, Q., Yuan, Y., Yang, X., et al. (2015). Genome-wide characterization of cis-acting DNA targets reveals the transcriptional regulatory framework of opaque2 in maize. Plant Cell 27, 532–545. doi: 10.1105/tpc.114. 134858

Liu, H., Shi, J., Sun, C., Gong, H., Fan, X., Qiu, F., et al. (2016). Gene duplication confers enhanced expression of 27-kDa γ-zein for endosperm modification in quality protein maize. Proc. Natl. Acad. Sci. U.S.A. 113, 4964–4969. doi:10.1073/pnas.1601352113

Lu, H. J., Bernardo, R., and Ohm, H. W. (2003). Mapping QTL for popping expansion volume in popcorn with simple sequence repeat markers. Theor. Appl. Genet. 106, 423–427. doi: 10.1007/s00122-002-1041-y

Lusas, R.W, and Rooney, L.W. (2001). Snack Foods Processing. New York, New York: CRC Press LLC.

MacGregor, C., Petersen, A., & Parker, C. (2018). Promoting a healthier, younger you: The media marketing of anti-ageing superfoods. *Journal Of Consumer Culture*, 146954051877382. https://doi.org/10.1177/1469540518773825

Mandal, B.N. (2019). ibd: Incomplete Block Designs. R package version 1.5. https://CRAN.R-project.org/package=ibd

Matz S.A. (1984) Snacks Based on Popcorn. In: Snack Food Technology. Springer, Dordrecht. https://doi.org/10.1007/978-94-010-9778-9_12

Mertz, E.T., Bates, L.S., Nelson, O.E. (1964). Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science. 145, 279-280. doi: 10.1126/science.145.3629.279

Meyerding, S., Kürzdörfer, A., & Gassler, B. (2018). Consumer Preferences for Superfood Ingredients—the Case of Bread in Germany. *Sustainability*, *10*(12), 4667. https://doi.org/10.3390/su10124667

Mordor Intelligence. (2018). POPCORN MARKET – GROWTH, TRENDS AND FORCAST (2020-2025). Hyderabad, India: Mordor Intelligence.

Murata, M. (2020). Browning and pigmentation in food through the Maillard reaction. *Glycoconjugate Journal*. https://doi.org/10.1007/s10719-020-09943-x

Murphy, S., Longhitano, C., Ayres, R., Cowen, P., & Harmer, C. (2006). Tryptophan supplementation induces a positive bias in the processing of emotional material in healthy female volunteers. *Psychopharmacology*, *187*(1), 121-130. https://doi.org/10.1007/s00213-006-0401-8

Nebraska Corn Board. (2019). Field Corn vs. Food Corn. Nebraska Corn Board. Available at: https://nebraskacorn.gov/issues-initiatives/your-food/field-corn-vs-food-corn/

Neuwirth, E. (2014). RColorBrewer: ColorBrewer Palettes. R package version 1.1-2. https://CRAN.R-project.org/package=RColorBrewer

Niva, M., & Mäkelä, J. (2005). Finns and functional foods: socio-demographics, health efforts, notions of technology and the acceptability of health-promoting foods. *International Journal Of Consumer Studies*, *31*(1). https://doi.org/10.1111/j.1470-6431.2005.00482.x

Noda, K., Yamada, S. & Murata, M (2015). Isolation, identification, and formation conditions of a novel Maillard yellowish pigment, pyrrolothiazolate. *Biosci. Biotechnol. Biochem.* 79, 1350-1355.

Noda, K., Terasawa, N. & Murata, M (2016). Formation scheme and antioxidant activity of a novel Maillard pigment, pyrrolothiazolate, formed from cysteine and glucose. *Food Funct.* 7, 2551-2556.

Nursten, H. (2005). *Maillard Reaction*. Royal Society of Chemistry.

Oltman, A., Lopetcharat, K., Bastian, E., & Drake, M. (2015). Identifying Key Attributes for Protein Beverages. *Journal Of Food Science*, *80*(6), S1383-S1390. https://doi.org/10.1111/1750-3841.12877

Paraginski, R.T., Souza, N.L., Alves, G.H., Ziegler, V., Oliveira, M., Elias, M.C. (2016). Sensory and nutritional evaluation of popcorn kernels with yellow, white and red pericarps expanded in different ways. Journ. of Cereal Sci. 383-391.

Parker, J. (2016). *Flavour development, analysis and perception in food and beverages* (pp. 151-185). WOODHEAD.

Parsons, L., Ren, Y., Yobi, A., Hurst, P., Angelovici, R., Rodriguez, O., & Holding, D. R. (2020). Production and Selection of Quality Protein Popcorn Hybrids Using a Novel Ranking System and Combining Ability Estimates. *Frontiers in Plant Sci*. 11, 698.

Paula, T., Gonçalves, L., Amaral Júnior, A., Oliveira, É., Silva, V., Scapim, C., & LopesII, A. (2010). Magnitude of the genetic base of commercial popcorn and in recommendation in Brazil. *Crop Breeding And Applied Biotechnology*, *10*(4). https://doi.org/https://doi.org/10.1590/S1984-70332010000400002

Payne, M., Stephens, T., Lim, K., Ball, R., Pencharz, P., & Elango, R. (2018). Lysine Requirements of Healthy Pregnant Women are Higher During Late Stages of Gestation Compared to Early Gestation. *The Journal Of Nutrition*, *148*(1), 94-99. https://doi.org/10.1093/jn/nxx034

Pereira, M.G., Amaral Júnior, A.T. (2001). Estimation of genetic components in popcorn based on the nested design. Crop Breeding and Applied Biotechnology. 1, 3-10.

R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Ritze, Y., Bárdos, G., Hubert, A., & Bischoff, S. (2013). PP053-SUN EFFECT OF TRYPTOPHAN SUPPLEMENTATION ON DIET-INDUCED NON-ALCOHOLIC

FATTY LIVER DISEASE IN MICE. *Clinical Nutrition*, *32*, S41. https://doi.org/10.1016/s0261-5614(13)60098-9

Ren, Y., Yobi, A., Marshall, L., Angelovici, R., Rodriguez, O., Holding, D. (2018). Generation and Evaluation of Modified *Opaque-2* Popcorn Suggests a Route to Quality Protein Popcorn. Front. Plant Sci. 9, 1803. doi: 10.3389/fpls.2018.01803

Robbins, W. A., and Ashman, R.B. (1984). Parent-offspring popping expansion correlations in progeny of dent corn x popcorn and flint corn x popcorn crosses. Crop Sci. 24, 119-121. doi: 10.2135/cropsci1984.0011183X002400010027x

Sailer, M.O. (2013). crossdes: Construction of Crossover Designs. R package version 1.1-1. https://CRAN.R-project.org/package=crossdes

dos Santos Alves, L., Lorenzo, J., Gonçalves, C., dos Santos, B., Heck, R., Cichoski, A., & Campagnol, P. (2017). Impact of lysine and liquid smoke as flavor enhancers on the quality of low-fat Bologna-type sausages with 50% replacement of NaCl by KCl. *Meat Science*, *123*, 50-56. https://doi.org/10.1016/j.meatsci.2016.09.001

Schloerke, B., Crowley, J., Cook, D., Briatte, F., Marbach, M., Edwin Thoen, E., Elberg A., and Larmarange J. (2020). GGally: Extension to 'ggplot2'. R package version 1.5.0. https://CRAN.R-project.org/package=GGally

Siró, I., Kápolna, E., Kápolna, B., & Lugasi, A. (2008). Functional food. Product development, marketing and consumer acceptance—A review. *Appetite*, *51*(3), 456-467. https://doi.org/10.1016/j.appet.2008.05.060

Sweley, J.C., Rose, D.J., and Jackson, D.S. (2011). Composition and Sensory Evaluation of Popcorn Flake Polymorphisms for a Select Butterfly-Type Hybrid. Fac. Pub. In Food Sci. and Tech. 109.

Sweley, J.C., Rose, D.J., and Jackson, D.S. (2013). Quality Traits and Popping Performance Considerations for Popcorn (Zea mays Everta). Food Reviews International. 29: 157-177.

Tahergorabi, R., Matak, K., & Jaczynski, J. (2015). Fish protein isolate: Development of functional foods with nutraceutical ingredients. *Journal Of Functional Foods*, *18*, 746-756. https://doi.org/10.1016/j.jff.2014.05.006

Tandjung, A.S. (2003). [Ph.D. Thesis] Improvement in popcorn popping performance through edible coatings and understanding inherent factors affecting moisture loss. Purdue University, West Lafayette, Indiana.

Traill, W., Arnoult, M., Chambers, S., Deaville, E., Gordon, M., & John, P. et al. (2008). The potential for competitive and healthy food chains of benefit to the countryside. *Trends In Food Science & Technology*, *19*(5), 248-254. https://doi.org/10.1016/j.tifs.2008.01.004

Vanhonacker, F., Kühne, B., Gellynck, X., Guerrero, L., Hersleth, M., & Verbeke, W. (2013). Innovations in traditional foods: Impact on perceived traditional character and consumer acceptance. *Food Research International*, *54*(2), 1828-1835. https://doi.org/10.1016/j.foodres.2013.10.027

Vasal, S.K. (2002). Quality Protein Maize: Overcoming the Hurdles. Journ. of Crop Prod. 6:1-2.

Vidal, V., Santana, J., Paglarini, C., da Silva, M., Freitas, M., & Esmerino, E. et al. (2020). Adding lysine and yeast extract improves sensory properties of low sodium salted meat. *Meat Science*, *159*, 107911. https://doi.org/10.1016/j.meatsci.2019.107911

Virág, D., Kiss, A., Forgó, P., Csutorás, C., & Molnár, S. (2013). Study on Maillard-reaction driven transformations and increase of antioxidant activity in lysine fortified biscuits. *Microchemical Journal*, *107*, 172-177. https://doi.org/10.1016/j.microc.2012.06.018

Weitkamp, E., & Eidsvaag, T. (2014). Agenda Building In Media Coverage of Food Research. *Journalism Practice*, *8*(6), 871-886. https://doi.org/10.1080/17512786.2013.865966

Wickham. H. (2016). ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.

Wickham, H. & Bryan, J. (2019). readxl: Read Excel Files. R package version 1.3.1. https://CRAN.R-project.org/package=readxl

Wickham, H (2020). forcats: Tools for Working with Categorical Variables (Factors). R package version 0.5.0. https://CRAN.R-project.org/package=forcats

Wickham, H. François, R., Henry L. & Müller K. (2020). dplyr: A Grammar of Data Manipulation. R package version 0.8.5. https://CRAN.R-project.org/package=dplyr

Wilke, C. (2019). cowplot: Streamlined Plot Theme and Plot Annotations for 'ggplot2'. R package version 1.0.0. https://CRAN.R-project.org/package=cowplot

Zhu, C., Zhao, J., Tian, W., Liu, Y., Li, M., & Zhao, G. (2017). Contribution of Histidine and Lysine to the Generation of Volatile Compounds in Jinhua Ham Exposed to Ripening Conditions Via Maillard Reaction. *Journal Of Food Science*, *83*(1), 46-52. https://doi.org/10.1111/1750-3841.13996

Ziegler, K.E., and Ashman, B. (1994). “Popcorn”, in Specialty corns, ed. A.R. Hallauer (New York, NY: CRC Press), 189-223.

CHAPTER 5: SUMMARY AND CONCLUSIONS

1. Final Remarks

The increase in global popcorn sales over the past decade has given private industries the opportunity to diversify product development and design strategies. The Ready-To-Eat popcorn sector has particularly experienced a proliferation of innovative product additives and coatings, marketing labeling, and new company competition as consumer awareness for more healthy, 'better for you' food products has grown. To stay relevant and competitive, ConAgra Brands® partnered with the University of Nebraska-Lincoln in 2012 to diversify their popcorn germplasm and bolster the protein quality of their popcorn. Utilizing Quality Protein Maize (QPM) varieties as parental lines to cross to elite proprietary popcorn lines enabled the homozygous introgression of the *opaque-2* mutant allele. This mutant *o2* allele, a transcription factor and key regulator for zein protein formation in its wild-type state, allowed for the popcorn endosperm protein to contain significantly higher amounts of lysine and tryptophan (essential amino acids customarily deficient in maize) than original lines. After years of inbreeding and selection, 12 Quality Protein Popcorn (QPP) inbred lines were developed in 2017 that had higher amounts of lysine, were highly vitreous (conferred a glassy, hard endosperm), and popped at varying levels.

These 12 QPP inbred lines were hybridized in a full diallel in the summer of 2018 and 44 hybrids were selected for further evaluation. In 2019, these 44 F_1 hybrids were grown to produce F_2 seed, and agronomic traits from the F_1 hybrid and seed traits from the F_2 seed were analyzed. Out of this analysis, five QPP crosses were selected as premier hybrids fit for potential commercialization.

Simultaneous to this crossing analysis, BC_3F_4 QPP inbred lines were derived in 2019 from the original BC_2F_5 QPP inbred lines cultivated in 2017. These BC_3F_4 lines were highly vitreous, popped adequately, conferred higher amounts of lysine in the endosperm, and were of the same pedigree as the BC_2F_5 inbred lines with the exception of one additional backcross to the proprietary popcorn lines. After identification of the best five BC_2F_5 QPP hybrids in 2019, the same hybrid crosses were made in the spring of 2020 with the BC_3F_4 inbred lines. These 10 hybrids were then grown alongside five ConAgra Brands® cultivars for comparative agronomic, popcorn quality, and protein quality analysis in the summer of 2020. Out of this analysis, six QPP hybrids, three BC_2F_5 and three BC_3F_4 crosses, were chosen for human evaluation. In November of 2020, a blind taste-test composed of 112 participants revealed that two particular QPP hybrids, termed H4 and H8 for simplification, ranked within the two ConAgra Brands® commercial line controls in overall likability.

The compilation of these results, from an agronomic comparison to human sensory trials, offer credible evidence that QPP would be competitive in the global popcorn market. Additionally, though the first and foremost objective of this study was to produce QPP lines and primitively evaluate marketable competency, this rapid inbred and hybrid breeding program serves as a blueprint for future successful popcorn by dent maize crosses. Moreover, the analyses involved in the agronomic and popcorn quality trait evaluations, namely the derived 2020 Ranking System, is publicly available and transferable to both plant and animal breeding programs. Finally, the general analyses required for producing QPP and assessing consumer approval, from the germplasm's initial production, inbred selection, hybridization, and hybrid selection to final varietal

determination, served as a small holistic example of the diverse resources necessary for successful product development and marketability potential.

CHAPTER 6: APPENDIX

1. R Script for 2020 Ranking System

Note – This script excludes the square root of the summation. The ranking order does not change if the sqrt is implemented but the final ranking values will. If desired, implement the square root function as: 2020FinalRank <- data.frame(IndexVariable = sqrt(2020FinalRank[,"IndexVariable"]) after the summation of all index values.

```
#2020 Ranking System; Y=Number of Lines tested, Z=Number of Traits
library(dplyr);library(ggplot2)
Location = c(If applicable)
Hybrid = c(1:Y)
Maternal = c(List Maternal Lines if applicable)
Paternal = c(List Paternal Lines if applicable)
Pedigree = c(List catagorical variables as applicable)
Trait1 = c(Data1)
Trait2 = c(Data2)
Trait3 = c(Data3)
TraitX = c(DataX)
2020RankingSystem =
data.frame(Location,Hybrid,Maternal,Paternal,Pedigree,Trait1, Trait2, Trait3,
TraitX)
options(max.print=1000000)
2020RankingSystem$Location=factor(2020RankingSystem$Location)
2020RankingSystem$Hybrid=factor(2020RankingSystem$Hybrid)
2020RankingSystem$Maternal=factor(2020RankingSystem$Maternal)
2020RankingSystem$Paternal=factor(2020RankingSystem$Paternal)
2020RankingSystem$Pedigree=factor(2020RankingSystem$Pedigree)
2020RankingSystem

sumTrait1 = tapply(Trait1,Hybrid,mean, na.rm=TRUE)
sumTrait2 = tapply(Trait2,Hybrid,mean, na.rm=TRUE)
sumTrait3 = tapply(Trait3,Hybrid,mean,na.rm=TRUE)
sumTraitX = tapply(TraitX,Hybrid,mean,na.rm=TRUE)


sdGerm = tapply(Trait1,Hybrid,sd,na.rm=TRUE)
sdTrait2 = tapply(Trait2,Hybrid,sd, na.rm=TRUE)
sdTrait3 = tapply(Trait3,Hybrid,sd,na.rm=TRUE)
sdTraitX = tapply(TraitX,Hybrid,sd,na.rm=TRUE)


maxGerm = max(sumGerm)
maxTrait2 = max(sumTrait2)
maxTrait3 = max(sumTrait3)
```

```
maxTraitX = max(sumTraitX)


maxsdGerm = max(sdGerm,na.rm = TRUE)
maxsdTrait2 = max(sdTrait2, na.rm=TRUE)
maxsdTrait3 = max(sdTrait3, na.rm=TRUE)
maxsdTraitX = max(sdTraitX, na.rm=TRUE)

IndexName = c("IndexTrait1","IndexTrait2","IndexTrait3","IndexTraitX")
IndexVariable = rep(IndexVariable,Y)
Hybrid2 = c(1:Y)
Hybrid2 = rep(Hybrid2,each=Z)
StackedChart = data.frame(Hybrid2,IndexVariable)
#Index Selection Intensities:  Between 0-1.  Numbers added for example, input as
needed.
SITrait1 = .8; SITrait2 = .1; SITrait3 = 0.5; SITrait4 = .9;
IndexTrait1 = ((((sumTrait1/maxTrait1)-1)^2)*(SITrait1*(sdTrait1/maxsdTrait1)))
IndexTrait2 = ((((sumTrait2/maxTrait2)-1)^2)*(SITrait2*(sdTrait2/maxsdTrait2)))
IndexTrait3 = ((((sumTrait3/maxTrait3)-1)^2)*(SITrait3*(sdTrait3/maxsdTrait3)))
IndexTraitX = ((((sumTraitX/maxTraitX)-
1)^2)*(SITraitX*(sdTraitX/maxsdTraitX)))

2020FinalRank1=data.frame(Hybrid2,IndexTrait1,IndexTrait2,IndexTrait3,Index
TraitX)
2020FinalRank <- data.frame(IndexVariable = c(2020FinalRank1[,"IndexTrait1"],
2020FinalRank1[,"IndexTrait2"]))
2020FinalRank <- data.frame(IndexVariable = c(2020FinalRank[,"IndexVariable"],
2020FinalRank1[,"IndexTrait3"]))
2020FinalRank <- data.frame(IndexVariable = c(2020FinalRank[,"IndexVariable"],
2020FinalRank1[,"IndexTraitX"]))
2020FinalRank

IndexName = c("IndexTrait1","IndexTrait2","IndexTrait3","IndexTraitX")
IndexName=rep(IndexName,each=Y)
Hybrid2 = c(1:Y)
Hybrid2=rep(Hybrid2,Z)
Hybrid2
DataframeFinal =
data.frame(Hybrid2,IndexName,IndexValue=2020FinalRank$IndexVariable)
DataframeFinal
DataframeFinal$Hybrid2=factor(DataframeFinal$Hybrid2)
DataframeFinal$IndexName=factor(DataframeFinal$IndexName)
Visual1= ggplot(data = DataframeFinal, aes(x = reorder(x=Hybrid2,IndexValue),
y = IndexValue, fill = IndexName)) +
```

```
  geom_bar(stat="identity")+theme_light()
Visual=Visual1+labs(color="Index Variable",x="___",y="Final Ranking",
fill="Trait") + scale_fill_discrete(labels = c("Trait1", "Trait2", "Trait3","TraitX"))
OrderedGraph= ggplot(data = DataframeFinal, aes(x =
reorder(x=Hybrid2,IndexValue), y = IndexValue, fill = IndexName)) +
geom_bar(stat="identity")
OrderedGraph
BlackWhiteOrderedGraph=OrderedGraph+labs(color="Index Variable",
x="_____", y="Final Ranking") + scale_fill_manual(values = c('IndexTrait1' =
'gray1', 'IndexTrait2' = 'gray100', 'IndexTrait3' = 'gray20', 'IndexTraitX'="gray80"))
BlackWhiteOrderedGraph
```