(Research Article)

Multiple patterns of heterotic grouping in temperate and tropical maize hybrids

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Abstract

Heatmap cluster figures that use correlation as distance function provide a powerful tool to visualize biologically meaningful information. By the use of an optimized R heatmap script to detect heterotic groups in corn, mexican tropical maize germplasm was analyzed, particularly some novel varieties of purple corn with high nutraceutical value (Vitamaize). Different genetic combinations (F1 hybrids) were evaluated in trials using an agricultural phenomic approach, and heterotic patterns were compared in two sets of temperate maize entries. Biostatistical analysis of grain yield data revealed various branches of genetically complementary inbred lines (groups with similar heterosis at the phenotypic level). In the set of Vitamaize germplasm, up to four heterotic groups were detected, in contrast to only two heterotic groups reported earlier in temperate yellow maize from the USA. Analysis of mixed germplasm from Argentina and the USA revealed five heterotic groups which demonstrated that heterotic patterns in maize are not restricted to only two (e.g. dent-flint, northern-southern, stifstalk-nonstifft, A-B), but can rather involve many more groups (e.g. ABCDEF). The occurrence of many heterotic groups in tropical germplasm, may explain the fact that in Mexico three way F2 hybrids have been the predominant form of released commercial varieties, whereas in temperate regions, one way F1 hybrids are much more common.

Keywords: Temperate Maize; Tropical Maize; Zea mays; Heterotic Grouping

1. Introduction

The bidirectional linkage between the genotype and the phenotype is one of the central challenges of experimental research in biological systems. The holistic measurement of the phenotype of a crop plant such as maize (Zea mays L.) or rice (Oryza sativa L.), is the main objective of agricultural phenomics, also called field-omics [1]. Currently, yield and phenotypic data obtained by public breeding institutions such as the International Maize and Wheat Improvement Center (CIMMYT) and the International Rice Research Institute (IRRI), are mostly provided as numbers within large tables in spreadsheet format. Numerical field trial data of phenomic experiments need to be converted into adequate charts and figures, to better visualize the major environmental and genetic effects [2,3]. One of the most powerful types of figures for that purpose are heatmap biclusters. These can be produced in the statistical programming environment R [4], which is widely used in the scientific community. Heatmaps can summarize data from large metabolomic [5] and transcriptomic experiments [6]. The data from columns and rows can be rearranged automatically employing various clustering algorithms [7], and the relationships between groups in rows and columns can be visualized through the hierarchical dendrograms. Since heatmaps have not yet been used for agricultural phenomics, new algorithms for plant breeding and other heterogenic phenotypic data types need to be implemented [8]. The default parameters of the R

heatmap function produces dendogram trees that are not congruent with the real biological context. An optimized R script that uses correlation as a distance measure for clustering, has been shown to be more useful in several scenarios and data sets that are typical of plant research, molecular breeding, and biochemical phenotyping. Heterosis in plants is reflected by the fact that plants from a cross between two parents (the F1 generation), have much more vigor and produce higher grain yield than the parental homocygotic lines. Heterosis in maize and other crop plants is caused by genetic [9,10] and epigenetic factors [11], both with additive and non-additive effects such as overdominance. Heterotic vigor of corn provides the economic basis of a multimillion dollar business for selling hybrid seeds with agrochemicals in a package [12]. Breeding programs of modern corn varieties in the USA and Europe, usually consider only two heterotic groups, the southern dent and the northern flint [13]. Inbred lines of these two heterotic groups are sexually crossed in order to produce a commercial F1 hybrid [14]. For example, the inbred B73 has typically been crossed with the inbred Mo17, resulting in a hybrid with larger cobs and > 300 % higher grain yield [15]. Mexico is a center of genetic diversity of maize and many other vegetative species [16]. Numerous Mexican corn varieties with different phenotypic properties, such as grain color, cob size, and biochemical profile are available in the gene pool [17]; therefore, it can be expected that heterotic patterns in tropical and subtropical maize, are different and not limited to only two groups such as in Europe and the USA. In an effort to produce more nutritious corn for human consumption, the Vitamaize breeding program of the department of genetic engineering of the Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV) in Irapuato, Mexico, has developed several tropical maize varieties that have grains with higher levels of antioxidants, such as carotenes, anthocyanins, and polyphenols. Through sexual breeding, a non-transgenic approach, inbred lines were generated that have dark purple grain color. The objective of this study was to address following questions: a) How well can the optimized R script reveal various heterotic patterns in maize?, b) are the heterotic groups in temperate maize from the USA different from those from Argentina?, c) are heterotic patterns in tropical maize restricted to two groups only as in germplasm from the USA?, and d) how many heterotic groups can be found in a set of 50 inbred Vitamaize lines?.

2. Materials and methods

2.1. Biological materials: Vitamaize lines and hybrids

Several new varieties of tropical purple corn were developed through a non-transgenic approach (sexual breeding), starting with the Mexican landraces Xoxocotla and Tepalcingo [18,19] as donor parents, and elite homocygotic lines from the subtropical and tropical breeding program of CIMMYT as recurrent parents. Near isogenic lines (NILs) were obtained by allele introgression, through repetitive backcrossing to recurrent parents (white and yellow elite lines from CIMMYT). Backcrossing was done for 4-6 generations and selfings were continued for 3-5 further generations. In each generation, segregating seeds were carefully screened for color, revealing the profile of biochemical compounds such as anthocyanins, polyphenols, and carotenes. Fifty-two inbred lines were obtained that had dark purple grain color, and 50 entries were selected for the genetic experiment and phenomic evaluation (holistic evaluation of the phenotype in the field). In order to measure heterosis and hybrid vigor in several Vitamaize varieties, female lines were planted in separate rows and sexually crossed with pollen of 3 male tester lines (VM311, VM321, and VM451) to generate enough seeds of the 150 hybrids. A replicated field trial was established in a tropical environment at sea level in La Esperanza ranch near Puerto Vallarta, Mexico, during the spring-summer season of 2014. Agronomic management was standard for the field station: machine sowing and cultivation, drip irrigation every week, fertilization with 100 kg of phosphorus + 400 kg of urea per ha, application of Cypervel 200 (Cypermethrin) at 0.35 L ha^{-1} [20] during the phenological stage V3 [21] for control of armyworm [*Spodoptera frugiperda* (J. E. Smith)] and Pounce (Permethrin) at 140 mL ha^{-1} [22] during V8 and V12. Grain yield of the 150 Vitamaize hybrids was measured for 8 replicate plots distributed across a homogenous field, and the average yield was calculated in t ha^{-1}.

2.2. Analysis of data from field experiments with temperate maize varieties

Data from a diallel type experiment was extracted from result tables previously published [23]. Fourteen inbred lines including key representatives of the Lancaster group like Mo17 and H102, and the Reid Dent USA group like B73 and B84, were included, thus covering most of the genetic diversity present in the USA germplasm [24]. The 14 homocigotic lines were crossed to each other, and the F1 hybrids and the corresponding parental inbreds, were evaluated in two locations in the USA over two years [23]. One hundred and eighty two hybrids were generated from all possible single crosses among 14 inbreds, including reciprocals. Since Dudley *et al*. [23] planted only one set of F1 hybrids which consisted of 91 entries, data for the second possible reciprocal crosses were not available in the report. In order to represent heatmaps in full square format, data had to be simulated of the reciprocal hybrids, and therefore, grain yield data had to be duplicated for all the reciprocal crosses, based on the premise that a cross A x B was phenotypically equivalent to the reciprocal B x A cross, since the maternal effects are negligible. In order to expand the survey with the improved heatmap correlation script, previously published data from field trials involving both inbred lines from the

USA and Argentina [25] was inspected. Delucchi *et al*. [25] crossed 20 female lines of mixed origin from the USA Dent and Argentina Flint with 5 male tester lines. They generated 95 crosses which were evaluated in two field experiments, but they managed to obtain seeds/data from 91 hybrids only. The data tables had missing data, so that missing values had to be imputed, based on linear functions and other estimations. Missing values of inbred lines in the experiment from Delucchi *et al*. [25], were replaced by the grain yield data of the inbred parents that were evaluated by Dudley *et al*. [23]. Other values were estimated using typical grain yields of inbreds in other nursery experiments (~ 4 t ha^{-1}) [26]. Since the result tables of Delucchi *et al*. [25] also contained missing values for some hybrid combinations such as Mo17 x B73 and B73 x LE3, they were replaced by interpolation. Overall, the USA and Argentinian datasets were complemented and curated, through reasonable assumptions and statistical imputing of numbers for construction of the heatmap figures.

2.3. Heatmap correlation script: Dendogram clustering with correlation as distance function

A detailed demonstration of the disadvantages of using the default function parameters of the R heatmap function can be found in a previous report [8]. The default dendogram is based on euclidean distance, whereas a scale independent function was developed that is based on the linear Pearson correlation coefficient. The script used in the present work starts with the definition of a special function for calculating branching distance, based on R correlation value: cor.dist<-function(x){ as.dist(1 -cor(t(x), use="pairwise.complete.obs"))}; with the above function, a short distance is generated for positive correlations R > 0.8, whereas an intermediate distance is calculated for pairs with zero correlation R≈0, whereas the longest distance is calculated for pairs with high negative correlation R < -0.8. The maximum distance is two and the minimum distance is zero. A heatmap figure with nonscaled data, optimized clustering and grayscale coloring can then be ploted with the command: hv <- heatmap(datam, distfun=cor.dist, col = gray(24:0/24), scale="none", main="R cor.dist "). The grain yield data needs to be previouly imported into the "datam" object as a numerical matrix for figure plotting. All statistical analysis and previous commands were executed using the programing environment R [27].

3. Results and discussion

3.1. Heterotic groups in Vitamaize hybrids

Table 1 shows the grain yield data from a representative set of 36 Vitamaize F1 hybrids. Most lines expressed a higher heterosis when crossed to one male line than to the other two. For example, VM311 crossed to itself (VM311a) resulted in low yield.

Table 1 Grain yield (t ha^{-1}) of a representative subset of 36 Vitamaize hybrids, evaluated during the spring-summer season 2014 in a field trial, in La Esperanza ranch near Puerto Vallarta, Mexico

Male progenitors	Female progenitors		
	VM311	VM321	VM451
Mz491492	5.47	5.84	4.63
MzATFW112	4.76	4.07	6.97
MzATFW1211	4.80	5.55	3.23
MzATFW1221	4.88	6.24	5.38
MzATFW1413	6.01	4.88	4.34
MzATFW512	5.33	6.14	5.70
MzATFW521	4.67	5.71	4.06
MzATFW641	2.67	5.18	2.93
MzDTP111	4.59	4.41	5.76
VM311a	0.40	4.74	5.53
VM321a	7.33	1.12	5.81
VM451	7.29	5.47	0.49

It expressed no heterosis as expected, since this cross is equivalent to an inbred line. The same effect was observed for the line MzATFW1211, which crossed best with VM321 and VM311, but poorly with VM451. A similar heterosis effect was observed for the line MzATFW641, which crossed best with VM321, but poorly with VM311 and VM451. Other crosses such as VM311 x VM321 and VM311 x VM451 demonstrated increased productivity up to 1400%, when comparing the F1 hybrid to each of the parental inbred lines, and showed the highest grain yield with 7.33 and 7.9 t ha^{-1}, respectively. The line VM451 worked both well as female as male parent, since the crosses VM451 x VM311 and MzATFW112 x VM451 had also good yield. In order to genetically classify the inbred lines, the full data matrix of grain yield of the 150 hybrids was used to prepare heatmap figures without scaling (Figure 1). The optimized script that uses the R value of correlation, produced a heatmap that grouped the samples in four groups. More groups can be expected if more male lines are tested and if materials from the highlands are included. This is in support of the hypothesis that heterosis in maize is due to additive genetic effects, caused by the presence-absence of specific genes across the chromosomes in some inbred lines compared to others, for example, missing genes in B73 that are present on Mo17 [28]. Four mayor branches of female lines appeared, which corresponded to the genotypes that worst crossed with either VM311, VM321, or VM451. Default clustering of R heatmap reveals mathematical proximity of the numerical values of grain yield, no biological meaning, whereas the optimized heatmap script based on correlation, better represented genetic patterns of heterosis in those 150 Vitamaize lines. Our script produced a dendogram tree, that revealed an important biological feature, which is the genetic correspondence to a specific heterotic group. The heatmap reveals the heterotic pattern of the general combining ability (GCA) and the specific combining ability (SCA) (genetic complementation). This information is further useful as a tool to continue and expand the breeding program by intercrossing lines of the same heterotic group, to improve their *per se* performance, and to cross them among complementary heterotic groups. It is expected that this procedure will generate a hybrid with high yield and improved nutritional quality (antioxidants), that can be commercially released to farmers.

3.2. Heterotic groups in maize varieties from temperate regions

The optimized heatmap script was used to investigate the patterns of heterotic groups of inbred lines from temperate regions. Maize germplasm from the USA (Figure 2) was first analyzed and then mixed germplasm from the USA and Argentina (Figure 3). Instead of repeating experiments that have been performed by other groups previously, the results published from several field trials were combined and the data was re-analyzed using the biostatistical algorithms.
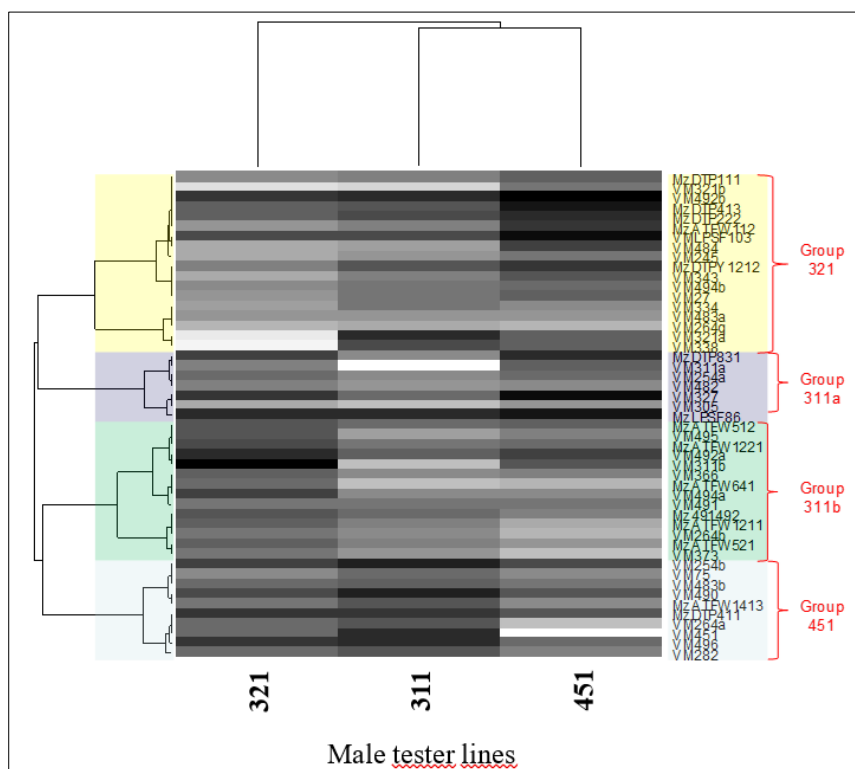

Figure 1 Heatmaps of grain yield (t ha^{-1}) across Vitamaize hybrids. The darker the color, the higher the yield. Female lines are shown in the rows, whereas the male tester lines in the columns. Color coding of female lines corresponds to the heterotic groups

Fourteen inbred lines including representatives of the Lancaster group and the Reid Dent USA group were crossed to each other in a diallel type of experiment [23]. Field trials were performed with hybrids and with inbred lines simultaneously as in the Vitamaize experiment (Figure 1). However, Dudley *et al.* [23] planted only one set of F1 hybrids. The reciprocal crosses were not evaluated in the field since they were considered to be equivalent. In order to represent the data in a full heatmap with square format, grain yield data was duplicated for all reciprocal crosses, for example, the hybrid Mo17 x B73 was mathematically converted to be fully equivalent to the reciprocal hybrid B73 x Mo17. The premise that maternal effects are negligible compared to the strong effect of heterosis, is a common assumption among maize breeding programs. The heatmap of grain yield data from the 105 hybrids revealed a characteristic pattern (Figure 2). The rows and the columns were arranged automatically by the clustering algorithm. A diagonal appeared composed of a series of white boxes, which corresponds to the yield of the inbred lines like B73 x B73. Heterosis may be calculated by the difference of the average of the color of the whole row or column compared to the color of the box in the diagonal. The hybrid with the highest yield corresponded to the cross between Mol7 x B84 (11.42 t ha^{-1}). Other crosses with higher yields than average were Pa91 x B73 (11.33 t ha^{-1}), Pa91 x B84 (11.05 t ha^{-1}), Mol7 x B73 (10.41 t ha^{-1}), B77x B73 (10.25 t ha^{-1}), H102 x H100 (10.25 t ha^{-1}). These results are represented as boxes of dark black color in the heatmap. Hybrid cross combinations with poor yield like H102 x Mo17 with 6.44 t ha^{-1} are represented as light grey colour. The dendogram tree revealed two major branches, heterotic groups, that belong either to the Reid Yellow USA Dent or the Northern Lancaster Sure Crop. Such simple heterotic pattern facilitates hybrid breeding programs, since a line from group A is always crossed to a line from group B in order to generate a commercial F1 hybrid. Either A x B or B x A are crossed, depending of the choice of the female and male lines. In comparison, tropical maize germplasm such as the Vitamaize hybrids, were classified into four groups by the heatmap correlation function (Figure 1). This complicates breeding programs of tropical germplasm, since there are more choices on how to perform the hybrid crosses. Then, the mixed germplasm from the USA and Argentina [25] was explored. Grain yield data of two field experiments was re-analyzed using algorithms (Figure 3). Delucchi *et al.* [25] crossed 20 female lines of mixed origin with 5 male tester lines, and generated 95 crosses for field evaluation; however, they managed to obtain seeds/data from 91 hybrids only. They reported grain yield from the hybrids, but they did not evaluate the inbreds in parallel. It is typical of maize breeding programs to focus on field trials of hybrids, but yield of inbreds is seldom measured in the experiments. Therefore, the data was curated by adding missing values through statistical imputing of numbers. Missing values of inbred lines in the experiment from Delucchi *et al.* [25] were replaced by the grain yield data reported by Dudley *et al.* [23]. Values were also estimated using typical grain yields of inbreds in other nursery experiments which range from 3 to 5 t ha^{-1} [29].
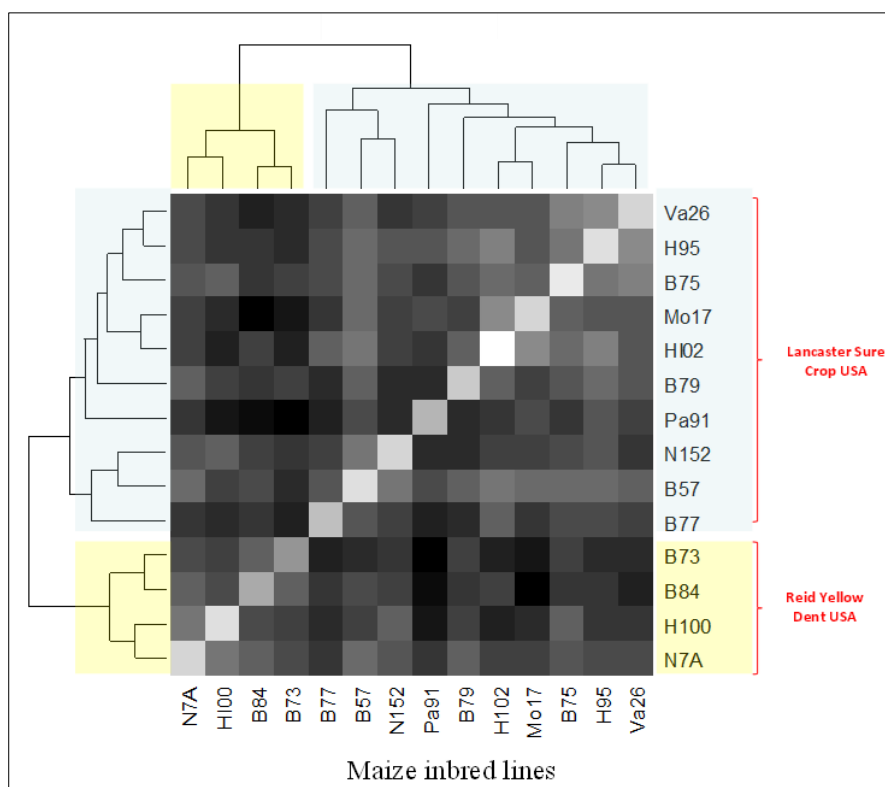

Figure 2 Heterotic groups of temperate maize from the USA. A grayscale coding was implemented, with darker color representing higher numerical values of grain yield

The heatmap of the Argentinian entries revealed also a characteristic pattern of heterosis. The hybrid with the highest yield corresponded to the cross between LP122.2 x B73 (9.22 t ha⁻¹). Other crosses with higher yields than average were ARZM03014 x LP612 (8.39 t ha⁻¹), and Mo17 x LP122.2 (8.67 t ha⁻¹). Dark color appeared in the given boxes of the heatmap, whereas cross combinations with poor yield appeared as boxes with light grey color (B73 x LE3 with 5 t ha⁻¹, LP612 x Mo17 with 6.45 t ha⁻¹, and ARZM02023 x LE3 with 6.75 t ha⁻¹).
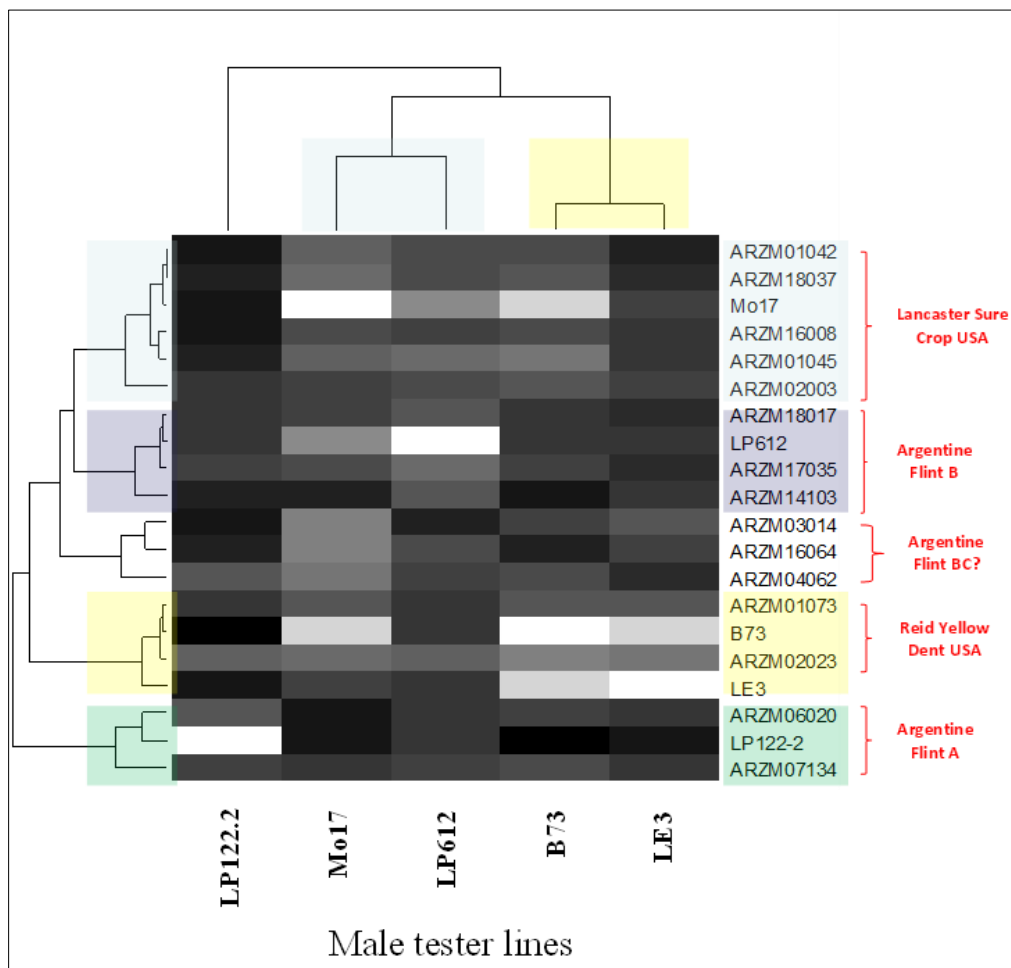

Figure 3 Heterotic groups of temperate maize of mixed sources from the USA and Argentina. Darker color represents higher values of grain yield, whereas white represents low yield (inbred)

In Figure 3 two dendograms appear: the vertical tree on top shows the relationship of the 5 lines used as male testers, while the horizontal one, the groups of lines used as female parents. In Figure 2, the top and right dendograms have identical branching, since data was duplicated for the reciprocal crosses, whereas in Figure 3, data for reciprocal crosses was available for statistical analysis; in this figure, the top dendogram shows the typical grouping in two major branches of USA Dent lines B73 and LE3, and the Lancaster lines Mo17 and LP612, whereas the Argentinian Flint A line LP122.2 appeared as a separate branch. This suggests that this Argentinian entry falls outside the heterotic pattern from the USA. Indeed, the horizontal dendogram of Figure 3 revealed five major branches, that is, female lines that correspond to distinct heterotic groups. In addition to the USA Dent and Lancaster, there appeared separate branches for the Argentinian Flint A and the Argentinian Flint B groups. Non-expectedly, a fifth heterotic group appeared that may be called the "Argentine Flint BC group". This fifth group is more similar the Flint B, whereas the Argentinian Flint A group, to which the line LP122.2 belongs, seems to be the most unrelated branch from the others. This pattern of heterotic patterning further strengthens the notion that genetic diversity of USA germplasm (only two major heterotic groups) is narrower compared to genetic diversity present in Argentinian lines. The fact that inbred lines from temperate regions belong to a limited pattern of two heterotic groups facilitates hybrid breeding programs, since it simplifies breeding decisions: a line A is always crossed to a line from group B in order to generate a good hybrid. It is a simple dichotomic decision that is true for the USA lines (Flint x Dent) as for the Argentinian inbreds (Flint A x Flint B). One of the two reciprocal hybrids are used, either A x B or B x A depending of the choice of the female and male lines. For a given F1 hybrid, the decision about which line is used as male or female parent is made mainly on the basis of technical

considerations, for example, cob size and pollen production determine which is used as male or female, respectively. In comparison, generation of hybrids from tropical corn and mixed germplasm sources, requires more complicated decisions for successful breeding, and subsequent release of commercial varieties. The Vitamaize inbreds were classified into many groups by the heatmap correlation function, whereas the dendogram of mixed USA-Argentinian germplasm revealed five heterotic groups. The existence of more than two heterotic groups opens more breeding possibilities to generate F1 and F2 hybrids. Different types of three way F2 hybrids are possible such as A x B /C, A x C /B, B x A /C, B x C /A, C x A /B, or C x B /A. The complex pattern of heterotic groups in tropical germplasm, may explain the fact that in Mexico three way F2 hybrids are the predominant form of released commercial varieties, whereas in temperate regions, maize lines are classified as Flint or Fent, one way F1 hybrids are much more common, if not, the only ones commercially available. Further, phenomic experiments will be performed with tropical germplasm to optimize the genotypes for production. The numerical data matrices of field experiments should be analyzed with optimized data mining algorithms and visualization tools, in order to better explain the complex link between genotype and phenotype, in a worldwide crop plant such as *Zea mays.* The optimized script for heatmap correlation bicluster will be useful not only for agricultural phenomics, but also, to improve the interpretation of other omic sciences, such as metabolomic fingerprints [30,31] and transcriptomic data sets [6]. The script can be used to prepare meaningful heatmap figures for molecular breeding programs, but it can also be applied for data matrices obtained from transcriptomic and/or metabolomic experiments [31,32,33] of any biological system. The knowledge of the molecular origin of heterotic groups may help scientists and engineers to adapt their breeding programs in order to obtain higher yielding varieties for each of the target environments [34]. Theoretically, high yielding maize hybrids may be obtained by polyploidization [35,36,37] or by genomic rearrangement, so that all favorable genes/alleles are accommodated linearly along the chromosome. Practically, it is difficult to obtain all favorable genes at once in a single chromosome, since recombination events during meiosis can occur only among highly homologous DNA regions (mainly coding determining sequences with high sequence identity). Therefore, novel methods of directional DNA reshuffling and selected gene re-arrangement may be promising tools to generate further improved maize varieties that can maintain high yields, even if they are in diploid and homocygotic state. Smart breeding protocols involving chromosome duplication, targeted DNA edition and gene shuffling by helitron-like transposons [38], may help to optimize heterotic grouping in tropical maize germplasm, so that molecular breeding strategies may provide more food in a sustainable fashion for a healthy human life. For Mesoamerican cultures, maize (*milpa*) is more than just an industrial product that can be commercially exploited or just be fed to cows or pigs. The *milpa* is not only a way of living and a source of biological and cultural diversity, but it also provides spiritual and physical well-being for humans. The Vitamaize varieties of purple corn represent a source of "good food" that have been generated by modern omic breeding, combining the best of Mexican landraces and the agronomic advantages of improved tropical corn hybrids. Instead of F1 hybrids, we expect to produce F2 hybrid combinations and open pollinated varieties (OPVs), using the preliminary knowledge of heterotic grouping reported here.

4. Conclusions

An optimized R heatmap script with cluster figures that uses correlation as distance function, was used to detect heterotic groups in corn.

In the set of Vitamaize germplasm, that is, novel varieties of purple corn with high nutraceutical value, up to four heterotic groups were detected, in contrast to only two heterotic groups reported earlier in temperate yellow maize from the USA.

The grain yield data from a representative set of 36 Vitamaize F1 hybrids out of 150, showed that VM321 x VM311 and VM451 x VM311 had the highest yield with 7.33 and 7.29 t ha^{-1}, respectively.

Analysis of mixed germplasm from Argentina and the USA revealed 5 heterotic groups, which demonstrated that heterotic patterns in maize are not restricted to only two, but can rather involve many more groups.

The Argentinian inbred lines showed a different heterotic pattern than germplasm from the USA.

The occurrence of many heterotic groups in tropical germplasm may explain the fact that in Mexico three way F2 hybrids have been the predominant form of released commercial varieties, whereas in temperate regions one way F1 hybrids are much more common

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that No conflict of interest.

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