frontiers

Characterization of the USDA *Cucurbita pepo*, *C. moschata*, and *C. maxima Germplasm* Collections

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ABSTRACT

The *Cucurbita* genus is home to a number of economically and culturally important species. We present the analysis of genotype data generated through genotyping-by-sequencing of the USDA germplasm collections of *Cucurbita pepo*, *C. moschata*, and *C. maxima*. These collections include a mixture of wild, landrace, and cultivated specimens from all over the world. Roughly 1,500 - 32,000 high-quality single nucleotide polymorphisms (SNPs) were called in each of the collections, which ranged in size from 314 to 829 accessions. Genomic analyses were conducted to characterize the diversity in each of the species and revealed extensive structure corresponding to a combination of geographical origin and morphotype/market class. Genome-wide associate study (GWAS) was conducted for each data set using both historical and contemporary data, and signals were detected for several traits. These data represent the largest collection of sequenced Cucurbita and can be used to direct the maintenance of genetic diversity and the development of breeding resources, and to help prioritize whole-genome re-sequencing for further GWAS and other genomics studies aimed at understanding the phenotypic and genetic diversity present in *Cucurbita*.

Keywords: Germplasm, Genotyping-by-sequencing, GWAS, Diversity, *Cucurbita*

1 INTRODUCTION

The *Cucurbitaceae* (Cucurbit) family is home to a number of vining species mostly cultivated for their fruits. This diverse and economically important family includes cucumber (*Cucumis sativus*), melon (*Cucumis melo*), watermelon (*Citrullus lanatus*), and squash (*Cucurbita ssp.*)(Ferriol and Picó, 2008). Like other cucurbits, squash exhibit diversity in growth habit, fruit morphology, metabolite content and disease resistance, and have a nuanced domestication story (Chomicki et al., 2020; Paris and Brown, 2005). The genomes of *Cucurbita ssp.* are small (roughly 400 Mb), but result from complex interactions between ancient genomes brought together through an allopolyploidization event (Sun et al., 2017). These factors make squash an excellent model for understanding the biology of genomes, fruit development, and domestication. Within *Cucurbita*, five species are recognized as domesticated. Three of these are broadly cultivated: *C. maxima*, *C. moschata*, and *C. pepo* (Ferriol and Picó, 2008). Few genomic resources have been available for these species; although, draft genomes and annotations, along with web-based tools and other genomics data are emerging (Yu et al., 2022). Already, these resources have been used to elucidate the genetics of fruit quality, growth habit, disease resistance, and to increase the efficiency of cucurbit improvement (Montero-Pau et al., 2017; Zhong et al., 2017; Kaźmińska et al., 2018; Wu et al., 2019a; Xanthopoulou et al., 2019; Hernandez et al., 2020); however, there has yet to be a comprehensive survey of the genetic diversity in the large diverse *Cucurbita* germplasm panels maintained by the USDA within the National Plant Germplasm System.

Germplasm collections play a vital role in maintaining and preserving genetic variation. These collections can be mined by breeders for valuable alleles and can also be used by geneticists and biologists for mapping studies (McCouch et al., 2020). Like many other orphan and specialty crops,there has been little effort put into developing community genetic resources for squash and other cucurbits. The Cucurbit Coordinated Agricultural Project (CucCAP project) was established to help close the knowledge gap in cucurbits. This collaborative project aims to provide genomics resources and tools that can aid in both applied breeding and basic research. The genetic and phenotypic diversity present in the USDA watermelon, melon, and cucumber collections has already been explored as part of the CucCap project, partially through the sequencing of USDA germplasm collections and development of core collections for whole-genome sequencing (Wang et al., 2021, 2018; Wu et al., 2019b). The diverse specimens of the USDA squash collections have yet to be well characterized at the genetic level;although, an elaborate system has been established for classifying squash based on species and various other characteristics.

The classification system used in squash is complex. Squash from each species can be classed as winter or summer squash depending on whether the fruit is consumed at an immature or mature stage, the latter is a winter squash (Loy, 2004). Squash are considered ornamental if they are used for decoration, and some irregularly shaped, inedible ornamental squash are called gourds; however, gourds include members of *Cucurbita* as well as some species from *Lagenaria*—not all gourds are squash (Paris, 2015). Many squash are known as pumpkins; the pumpkin designation is a culture dependent colloquialism that can refer to jack O' lantern types, squash used for desserts or, in some Latin American countries, to eating squash from *C. moschata* known locally as Calabaza (Ferriol and Picó, 2008). Cultivars deemed as pumpkins can be found in all widely cultivated squash species. Unlike the previous groupings, morphotypes/market classes are defined within species. For example, a Zucchini is reliably a member of *C. pepo* and a Buttercups are from *C. maxima*. Adding to the complexity of their classification, the *Cucurbita* species are believed to have arisen from independent domestication events and the relationships between cultivated and wild species remains poorly understood (Kates et al., 2017).

C. pepo is the most economically important of the *Cucurbita* species and is split into two different subspecies: *C. pepo* subsp. *pepo* and *C. pepo* subsp. *ovifera* (Xanthopoulou et al., 2019). Evidence points to Mexico as the center of origin for *pepo* and southwest/central United States as the origin of *ovifera*. The progenitor of *ovifera* is considered by some to be subsp. *ovifera* var. *texana*, whereas subsp. *fraterna* is a candidate progenitor for *pepo* (Kates et al., 2017). Europe played a crucial role as a secondary center of diversification for subsp. *pepo*, but not subsp. (Lust and Paris, 2016). Important morphoptypes of *pepo* include Zucchini, Spaghetti squash, Cocozelle, Vegetable Marrow, and some ornamental pumpkins. *C. pepo* subsp. *ovifera* includes summer squash from the Crookneck, Scallop, and Straightneck group, and winter squash such as Delicata and Acorn (Paris et al., 2012).

The origin of *C. moschata* is more uncertain than *C. pepo*; it is unclear whether *C. moschata* has a South or North American origin (Chomicki et al., 2020). Where and when domestication occurred for this species is also unknown; however, it is known that *C. moschata* had an India-Myanmar secondary center of origin where the species was further diversified (Sun et al., 2017). *C. moschata* plays an important role in squash breeding as it is cross-fertile to various degrees with *C. pepo* and *C. maxima*, and can thus be used as a bridge to move genes across species (Sun et al., 2017). Popular market classes of *C. moschata* include cheese types like dickenson, which is widely used for canned pumpkin products, Butternut (Neck) types, Japonica, and tropical pumpkins known as Calabaza (Ferriol and Picó, 2008).

C. maxima contains many popular winter squash including Buttercup/Kabocha types, Kuri, Hubbard, and Banana squash (Ferriol and Picó, 2008). This species also sports the world's largest fruit, the giant pumpkin whose fruit are grown for competition and can reach well over 1000 Kg (Savage et al., 2015). Although this species exhibits a wide range of phenotypic diversity in terms of fruit characteristics, it appears to be the least genetically diverse of the three species described (Kates et al., 2017). *C. maxima* is believed to have a South American origin, and was likely domesticated near Peru, with a secondary center of domestication in Japan and China (Nee, 1990; Sun et al., 2017).

In this study, we set out to characterize the genetic diversity present in the USDA *Cucurbita* germplasm collections for *C. pepo*, *C. moschata*, and *C. maxima*. We present genotyping-by-sequencing (GBS) data from each of these collections, population genomics analysis, results from genome-wide association study (GWAS) using historical and contemporary phenotypes, and suggest a core panel for re-sequencing.

2 MATERIALS AND METHODS

2.1 Plant Materials and Genotyping

All available germplasm were requested from USDA cooperators for C. maxima (534 accessions from Geneva, NY), C. moschata (314 accessions from Griffin, GA), and C. pepo (829 accessions from Ames, IA) respectively. Seeds were planted in 50-cell trays and two 3/4 inch punches of tissue (approximately 80-150 mg) was sampled from the first true leaf of each seedling. DNA was extracted using Omega Mag-Bind Plant DNA DS kits (M1130, Omega Bio-Tek, Norcross, GA) and quantified using Quant-iT PicoGreen dsDNA Kit (Invitrogen, Carlsbad, CA). Purified DNA was shipped to Cornell's Genomic Diversity Facility for GBS library preparation using protocols optimized for each species. Libraries were sequenced at either 96, 192, or 384-plex on the HiSeq 2500 (Illumina Inc., USA) with single-end mode and a read length of 101 bp.

2.2 Variant Calling and Filtering

SNP calling was conducted using the TASSEL-GBS V5 pipeline (Glaubitz et al., 2014). Tags produced by this pipeline were aligned using the default settings of the BWA aligner (Li and Durbin, 2009). Raw variants were filtered using BCFtools (Danecek et al., 2021). Settings for filtering SNPs were as follows, minor allele frequency (MAF) ≥ 0.05, missingness ≤ 0.4, and biallelic. Three outlier genotypes were found in an initial principal components analysis (PCA) of the C. maxima data and were removed, as they were likely not C. maxima. Variants were further filtered for specific uses as described below.

2.3 Population Genomics Analysis

ADMIXTURE (Alexander and Lange, 2011), which uses a model-based approach to infer ancestral populations (k) and admixture proportions in a given sample, was used to explore population structure in each dataset. ADMIXTURE does not model linkage disequilibrium (LD); thus, marker sets were further filtered to obtain SNPs in approximate linkage equilibrium using the "–indep-pairwise" option in PLINK (Purcell et al., 2007) with r^2 set to 0.1, a window size of 50 SNPs, and a 10 SNP step size . All samples labeled as cultivars or breeding material were removed from the data prior to running ADMIXTURE. Cross-validation was used to determine the best value for each species. Briefly, ADMIXTURE was run with different values (1-20) and the cross-validation error was reported for each k. The k value with minimal cross-validation error was chosen for each species (Supplemental Figures, Figure 2). Ancestral populations were then assigned to cultivars using the program's projection feature.

Principal components analysis (PCA) was used as a model-free way of determining population structure. PCA was conducted using SNPRelate () on the same LD-pruned data used by ADMIXTURE.

Linkage disequilibrium was calculated in each germplasm panel, and within each subgroup identified by ADMIXTURE.

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2.4 Analysis of Phenotypic Data

Historical data were obtained from the USDA Germplasm Resources Information Network (GRIN; www.ars-grin.gov) for C. maxima, C. pepo, and C. moschata. All duplicated entries were removed for qualitative traits, where categories are mutually exclusive, leaving only samples with unique entries for analysis. Two traits: adult and nymph squash bug damage in C. pepo where transformed using the boxcox procedure. Contemporary phenotypic data were collected from a subset of the C. pepo collection grown in the summer of 2018 in Ithaca, NY. Field-grown plants were phenotyped for vining bush habit at three different stages during the growing seasons to confirm bush, semi-bush or vining growth habit. Plants that had a bush habit early in the season but started to vine at the end of the season were considered semi-bush.

2.5 GWAS

Variant data were filtered to MAF and missingness , and then imputed prior to association analysis. LinkImpute (Money et al., 2015), as implemented by the TASSEL (Bradbury et al., 2007) "LDKNNiImputatioHetV2Plugin" plugin was used for imputation with default settings. Any data still missing after this process were mean imputed. The GENESIS () R package, which can model both binary and continuous traits. All models included the first two PCs of the marker matrix as fixed effects and modeled genotype effect () as a random effect distributed according to the kinship () matrix (). Binary traits

were modeled using a logistic regression with GENESIS. The kinship matrix was calculated using A.mat from rrBLUP (Endelman, 2011) with mean imputation.

2.6 Genomic Heritability

An estimate of genomic heritability (de los Campos et al., 2015) was calculated for all ordinal and quantitative traits using a similar model an equivalent model to what was used for GWAS. Variance components from the random genetic effect and error were then used to calculate the heritability as .

2.7 Syntenty of Bu putative region in C. pepo and C. maxima

All tools used in the analysis can be found on the Cucurbit Genomics website (http://cucurbitgenomics.org/). A candidate gene for dwarfism, Bu, in C. maxima was elucidated by a previous study23 and was named Cma_004516. The Cucurbit Genomics Database gene ID of Cma_004516 was identified by using the BLAST tool to align primer sequences used for RT-QPCR in the previous study against the C. maxima reference genome. The synteny analysis was done by using the Synteny Viewer tool and evaluating C. maxima's chromosome 3 with C. pepo's chromosome 10 and searching for an ortholog to the candidate gene. The physical position of the C. pepo ortholog was identified by searching the gene using the Search tool. All tools used in the analysis can be found on the Cucurbit Genomics Database at cucurbitgenomics.org.

2.8 Identification of a Core Collection

Subsets representative of each panel's genetic diversity were identified through running GenoCore (Jeong et al., 2017) using the filtered SNP sets. The GenoCore settings were "-cv 99 -d 0.001".

3 RESULTS

4 DISCUSSION

CONFLICT OF INTEREST STATEMENT

Michael Mazourek is a co-founder of Row 7 Seeds, but neither receives compensation nor holds equity.

AUTHOR CONTRIBUTIONS

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DATA AVAILABILITY STATEMENT

The datasets generated for this study includeing variant and raw sequence data are available on the Cucurbit Genomics Dataase at cucurbitgenomics.org. The phenotypic data used are available for download from

the USDA Germplasm Resources Information Network (GRIN; www.ars-grin.gov). Intermediate files and code used in the study are available on Github at www.github.com/ch728/Cucurbita-USDA.

REFERENCES

Alexander, D. H. and Lange, K. (2011). Enhancements to the ADMIXTURE algorithm for individual ancestry estimation. *BMC Bioinformatics* 12. doi:10.1186/1471-2105-12-246

Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y., and Buckler, E. S. (2007). TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics* 23, 2633–2635. doi:10.1093/bioinformatics/btm308

Chomicki, G., Schaefer, H., and Renner, S. S. (2020). Origin and domestication of Cucurbitaceae crops: insights from phylogenies, genomics and archaeology. *New Phytologist* 226, 1240–1255. doi:10.1111/nph.16015

Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., et al. (2021). Twelve years of SAMtools and BCFtools. *GigaScience* 10. doi:10.1093/gigascience/giab008

de los Campos, G., Sorensen, D., and Gianola, D. (2015). Genomic heritability: What is it? *PLOS Genetics* 11, e1005048. doi:10.1371/journal.pgen.1005048

Endelman, J. B. (2011). Ridge regression and other kernels for genomic selection with r package rrBLUP. *The Plant Genome* 4, 250–255. doi:10.3835/plantgenome2011.08.0024

Ferriol, M. and Picó, B. (2008). Pumpkin and winter squash. In *Handbook of Plant Breeding* (Springer New York). 317–349. doi:10.1007/978-0-387-30443-4_10

Glaubitz, J. C., Casstevens, T. M., Lu, F., Harriman, J., Elshire, R. J., Sun, Q., et al. (2014). TASSEL-GBS: A high capacity genotyping by sequencing analysis pipeline. *PLoS ONE* 9, e90346. doi:10.1371/journal.pone.0090346

Hernandez, C. O., Wyatt, L. E., and Mazourek, M. R. (2020). Genomic Prediction and Selection for Fruit Traits in Winter Squash. *G3 Genes|Genomes|Genetics* 10, 3601–3610. doi:10.1534/g3.120.401215

Jeong, S., Kim, J.-Y., Jeong, S.-C., Kang, S.-T., Moon, J.-K., and Kim, N. (2017). GenoCore: A simple and fast algorithm for core subset selection from large genotype datasets. *PLOS ONE* 12, e0181420. doi:10.1371/journal.pone.0181420

Kates, H. R., Soltis, P. S., and Soltis, D. E. (2017). Evolutionary and domestication history of cucurbita (pumpkin and squash) species inferred from 44 nuclear loci. *Molecular Phylogenetics and Evolution* 111, 98–109. doi:10.1016/j.ympev.2017.03.002

Kaźmińska, K., Hallmann, E., Rusaczonek, A., Korzeniewska, A., Sobczak, M., Filipczak, J., et al. (2018). Genetic mapping of ovary colour and quantitative trait loci for carotenoid content in the fruit of Cucurbita maxima Duchesne. *Molecular Breeding* 38, 114. doi:10.1007/s11032-018-0869-z

Li, H. and Durbin, R. (2009). Fast and accurate short read alignment with burrows-wheeler transform. *Bioinformatics* 25, 1754–1760. doi:10.1093/bioinformatics/btp324

Loy, J. B. (2004). Morpho-physiological aspects of productivity and quality in squash and pumpkins (cucurbita spp.). *Critical Reviews in Plant Sciences* 23, 337–363. doi:10.1080/07352680490490733

Lust, T. A. and Paris, H. S. (2016). Italian horticultural and culinary records of summer squash (cucurbita pepo, cucurbitaceae) and emergence of the zucchini in 19th-century milan. *Annals of Botany* 118, 53–69. doi:10.1093/aob/mcw080

McCouch, S., Navabi, Z. K., Abberton, M., Anglin, N. L., Barbieri, R. L., Baum, M., et al. (2020). Mobilizing crop biodiversity. *Molecular Plant* 13, 1341–1344. doi:10.1016/j.molp.2020.08.011

Money, D., Gardner, K., Migicovsky, Z., Schwaninger, H., Zhong, G.-Y., and Myles, S. (2015). LinkImpute: Fast and accurate genotype imputation for nonmodel organisms. *G3 Genes|Genomes|Genetics* 5, 2383–2390. doi:10.1534/g3.115.021667

Montero-Pau, J., Blanca, J., Esteras, C., Martínez-Pérez, E. M., Gómez, P., Monforte, A. J., et al. (2017). An SNP-based saturated genetic map and QTL analysis of fruit-related traits in Zucchini using Genotyping-by-sequencing. *BMC Genomics* 18, 94. doi:10.1186/s12864-016-3439-y

Nee, M. (1990). The domestication ofcucurbita (cucurbitaceae). *Economic Botany* 44, 56–68. doi:10.1007/bf02860475

Paris, H. S. (2015). Germplasm enhancement of cucurbita pepo (pumpkin, squash, gourd: Cucurbitaceae): progress and challenges. *Euphytica* 208, 415–438. doi:10.1007/s10681-015-1605-y

Paris, H. S. and Brown, R. N. (2005). The genes of pumpkin and squash. *HortScience* 40, 1620–1630. doi:10.21273/hortsci.40.6.1620

Paris, H. S., Lebeda, A., Křistkova, E., Andres, T. C., and Nee, M. H. (2012). Parallel evolution under domestication and phenotypic differentiation of the cultivated subspecies of cucurbita pepo (cucurbitaceae). *Economic Botany* 66, 71–90. doi:10.1007/s12231-012-9186-3

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A., Bender, D., et al. (2007). PLINK: A tool set for whole-genome association and population-based linkage analyses. *The American Journal of Human Genetics* 81, 559–575. doi:10.1086/519795

Savage, J. A., Haines, D. F., and Holbrook, M. N. (2015). The making of giant pumpkins: how selective breeding changed the phloem of cucurbita maxima from source to sink. *Plant, Cell & Environment* 38, 1543–1554. doi:10.1111/pce.12502

Sun, H., Wu, S., Zhang, G., Jiao, C., Guo, S., Ren, Y., et al. (2017). Karyotype Stability and Unbiased Fractionation in the Paleo-Allotetraploid Cucurbita Genomes. *Molecular Plant* 10, 1293–1306. doi:10.1016/j.molp.2017.09.003

Wang, X., Ando, K., Wu, S., Reddy, U. K., Tamang, P., Bao, K., et al. (2021). Genetic characterization of melon accessions in the u.s. national plant germplasm system and construction of a melon core collection. *Molecular Horticulture* 1. doi:10.1186/s43897-021-00014-9

Wang, X., Bao, K., Reddy, U. K., Bai, Y., Hammar, S. A., Jiao, C., et al. (2018). The USDA cucumber (cucumis sativus l.) collection: genetic diversity, population structure, genome-wide association studies, and core collection development. *Horticulture Research* 5. doi:10.1038/s41438-018-0080-8

Wu, P.-Y., Tung, C.-W., Lee, C.-Y., and Liao, C.-T. (2019a). Genomic Prediction of Pumpkin Hybrid Performance. *The Plant Genome* 12, 180082. doi:10.3835/plantgenome2018.10.0082

Wu, S., Wang, X., Reddy, U., Sun, H., Bao, K., Gao, L., et al. (2019b). Genome of 'charleston gray', the principal american watermelon cultivar, and genetic characterization of 1,365 accessions in the u.s. national plant germplasm system watermelon collection. *Plant Biotechnology Journal* 17, 2246–2258. doi:10.1111/pbi.13136

Xanthopoulou, A., Montero Pau, J., Mellidou, I., Kissoudis, C., Blanca, J., Picó, B., et al. (2019). Whole-genome resequencing of Cucurbita pepo morphotypes to discover genomic variants associated with morphology and horticulturally valuable traits. *Horticulture Research* 6, 94. doi:10.1038/s41438-019-0176-9

Yu, J., Wu, S., Sun, H., Wang, X., Tang, X., Guo, S., et al. (2022). CuGenDBv2: an updated database for cucurbit genomics. *Nucleic Acids Research* doi:10.1093/nar/gkac921

Zhong, Y.-J., Zhou, Y.-Y., Li, J.-X., Yu, T., Wu, T.-Q., Luo, J.-N., et al. (2017). A high-density linkage map and QTL mapping of fruit-related traits in pumpkin (Cucurbita moschata Duch.). *Scientific Reports* 7, 12785. doi:10.1038/s41598-017-13216-3

FIGURE CAPTIONS

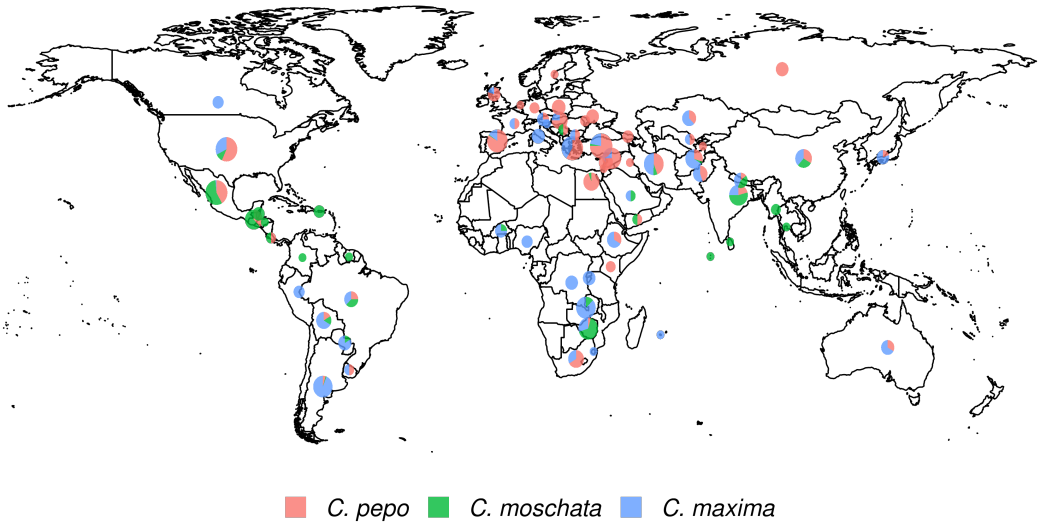

Figure 1. Geographical distribution of the USDA Cucurbita ssp. collection. The size of the pie chart is scaled according to the number of accessions and sector areas correspond to the proportion of the three species.

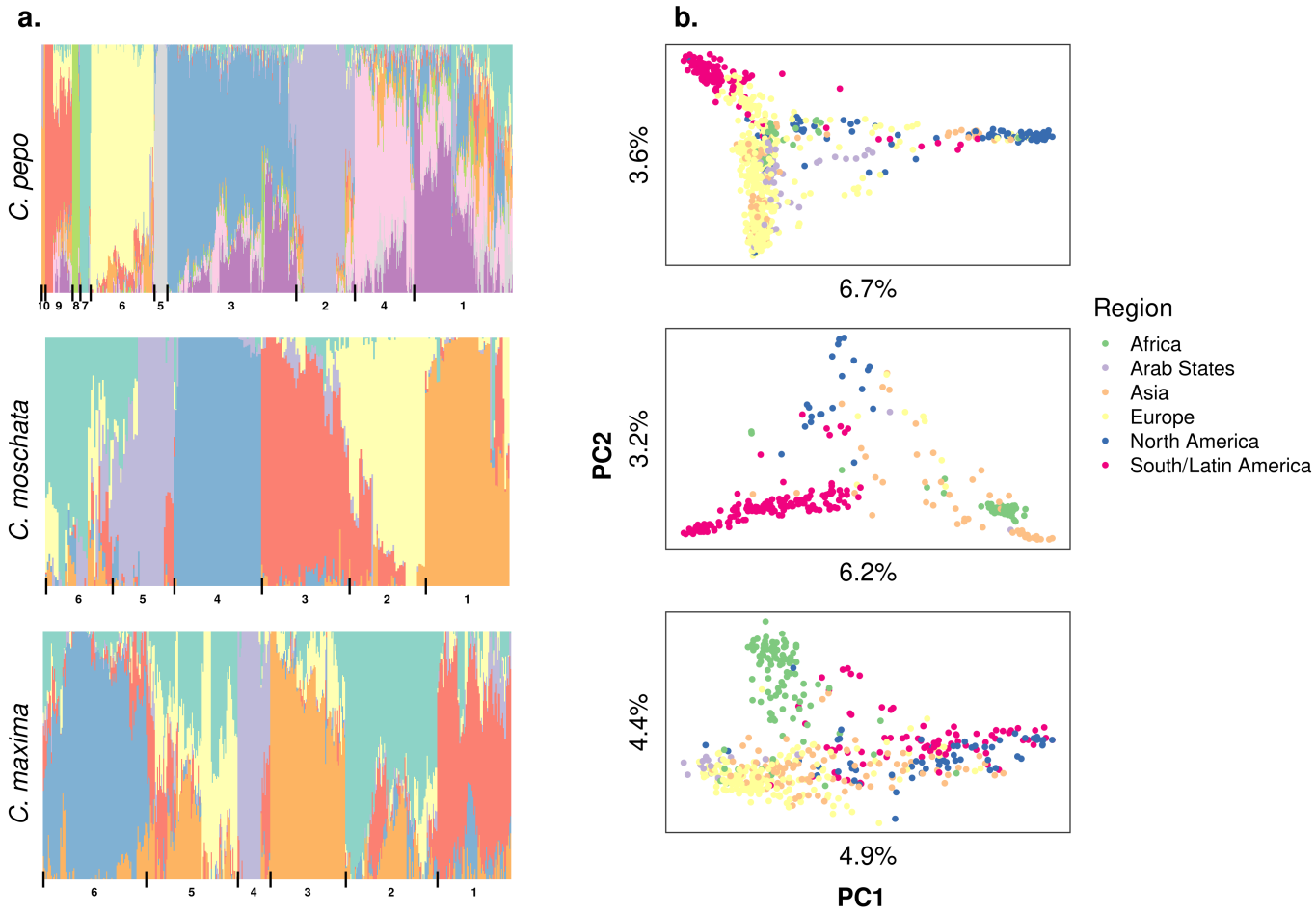

Figure 2. Population structure results aligned vertically by species. Panel a. Admixture plots: each stacked barplot represents an accession colored by proportion of inferred ancestral population. Groups based on hierarchical clustering are delimited by vertical bars and labeled with numbers along the bottom. Panel b. Plots of the first two principal components (PC) of accessions colored by region, variation explained by PCs is labeled on each axis.

TABLES

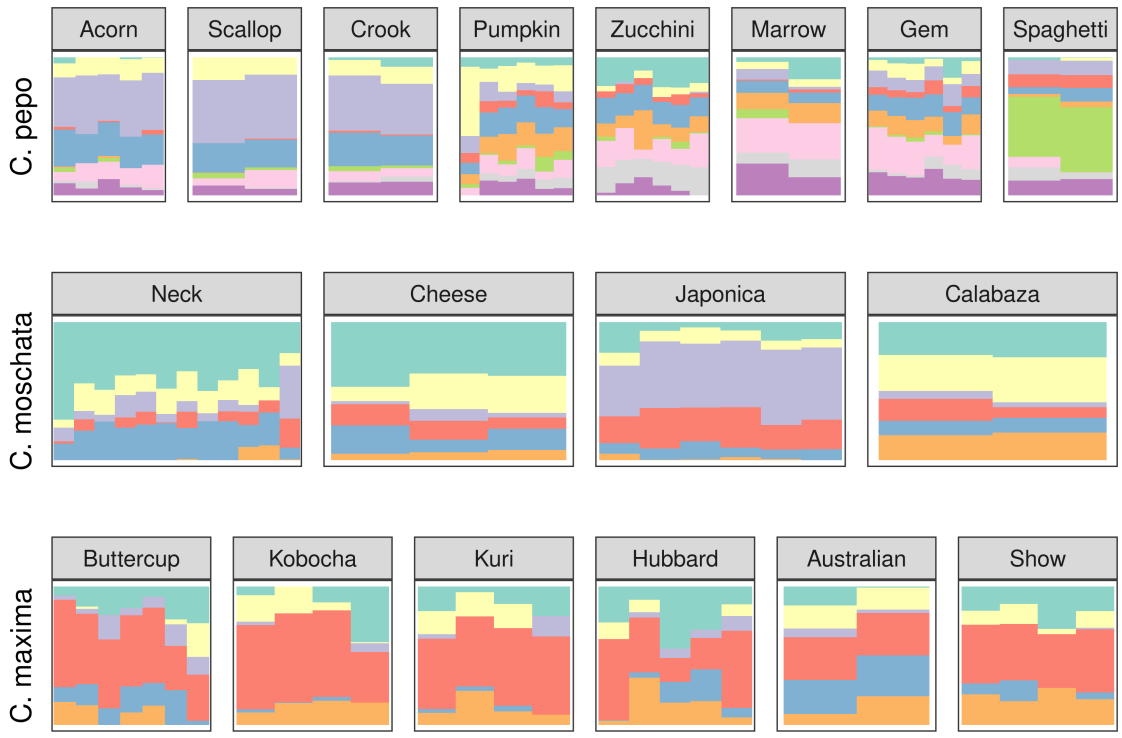

Figure 3. Ancestry coefficients projected on cultivars from each species. Results are shown grouped by market/varietal class.

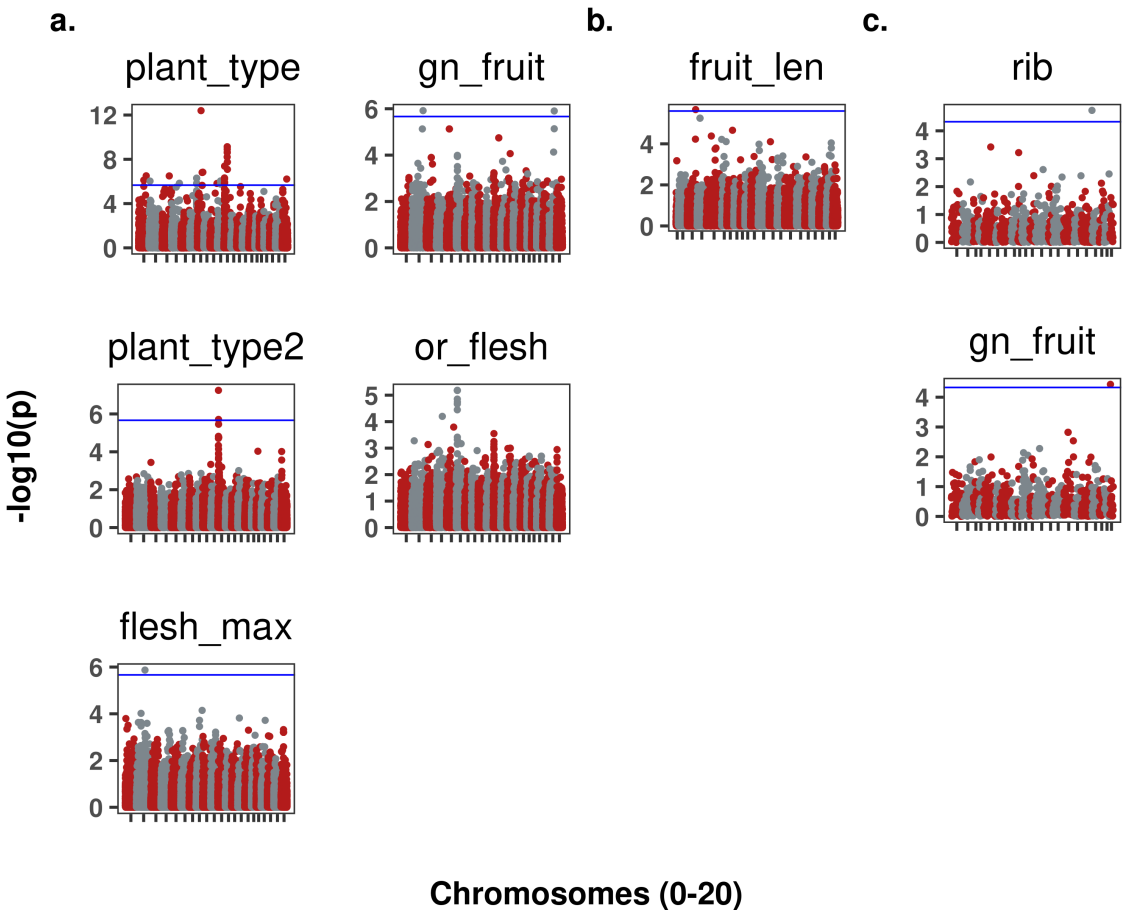

Figure 4. GWAS results: panel a. C. pepo plant_type (bush or vine historical data, Bu gene), plant_type2(bush or vine contemporary data, Bu), flesh_max (flesh maximum thickness), gn_fruit (green fruit color), or_flesh (orange flesh color); panel b. C. moschata fruit_len (fruit length); panel c. C. maxima rib (degree of fruit ribbing); gn_fruit (green fruit color)