Evolutionary and domestication history of *Cucurbita* (pumpkin and squash) species inferred from 44 nuclear loci

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Abstract

Phylogenetics can facilitate the study of plant domestication by resolving sister relationships between crops and their wild relatives, thereby identifying the ancestors of cultivated plants. Previous phylogenetic studies of the six *Cucurbita* crop lineages (pumpkins and squashes) and their wild relatives suggest histories of deep coalescence that complicate uncovering the genetic origins of the six crop taxa. We investigated the evolution of wild and domesticated *Cucurbita* using the most comprehensive and robust molecular-based phylogeny for *Cucurbita* to date based on 44 loci derived from introns of single-copy nuclear genes. We discovered novel relationships among *Cucurbita* species and recovered the first *Cucurbita* tree with well-supported resolution within species. *Cucurbita* comprises a clade of mesophytic annual species that includes all six crop taxa and a grade of xerophytic perennial species that represent the ancestral xerophytic habit of the genus. Based on phylogenetic resolution within-species we

hypothesize that the magnitude of domestication bottlenecks varies among *Cucurbita* crop lineages. Our phylogeny clarifies how wild *Cucurbita* species are related to the domesticated taxa. We find close relationships between two wild species and crop lineages not previously identified. Expanded geographic sampling of key wild species is needed for improved understanding of the evolution of domesticated *Cucurbita*.

Keywords: *Cucurbita* (pumpkin), domestication, evolution, phylogeny, single-copy nuclear genes, crop wild relatives

1. Introduction

The domestication of *Cucurbita* (squashes and pumpkins) is among the earliest on record, with archaeological evidence indicating that *Cucurbita* species were used by humans as early as *ca.* 10,000 B.P. (Smith, 2006). With an incredibly rich and long history of domestication and cultivation, *Cucurbita* crops are unrivalled in fruit morphological diversity and their wide range of adaptation to cultivation. Among all plants cultivated for edible fruit, squashes and pumpkins have the most dramatic increase in fruit size compared with their wild relatives (Savage *et al.*, 2015).

Each of the six cultivated species of squashes and pumpkins exhibits the domestication traits noted above; different genetic changes could underlie their convergent morphologies in that each crop appears to have originated from a different wild species. Furthermore, the cultivated taxa exhibit different adaptations to cultivation (Nee, 1990). For example, *C. ficifolia* (figleaf gourd) is adapted to cool temperatures and short days whereas *C. moschata* (butternut squash, Seminole pumpkin, canned pumpkin, and others) grows best in the humid tropics. *Cucurbita pepo* ssp. *pepo* (jack-o-lantern pumpkin, zucchini, summer squash, spaghetti squash, and others) is adapted to the widest range of habitats and is the most widely cultivated taxon (Nee, 1990). The multiple domestication events from diverse wild ancestors in *Cucurbita* serve as replicates of domestication and therefore make *Cucurbita* an especially valuable system in which to study crop evolution.

Cucurbita ($2n = 40$) comprises 14 species, with six subspecies and two wild varieties (Nesom, 2011) (Table 1). The wild taxa occupy diverse elevational and latitudinal habitats

62 ranging from the Midwestern United States to southern Argentina, with a center of diversity in
63 Mexico (Fig. 1). Five of the wild *Cucurbita* species are xerophytic perennials that occur in
64 Mexico and the southwestern United States; seven wild species are mesophytic annuals that
65 occur in Mexico, the southeastern and southcentral United States, and Central and South
66 America (Nee, 1990). The mesophytic annuals are apparently the result of a very recent radiation
67 (~7 mya) from a common ancestor in Central or South America (Schaefer *et al.*, 2009).

68 The six *Cucurbita* crop lineages are hypothesized to be of independent origin from
69 different wild ancestors in different geographic regions (Nee, 1990) (Fig. 1). Whereas the origins
70 of many similarly valuable crops (e.g. common bean: Gepts, 1996; kiwi: Atkinson *et al.*, 1997;
71 cassava: Olsen *et al.*, 1999; pea: Jing *et al.*, 2011; eggplant: Meyer *et al.*, 2012) have been
72 identified, the ancestors of four of the six *Cucurbita* crop lineages, including the two most
73 economically important, *C. pepo* ssp. *pepo* and *C. moschata*, are still unknown, despite repeated
74 efforts to identify them (e.g. Sanjur *et al.*, 2002; Zheng *et al.*, 2013; Kistler *et al.*, 2015).

75 Phylogenetic studies of crops and their wild relatives are a necessary crucial first step in
76 investigating the genetic basis of domestication, as interpretation of any phenotype in an
77 evolutionary context requires a phylogeny (Kellogg & Birchler, 1993; Olsen
78 & Purugganan, 2002; Caicedo & Schaal, 2004; Collins *et al.*, 2005). Furthermore, resolving
79 sister relationships between crops and their wild relatives (e.g. Hoffman *et al.*, 1986; Mayer
80 & Soltis, 1994) is key to identifying the ancestors of cultivated plants. Moreover, studies that
81 include sufficiently broad geographic sampling can point to geographic regions where close
82 relationships between wild populations and the domesticate suggest a center of domestication
83 (e.g. Smykal *et al.*, 2010; Huang *et al.*, 2012; Meyer *et al.*, 2012).

84 Studies of evolutionary relationships within domesticated groups have contributed greatly
85 to our understanding of the history and origin of cultivated plants. For example, researchers
86 argued for decades about whether the distinctive architecture of maize could be the product of a
87 single vs. multiple origins (e.g. Wilkes, 1977; Doebley & Iltis, 1980; Doebley, 1990). However,
88 a genetic study of relationships within *Zea* (Matsuoka, 2002) settled this debate by showing
89 that cultivated maize is most closely related to teosinte from Oaxaca, Mexico, with all other
90 wild *Zea* species outside of this clade.

91 Much of what is known about the origins of domestication in *Cucurbita* comes from a
92 combination of archaeological findings (e.g. Piperno & Pearsall, 1998; Smith, 2006), studies of

the genetic diversity within individual lineages in the genus (e.g. Decker-Walters *et al.*, 2002; Ferriol *et al.*, 2003; Paris *et al.*, 2003; Andres, 2006; Heikal *et al.*, 2008, Gong *et al.*, 2012), and extensive observations of wild and cultivated *Cucurbita* taxa in their native ranges throughout North, Central, and South America (e.g. Nee, 1990). Some longstanding ideas about squash and pumpkin domestication therefore represent hypotheses that have not been supported with genetic data. Sanjur *et al.*, (2002) hypothesized that the ancestor of *C. pepo* ssp. *pepo* is a close relative of the wild *C. pepo* ssp. *fraterna*, but no phylogeny has resolved the *C. pepo* clade to support this suggestion (e.g. Sanjur *et al.*, 2002; Zheng *et al.*, 2013; Kistler *et al.*, 2015). Nee (1990) proposed northern Colombia as the likely site of origin of domestication for *C. moschata*, but no published phylogeny has included sufficient samples of this taxon to find a geographic center of phylogenetic diversity that allows for further investigation of this hypothesis with additional phylogeographic analyses.

Molecular phylogenetic studies of *Cucurbita* support the proposed ancestry of two of the six crops: *C. maxima* ssp. *maxima* (giant pumpkin, hubbard squash, kabocha squash) and *C. argyrosperma* ssp. *argyrosperma* (cushaw pumpkin, Japanese pie pumpkin) (Sanjur *et al.*, 2002; Zheng *et al.*, 2013; Kistler *et al.*, 2015). However, the crop and breeding communities continue to rely on putative relationships such as those described above, and phylogenetic studies have thus far been unable to offer alternative hypotheses for the wild ancestors of four of the crop lineages (Sanjur *et al.*, 2002; Zheng *et al.*, 2013; Kistler *et al.*, 2015). Although it is possible that the wild ancestors of crops with unclear ancestry are extinct, previous studies have also been limited by sparse taxonomic, geographic, and genetic sampling. Furthermore, the very recent and rapid diversification of the wild species and potential hybridization among *Cucurbita* species (Bemis & Nelson, 1963) may impede phylogenetic inference using slow-evolving and maternally inherited chloroplast DNA sequence data.

Here we improve upon previous phylogenetic knowledge of *Cucurbita* through increased taxonomic, geographic, and genetic sampling. We present a phylogenetic reconstruction of all cultivated squashes, pumpkins, and gourds and 13 of the 14 crop wild relatives based on multiple, unlinked nuclear loci that provides the first support for close relationships between wild species and two of the six crop taxa. Through our study, we hope to enhance *Cucurbita* as a study system for investigating adaptive response to selection under domestication.

2. Materials and methods

2.1 Plant material and DNA extraction

When species divergences are recent (resulting in incomplete lineage sorting), sampling more individuals per species leads to the reconstruction of a more accurate species tree (Maddison & Knowles, 2006). Our analysis is the first reconstruction of *Cucurbita* phylogeny to include multiple individuals of 19 taxa, with 92 individuals from 19 of the 20 species, subspecies, and wild varieties of *Cucurbita* (all taxon names following Nesom, 2011). However, we were unable to obtain material from *C. radicans*, a xerophytic species narrowly-distributed in western Mexico. Two outgroup species, *Citrullus lanatus* and *Cucumis sativus*, were selected based on phylogenetic results for Cucurbitaceae (Schaefer *et al.*, 2011) (Supplementary Table 1). Leaf samples for DNA extraction were obtained from seedlings grown at the University of Florida, Gainesville, FL, from seeds obtained from the USDA or from herbarium specimens (Supplementary Table 1). DNA was extracted from fresh or dried leaf tissue using a modified 2X CTAB method (Doyle & Doyle, 1987) yielding ~50-120 ng/μl of DNA per sample. Modifications to the Doyle & Doyle (1987) protocol include a second addition of chloroform-isoamyl alcohol to the aqueous phase immediately following the first addition, and precipitation of DNA at -20°C instead of room temperature. We used a precipitation time of thirty-minutes for fresh tissue and overnight for dried tissue.

2.2 Selection of nuclear loci

To improve the resolution of relationships within and among closely related taxa (following e.g. Parks *et al.*, 2009; Steane *et al.*, 2011), we sampled loci that are unlinked and more variable than those used in previous analyses of *Cucurbita* phylogeny (Sanjurjo *et al.*, 2002; Zheng *et al.*, 2013; Kistler *et al.*, 2015). We identified 85 putatively single-copy, highly variable nuclear loci from a set of four reference genomes and transcriptomes (watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), melon (*Cucumis melon*), and pumpkin (*Cucurbita pepo* ssp. *pepo*; icugi.org), hereafter “cucurbit references”) using a custom pipeline (Supplementary Fig. 1). Our pipeline selected intron sequences (400-700 bp) flanked by conserved exons with no highly similar sequence(s) likely to correspond to a paralog present in the same genome or transcriptome. Sequence similarity among putative genes in the four references was determined by BLAST with an e-value cutoff of 1.0e-5.

2.3 Microfluidic PCR amplification and Illumina sequencing

To amplify the 85 potentially phylogenetically informative loci, we used primer3 (Untergasser *et al.*, 2012) in Geneious ver. 7.1.9 (<http://www.geneious.com>, Kears *et al.*, 2012) to design primers with uniform 60°C annealing temperatures and no homopolymers of length greater than three, as recommended for the Fluidigm Access Array™ System (Fluidigm, San Francisco, CA, USA). We used alignments of the four cucurbit references to identify exon regions conserved across all cucurbit references and designed primers from *C. pepo* ssp. *pepo* exon sequences in these regions to amplify intron sequences (Supplementary Table 2).

In addition to the target-specific primer sequences identified, two conserved sequences (CS1 and CS2, Access Array™ System for Illumina Sequencing Systems User Guide) were added to the 5' end of both the forward and reverse primers to provide an annealing site for a second pair of custom-designed primers composed of the complementary CS sequence, Illumina adapters, and a sample-specific forward and reverse barcode to allow for all samples to be multiplexed in a fraction of a sequencing run.

Each primer set was validated prior to microfluidic PCR amplification to ensure success under the specific conditions on the Fluidigm Access Array™ (Uribe-Convers *et al.*, 2016). We performed primer validation reactions on one of the species used for primer design, *C. pepo*, including both fresh and herbarium-derived samples, and on *C. foetidissima*, which is thought to be among the most phylogenetically distant species from *C. pepo*, based on previous phylogenetic studies (Zheng *et al.*, 2013). We performed primer validation for each primer set in 10-μL reactions following the Access Array™ System for Illumina Sequencing Systems User Guide. Reactions were as follows: 1 μL of 10X FastStart High Fidelity Reaction Buffer without MgCl₂ (Roche Diagnostic Corp., Indianapolis, Indiana, USA), 1.8 μL of 25 mM MgCl₂ (Roche), 0.5 μL DMSO (Roche), 0.2 μL 10mM PCR Grade Nucleotide Mix (Roche), 0.1 μL of 5 U/μL FastStart High Fidelity Enzyme Blend (Roche), 0.5 μL of 20X Access Array™ Loading Reagent (Fluidigm), 2 μL of 2 μM barcoded primers, 2 μL of 50 nM target-specific primers, 0.5 μL of 25-50 ng/μL genomic DNA, and 1.4 μL of PCR Certified Water. Resulting amplicons from successful primer validation reactions were analyzed using a Bioanalyzer High-Sensitivity Chip (Agilent Technologies, Santa Clara, California, USA), and the 48 primer pairs that produced a single amplicon of the expected size and that had no primer dimers were selected (Supplementary Table 2) for microfluidic PCR.

Access Array™ System (Fluidigm) Microfluidic PCR, Illumina MiSeq sequencing, and quality-control steps were performed at the University of Idaho Institute for Bioinformatics and Evolutionary Studies Genomics Resources Core Facility. Microfluidic PCR was performed in an Access Array™ System (Fluidigm) using two 48.48 Access Array™ Integrated Fluidic Circuits (Fluidigm) following the manufacturer's protocols. On this array, 48 samples were simultaneously amplified using the 48 unique primer pairs described above. The resulting ~2,304 unique PCR amplicons were harvested and pooled by individual in equal volumes and sequenced on 1/10th of a run of an Illumina MiSeq using 300-bp paired-end reads.

2.4 Assembly of sequencing reads into targeted nuclear loci

Demultiplexing and assembly of Illumina sequencing reads followed Uribe-Convers *et al.* (2016). Briefly, Illumina sequencing reads were demultiplexed with the python preprocessing application dbcAmplicons (<https://github.com/msettles/dbcAmplicons>) by individual using the individual-specific dual barcode combinations and by locus using the target-specific primers. To address heterozygosity of nuclear loci, we performed two assemblies of Illumina reads into representative sequences for each of the 48 amplicons using the reduce_amplicons R script (https://github.com/msettles/dbcAmplicons/blob/master/scripts/R/reduce_amplicons.R) described in Uribe-Convers *et al.* (2016). In cases where the target locus was longer than the paired-end read length, the two reads were concatenated into a single sequence prior to assembly with reduce_amplicons. One assembly used the “-p ambiguity” option to generate consensus sequences with IUPAC ambiguity codes, and the second assembly used the “-p occurrence” option to generate allelic occurrences for every individual at every locus. Briefly, “-p ambiguity” calls an ambiguity code for any site represented by more than one base when each variant is present in at least 5 reads and 5% of the total of reads. The “-p occurrence” option assembles reads into two alleles at heterozygous loci by reducing reads to counts of identical reads if the count represents at least 5% of the total of reads and contains at least 5 reads. This method of assembly recovers true alleles if the following assumptions are met: sequencing errors are mostly random, and reads containing errors are present at much lower frequencies than real biological variation (Uribe-Convers *et al.*, 2016). These two methods of assembly resulted in two data matrices for downstream analysis: 1) consensus sequences with IUPAC ambiguity codes (“ambiguity data set”), and 2) consensus sequences with two alleles per individual for any

individual heterozygous at a locus (“allelic data set”) such that each heterozygous individual was represented by two tips in gene trees.

2.5 Sequence alignment and phylogenetic analyses

Each locus was aligned with MUSCLE v 3.8.31 (Edgar, 2004) using default settings; alignments were visually inspected and edited in Geneious ver. 7.1.9 (<http://www.geneious.com>, Kearse *et al.*, 2012). Alignments with data for fewer than 20 individuals were not included in the analyses, resulting in 42 alignments for both the ambiguity and allelic data sets. Two alignments were inferred to include paralogous loci based on the occurrence of more than two alleles per individual and preliminary maximum likelihood (ML) phylogeny reconstruction of each locus using RAxML v 8.2.3 (Stamatakis, 2014) and 1,000 bootstrap replicates under the GTRCAT model. Paralogy was inferred using gene phylogenies reconstructed as described above -- if two major clades were resolved that each contained a set of sequences that represented all or most sampled taxa then this was considered to represent paralogy. The paralogous loci were separated into four putative loci based on the major clades described above, resulting in data sets of 44 loci with a total aligned length of 20.3 kb. Observed heterozygosity was calculated as the proportion of individuals with two alleles at a locus, averaged across 44 loci.

To compare alternative strategies for phylogenetic analyses of a multi-locus nuclear data set, we performed a concatenated analysis using ML (“CA-ML”) of the ambiguity data set and species tree estimation using the gene trees inferred from the ambiguity and allelic data sets. Prior to concatenation and species tree estimation, we conducted ML analysis of each locus using RAxML as described above for both the ambiguity and allelic alignments. We assessed taxon-level congruence of the resulting gene trees (with nodes with less than 50% bootstrap support (“BS”) collapsed) by visual inspection of each topology, rather than standard congruence tests, to avoid erroneous detection of incongruence due to missing data (Leigh *et al.*, 2011).

Two likely sources of observed gene tree incongruence in our dataset are error in gene trees reconstructed from short loci and incomplete lineage sorting. We proceeded with CA-ML analysis despite gene tree incongruence because concatenation methods are robust to short loci but cannot account for incomplete lineage sorting. Thus comparison of CA-ML and species tree estimation results may aid in distinguishing incongruence due to gene tree error from that due to incomplete lineage sorting. Because there is no straightforward way to concatenate multiple alleles across alignments in the absence of a chromosomal linkage map (e.g. Edwards *et al.*,

2008), we did not analyze the allelic data set using the concatenated approach. ML analysis of the concatenated ambiguity data set was conducted using the hybrid MPI/Pthreads version of RAxML v 8.2.3 (Stamatakis, 2014) with 1,000 bootstrap replicates under the GTRCAT model. The GTRCAT model was selected using JModelTest2 (Darriba *et al.*, 2012; Guindon and Gascuel 2003) and the CAT approximation of rate heterogeneity was used to accelerate computation (Stamatakis 2006).

We used a coalescent-based species tree approach as implemented in ASTRAL v 4.7.4 (Mirarab & Warnow, 2015) to explore the effects of incongruence among gene trees on the species topology. ASTRAL is statistically consistent under the multi-species coalescent model and estimates a species tree given a set of unrooted gene trees (Mirarab & Warnow, 2015). We present two results of ASTRAL species tree estimation: one from the allelic data set ("Sp-TR-alleles") and one from the ambiguity data set ("Sp-TR-amb"). Each species tree was estimated from a set of 44 ML Majority-Rule-Consensus gene trees to reduce the effect of erroneous signals that may be present in poorly supported branches of best ML trees. For the allelic data set, we used a multi-individual mapping file (ASTRAL -a option) to map alleles to individuals in the case of heterozygotes as there is currently no feasible way to estimate a species tree from gene trees with multiple alleles per individual without mapping alleles to individuals. We performed multi-locus bootstrapping with 1,000 replicates for both trees.

2.6 Ancestral state reconstruction

We examined the evolution of mesophytic and xerophytic habits using a topology we manually constructed to represent species-level relationships all of which were resolved congruently in both the CA-ML and species trees. We used two outgroup taxa, *Peponopsis adhaerens* and *Polyclathra cucumerina*, placed based on the phylogeny of the entire family Cucurbitaceae (Schaefer *et al.* 2009) to polarize the reconstruction. To create a bifurcating tree for ancestral character state reconstruction, we collapsed xerophytic species into two terminals (Xerophytes 1 and Xerophytes 2) that represent the two xerophytic clades of non-monophyletic species resolved in the species trees and CA-ML tree. Branch lengths for this tree were optimized in RAxML v 8.2.3 (Stamatakis, 2014) using an alignment of GenBank sequence data of four chloroplast loci with one representative for each terminal.

Habit (xerophytic or mesophytic) was scored from the literature (Schaefer & Renner, 2011). We conducted ML reconstructions using the ACE (ancestral character estimations)

function of the ape R package (Paradis *et al.*, 2004; R Development Core Team, 2010). We tested two alternative models, ER (equal rates; assuming equal transition rates between the character states) and ARD (all-rates-different; transitions between the character states allowed to vary freely). The ARD model had a higher log likelihood value of -4.54 compared with -4.61. However, a chi square test of the difference in log likelihood between the two models yielded a p-value of 0.7. We therefore did not accept the more heavily parameterized ARD model, and instead chose the single-parameter ER model.

3. Results

3.1 Sequence data

Success of microfluidic PCR amplification was correlated with phylogenetic distance of a taxon to *Cucurbita pepo* ssp. *pepo*, from which the PCR primers were designed (Fig. 2). We did not observe a correlation between primer length and amplification success (Supplementary Fig. 2), and although we did observe a lower proportion of amplification success for samples derived from herbarium specimens (Supplementary Fig. 2), our sample set included four herbarium samples and ninety-four fresh leaf samples, so this comparison may be biased. The variable amplification success across taxa resulted in an initial data matrix with 42% missing data and 81% taxon coverage density (Fig. 2). However, substantially more missing data have been shown to be non-problematic in simulation and empirical studies (e.g. Driskell *et al.*, 2004; Philippe *et al.*, 2004; Wiens & Moen, 2008; Wiens *et al.*, 2010). In fact, including taxa with highly incomplete, non-random character sampling in phylogenetic analyses has been shown to dramatically improve results in some cases (Wiens 2006). Furthermore, some species tree methods, including ASTRAL, are robust to issues that arise because of non-random missing data when concatenation is used (Zhengxiang Xi *et al.* 2015). Nonetheless, to be cautious, we removed samples that occupied less than four gene alignments yielding a final data matrix of 94 samples with only 24% missing data. The numbers of aligned bases, including gaps, for 44 loci totaled 20.3 kb; 19.9 kb nucleotide positions were variable. Heterozygosity varied among species between ~7% in *C. lundelliana* and *C. palmata* to ~43% in *C. foetidissima*, and observed heterozygosity was 22% overall.

3.2 Phylogeny of *Cucurbita* based on CA-ML and species tree methods

The phylogeny of *Cucurbita* species provides strong support (BS 100%) for the monophyly of the mesophytic species, including the domesticated figleaf gourd, *C. ficifolia*. The xerophytic species form a grade with *C. digitata* + *C. cordata* sister to the rest of *Cucurbita*, and a clade formed by accessions of *C. foetidissima* and *C. pedatifolia* (neither of which is monophyletic) sister to all mesophytic species (Fig. 3). An accession of the xerophytic *C. palmata* is a member of each of these xerophytic clades.

The clade of mesophytic species includes the six *Cucurbita* crop lineages and their close relatives (Fig. 3). We recovered *C. pepo* (domesticated pumpkin, domesticated ornamental gourd, and their close wild relatives) sister to *C. okeechobeensis* + *C. lundelliana*. Together these three species are sister to the *C. moschata* (butternut squash) + *C. argyrosperma* (domesticated cushaw pumpkin and its close wild relative) clade. We resolved the species pair of *C. maxima* (giant pumpkin and its close wild relative) + *C. ecuadorensis* as sister to the rest of the mesophytic species.

For considerations of within-species resolution we will refer to the CA-ML tree, although we note that the concatenation method used can increase phylogenetic resolution despite incongruent within-species relationships among gene trees (cite). We define percent resolution within a clade as the number of nodes with > 50% BS divided by the number of possible bifurcations (two less than the number of tips). *Cucurbita maxima* is 42% resolved in the CA-ML tree (Fig. 3) and is the only species in which the cultivated and wild accessions form separate clades, with 100% and 92% BS, respectively. We did not observe a geographic association within the clade of cultivated accessions of *C. maxima* ssp. *maxima*.

Cucurbita argyrosperma is 31% resolved in the CA-ML tree (Fig. 3). In contrast to the pattern in *C. maxima*, the wild and cultivated accessions of *C. argyrosperma* do not form separate clades. Three of the five subclades recovered (BS > 50%) include both wild and cultivated accessions. We included wild and cultivated accessions of *C. argyrosperma* from throughout both the native range and the range of cultivation in northwestern Mexico, eastern Mexico, southwestern Mexico, western Mexico, Central America, and the United States and found no geographic pattern associated with phylogenetic resolution within *C. argyrosperma*.

The *C. moschata* clade is the best-resolved species clade in our phylogeny (71% resolved in the CA-ML tree) (Fig. 3). We included accessions of *C. moschata* from throughout its range

of cultivation in Mexico, Central America, South America, and the United States and found no clear geographic pattern to the relationships.

Cucurbita pepo is 48% resolved in the CA-ML tree (Fig. 3). We did not find internal support (i.e., BS > 50%) for the monophyly of either of the two wild varieties of *C. pepo* ssp. *ovifera*, and the resolution among accessions of *C. pepo* ssp. *ovifera* was not correlated with geographic origin or taxonomy. The CA-ML tree resolves accessions of cultivated *C. pepo* ssp. *ovifera* in a clade with the three wild *C. pepo* taxa. The most widely cultivated pumpkin and squash, *C. pepo* ssp. *pepo*, forms a clade (BS = 91%) sister to the rest of wild and cultivated *C. pepo* and thus is not sister to any wild accessions. *Cucurbita pepo* ssp. *fraterna* (wild) forms a clade (BS = 100%) within the *C. pepo* minus *C. pepo* ssp. *pepo* (crop) clade.

Our phylogeny does not resolve accessions of *C. okeechobeensis* as a clade, and accessions of *C. lundelliana* form a clade in the Sp-TR-amb tree (BS = 89%), but not in the CA-ML tree. There is lower support for relationships within the *C. okeechobeensis* + *C. lundelliana* clade in the CA-ML tree (Fig. 3) than in the Sp-TR-amb (Supplementary Fig. 3).

3.3 Ancestral character state reconstruction

The maximum likelihood reconstruction under the ER model showed that the xerophytic habit is the more likely character state for the ancestor of *Cucurbita* (ancestral state xerophytic: likelihood value = 59%, ancestral state mesophytic = 41%).

3.4 Differences in topology and support among the gene trees, CA-ML tree, and species trees

The branching order of species we recovered here is congruent between both species trees and the CA-ML tree (Fig. 5). Bootstrap support for between and among species relationships is similar between the CA-ML tree and Sp-TR-amb tree (Fig. 5) and lower for some nodes in the Sp-TR-alleles tree, with two exceptions: 1) *C. pepo* sister to *C. lundelliana* + *C. okeechobeensis* has 100% BS in the CA-ML tree compared with 72% in the Sp-TR-amb tree and 82% in the Sp-TR-alleles tree, and 2) the placement of the second-branching clade of xerophytic species sister to all mesophytic species has 75% BS in the CA-ML tree compared with 100% in the Sp-TR-amb tree and 97% in the Sp-TR-alleles tree. The CA-ML tree has greatest within-species resolution and the Sp-TR-alleles tree has the least within-species resolution. For example, *C. pepo* is 48% resolved in the CA-ML tree (Table 1), 36% resolved in the Sp-TR-amb tree, and 16% resolved in the Sp-TR-alleles tree. All species exhibit within-species incongruence to varying degrees between the CA-ML tree and the Sp-TR-amb tree (Fig. 5), and the low

within-species resolution in the species trees is likely due in part to the use of majority-rule consensus gene trees to estimate these trees.

All gene trees (reconstructed from both ambiguity and allele sequences) had lower resolution than the CA-ML or species trees. The 44 ambiguity gene trees had an average of 80 tips and 29 bifurcations when branch lengths $< 1e-5$ were collapsed to reduce forced bifurcations. Out of the 44 ambiguity gene trees, six gene trees do not resolve most species as clades (with BS $> 50\%$) and thus do not support any species-level topology, 15 gene trees resolve a species-level topology congruent with the CA-ML tree and the species trees, and the remaining 23 gene trees support (BS $> 50\%$) alternative topologies (Supplementary Figs. 5-48). Gene tree incongruence with the CA-ML and species tree topologies is due to the variable position of *C. ficifolia* in ten of the gene trees and *C. pepo* in eight gene trees. The cause of incongruence in the remaining five gene trees varied. The 44 allele gene trees (Supplementary Figs. 49-92) were generally less resolved than the ambiguity gene trees, but showed a similar pattern of incongruence.

4. Discussion

4.1 New *Cucurbita* species topology

The species-level topology presented here (Fig. 3) includes two major clades of mesophytic species: five-species clade from North and Central America and a two-species clade from South America. The branching order within the five-species clade differs from that in previous studies (Fig. 4b,c) (Sanjur *et al.*, 2002; Zheng *et al.*, 2013; Kistler *et al.*, 2015) and Zheng *et al.* (2013) did not recover these two clades (Fig. 4d). Only two relationships among the seven species in the mesophytic clades are consistent across all molecular phylogenetic studies conducted to date: *C. maxima* as sister to *C. ecuadorensis*, and *C. lundelliana*/*C. okeechobeensis*. The lack of reciprocal monophyly of *C. lundelliana* and *C. okeechobeensis* in our trees may be due to hybridization between the cross-compatible Mexican taxa, *C. okeechobeensis* ssp. *martinezii* and *C. lundelliana* (Whitaker & Bemis, 1964). We also note however that *C. okeechobeensis* had the highest within-species proportion of missing data (65%), and *C. lundelliana* had among the lowest proportion of within-species variable sites (22%) and the characteristics of our data for these taxa could affect this result. The position of *C. pepo* (including the most widely cultivated *Cucurbita* crops) differs in each study conducted to date

(Sanjur *et al.*, 2002; Zheng *et al.*, 2013; Kistler *et al.*, 2015) and varies among our gene trees (Supplementary Figs. 5-92). Other studies resolved *C. ficifolia*, a mesophytic crop species that may live longer than a year without enlarged storage roots (Nee, 1990), as part of the grade of xerophytic species (Sanjur *et al.*, 2002; Zheng *et al.*, 2013; Kistler *et al.*, 2015), in contrast to our study's placement of *C. ficifolia* as sister to the rest of the mesophytic species (Fig. 3, Supplementary Figs. 3,4).

The xerophytic species have not been evenly sampled in previous phylogenetic studies of *Cucurbita*, so we cannot directly compare the relationships among these species found here with previous analyses. In our trees, three morphologically and geographically distinct xerophytic species (Bemis & Whitaker, 1969) are each reconstructed as non-monophyletic (*C. foetidissima*: paraphyletic, *C. pedatifolia* and *C. palmata*: polyphyletic). Although our sampling of these species is limited (Table 1), our results suggest the possibility of hybridization among xerophytic species. The three non-monophyletic xerophytic species are interfertile (*C. pedatifolia* and *C. foetidissima*, *C. foetidissima* and *C. palmata*), and where these species are sympatric they are known to hybridize (Bemis & Whitaker 1969). The samples included here were not from the same geographic region (Supplementary Table 1), but past hybridization is a possibility as *Cucurbita* fruits can disperse long distances (Schaefer *et al.*, 2009) and historical distributions of wild *Cucurbita* species were likely broader than they are currently (Kistler *et al.*, 2015). Non-monophyly of xerophytic species could also be due to lower variation in phylogenetic loci for xerophytic species compared with mesophytic annual *Cucurbita* species. Xerophytic *Cucurbita* are perennial species (Bemis & Whitaker, 1969) and thus may have a slower rate of molecular evolution than the annual species included in this study (Andreasen & Baldwin, 2001).

Phylogenetic studies of *Cucurbita*, including our results, have been based on DNA sequence data from one of three plant genomes (Sanjur *et al.*, 2002: mitochondria; Zheng *et al.*, 2013 and Kistler *et al.*, 2015: chloroplast; our study: nuclear). A lack of congruence among phylogenetic trees across these three genomes may be due to reticulate evolution and/or a lack of phylogenetically informative characters in one or more of the trees (Knowles *et al.*, 2011). Our phylogeny and that using whole plastomes (Kistler *et al.*, 2015) were reconstructed from many variable DNA characters, but despite its large number of characters, the plastome is inherited as a linked unit and therefore must be regarded as a single locus (Doyle *et al.*, 1990; Sun *et al.*, 2016).

Incongruence between our nuclear and the plastome phylogeny and among our gene trees is unsurprising as incongruence among gene trees is expected in closely related species (e.g. Maddison, 1997; Degnan & Rosenberg, 2009; Knowles, 2009), particularly groups suspected of hybridization and/or incomplete lineage sorting (Maureira-Butler *et al.*, 2008; Gerard *et al.*, 2011; Blanco-Pastor *et al.*, 2012). Some gene trees reconstructed in this study (Supplementary Figs. 5-92) resolve topologies congruent with Kistler *et al.* (2015) (four gene trees), Zheng *et al.* (2013) (four gene trees), and Sanjur *et al.* (2002) (four gene trees) in part due to the variable placement of *C. pepo* and of *C. ficifolia*. The variable position of these species among gene trees suggests possible ancient hybridization or incomplete lineage sorting. Although we do not see clear evidence of widespread hybridization in the incongruence among alternative topologies or gene trees, the possibility of hybridization and hybrid origins in *Cucurbita* should be further studied with methods that model reticulate evolution (Shizhong Xu, 2000; Linder and Rieseberg 2004).

Species tree estimation is expected to be consistent under incomplete lineage sorting (Mirarab & Warnow, 2015), therefore the higher BS value for the stem node of the mesophytic clade in the species trees (100%) than in the CA-ML tree (75%) (Fig. 5) supports incomplete lineage sorting as a probable cause of the incongruent position of the first-branching mesophytic species *C. ficifolia* among gene trees. In contrast, the lower BS value for the stem node of *C. pepo* in the Sp-TR-amb (73%) than in the CA-ML tree (100%) (Fig. 5) suggests the variable position of *C. pepo* may be due to weak phylogenetic signal associated with the low number of variable sites per gene (Patel *et al.*, 2013; Smith *et al.*, 2015) resulting in incongruence among gene trees due to error.

Whereas coalescent methods for species tree estimation assume that all gene trees used for the species tree estimation are correct and that any incongruence among the gene trees is due to incomplete lineage sorting, concatenation of loci overcomes the problem of short genes that may yield high-error gene trees by combining them into one longer locus with many variable sites (e.g. Gadagkar *et al.*, 2005). This increase in variable sites is often needed to resolve relationships among closely related species (e.g. Edwards *et al.*, 2008; Parks *et al.*, 2009; Crowl *et al.*, 2015; Uribe-Convers *et al.*, 2016), and the *Cucurbita* CA-ML tree (Fig. 3) has high tree-wide support compared with the species trees (Supplementary Figs. 3, 4).

4.2 Evolution of mesophytic habit in *Cucurbita* species

Ancestral character-state reconstruction using the species-level topology (Fig. 3, Supplementary Figs. 3,4) support a xerophytic ancestor of *Cucurbita* and a single loss of the xerophytic habit during the evolution of *Cucurbita* species (Fig. 6). This finding differs from the hypothesis of Zheng *et al.*, (2013) of multiple derivations of the mesophytic habit within *Cucurbita* and/or a mesophytic ancestor in part due to the position of *C. ficifolia* in our phylogeny. *Cucurbita ficifolia* is a crop that lacks drought-resistance and enlarged storage roots but has been resolved within the xerophytic grade in previous phylogenetic trees (Sanjur *et al.*, 2002; Zheng *et al.*, 2013; Kistler *et al.*, 2015). Although our results point to a more parsimonious trait evolution than do previous studies, a set of the 44 gene trees resolved *C. ficifolia* as sister to xerophytic species as described above.

The placement of the South American *C. maxima* + *C. ecuadorensis* clade as sister to the rest of the North American mesophytic species suggests a period of diversification within the ancestral range of *Cucurbita*, as well as by dispersal into new areas. This diversification and dispersal appears to follow the evolution of the mesophytic habit perhaps as mesophytic forms occupied new ecological niches in wetter climates south of the center of diversity of *Cucurbita*. The poor resolution of the backbone of the *Cucurbita* tree in individual gene trees points to rapid diversification following the evolution of the mesophytic habit. This hypothesis of recent and rapid radiation could explain in part why different *Cucurbita* phylogenies do not converge on a single topology of the mesophytic species. Biogeographic and diversification analyses of a *Cucurbita* phylogeny reconstructed with broader geographic sampling than we have included in this study are needed to address the above hypotheses.

4.3 Phylogenetic resolution within clades of crops and their wild relatives

We found variable patterns of resolution within each of five clades that include domesticates (domesticated *C. ficifolia* is excluded here due to insufficient sampling) to inform hypotheses for the number and magnitude of initial domestications for the *Cucurbita* crops. Accessions of *C. pepo* ssp. *ovifera* var. *ovifera* (ornamental gourd) and *C. argyrosperma* ssp. *argyrosperma* (cushaw pumpkin) included here both form multiple clusters that include both wild and domesticated accessions (three wild *C. pepo* taxa and *C. argyrosperma* ssp. *sororia*, respectively) (Fig. 3). This pattern is similar to that observed in crops found to have multiple centers of diversification, broad domestication bottlenecks, and/or introgression between wild

and domesticated species in other crops (e.g. African rice: Li *et al.*, 2011; olive: Diez *et al.*, 2015; *kabuli* chickpea: Penmetsa *et al.*, 2016).

These two crop-wild clades are each sister to a second crop clade: *C. pepo* ssp. *ovifera*/*C. pepo* ssp. *fraterna* (crop-wild) is sister to *C. pepo* ssp. *pepo* (crop), and *C. argyrosperma* (crop-wild) is sister to *C. moschata* (crop) (Fig. 3). The possibility of a related domestication history for each of these two crop/crop-wild pairs may need to be considered. A shared domestication history between two *Cucurbita* crops has only been considered (though not supported by molecular studies: Decker, 1987; Decker, 1988) in *C. pepo* because *C. argyrosperma* and *C. moschata*, though interfertile, did not appear to hybridize when grown together (Merrick, 1990). In addition, the wide diversity of fruit types in *C. moschata* cultivated in South America led to hypotheses that South America is the more likely range of its wild ancestor (Nee, 1990; Wessel-Beaver, 2000). However, our phylogeny resolves separate South American and North American/Central American *Cucurbita* clades, and the membership of *C. moschata* in the North America/Central America clade (Fig. 3) makes it unlikely that the closest wild ancestor of *C. moschata*, if extinct or uncollected, would be found in South America. Furthermore, at least one study has now confirmed natural hybridization between *C. argyrosperma* and *C. moschata* (Wessel-Beaver, 2000).

In contrast to the similar pattern of relatedness within and between the four *Cucurbita* crops included in *C. pepo* and *C. argyrosperma* + *C. moschata*, the crop and wild accessions of *C. maxima* (giant pumpkin) form two sister clades and this crop-wild clade is not sister to another crop clade (Fig. 3). The resolution of *C. maxima* into separate wild and crop clades is similar to patterns seen in crops for which single domestications and low introgression between crop and wild taxa have been hypothesized (e.g. Einkorn wheat: Heun *et al.*, 1997; maize: Matsuoka *et al.*, 2002; soybean: Guo *et al.*, 2010). The phylogenetic resolution and observed heterozygosity among accessions of cultivated *C. maxima* ssp. *maxima* included in our study are the lowest of all the *Cucurbita* crops, further supporting the hypothesis of a narrow domestication bottleneck and a single domestication and limited post-domestication diversification for *C. maxima*. The within-species resolution in our *Cucurbita* phylogeny provides an initial framework for domestication-genetics studies to test these and other hypotheses of *Cucurbita* domestication.

5. Conclusions

Advances in methods for phylogeny reconstruction and the expansion of expert-curated seed banks of crops and crop-wild-relatives have enabled rigorous investigations into the genetic origins of domesticated species. Ours is the first phylogenetic study to include multiple accessions of all known wild *Cucurbita* species and to use many, unlinked genetic loci to reconstruct relationships between these species and the six *Cucurbita* crop lineages. The novel relationships recovered provide new information about the evolution of *Cucurbita* and the patterns of diversity within *Cucurbita* crops and their wild relatives. We also demonstrate the need to study the evolution of closely related species using multiple, unlinked nuclear genes, as the relationships recovered in *Cucurbita* using these loci differ from those recovered using organellar loci.

The lack of close wild relatives identified in this study for *C. moschata*, *C. ficifolia*, and *C. pepo* ssp. *pepo* may be due in part to widespread extinction of wild *Cucurbita*, as proposed by Kistler *et al.* (2015) and to inadequate sampling of wild *Cucurbita* from regions of possible domestication. Future work to uncover the origins of these three *Cucurbita* crops must begin with an extensive effort to survey and collect wild *Cucurbita* from these geographic regions. The three *Cucurbita* crops for which we have recovered well-supported relationships to wild species, *C. maxima* ssp. *maxima*, *C. argyrosperma* ssp. *sororia*, and *C. pepo* ssp. *ovifera* var. *ovifera*, present ideal models in which to study the genetic and demographic processes of plant domestication.

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Figure legends

Figure 1 Distribution map of wild species of *Cucurbita* with occurrences based on ~7000 records from GBIF (www.gbif.org). Hypothesized origins of domestication for the six *Cucurbita* crop lineages are indicated by open circles. Colors correspond to tip colors in Figure 3: *Cucurbita pepo* ssp. *ovifera* var. *ovifera*: dark green; *C. pepo* ssp. *pepo*: light green; *C. argyrosperma* ssp. *argyrosperma*: blue; *C. moschata*: magenta; *C. ficifolia*: light blue; *C. maxima* ssp. *andreana*: orange.

Figure 2 Success of microfluidic PCR amplification of loci across taxa. Highlighted taxon *C. pepo* ssp. *pepo* was used for locus-selection and primer design. Black indicates that a locus was amplified for all individuals of the corresponding taxon, white indicates the locus was not successfully amplified for any individuals of the corresponding taxon. Tree illustrates relationships among these taxa as reconstructed in this study. Not all samples and loci presented in this heatmap of amplification success were ultimately included in the analyses presented in this paper.

Figure 3 Best maximum-likelihood tree reconstructed with RAxML using a matrix of concatenated ambiguity-coded gene sequences under the GTRCAT model. Support values are from 1,000 bootstrap replicates. BS values < 50% are not shown. Outgroup branch lengths are scaled to 1/10 of actual length to accommodate viewing of ingroup.

Figure 4 Comparison of alternative branching patterns among seven mesophytic *Cucurbita* species resolved by four phylogenetic studies of *Cucurbita*. a. The topology found in this study; b. Sanjur *et al.* (2002); c. Kistler *et al.* (2015); d. Zheng *et al.* (2013). Mesophytic species *Cucurbita ficifolia* is excluded due its position outside of this clade in some previous studies.

Figure 5. Comparison of the ML phylogeny inferred from the concatenated matrix and ASTRAL species tree estimated from ambiguity-coded gene trees, with branches not found in both trees highlighted in red. Nodes with <50% BS are collapsed in both trees. Support values are shown in black for incongruent nodes and in red where BS values disagree between the two trees by more than ten points.

Figure 6 Ancestral character-state reconstruction of habit using maximum likelihood. Possible states are mesophytic habit (black) and xerophytic habit (gray). Relative likelihood probabilities for each character state are represented with a pie chart at nodes.

References

- Andreasen K, Baldwin BG. 2001. Unequal Evolutionary Rates Between Annual and Perennial Lineages of Checker Mallows (*Sidalcea*, Malvaceae): Evidence from 18S–26S rDNA Internal and External Transcribed Spacers. *Mol Biol Evol* 18 (6): 936-944.
- Andres TC., 2006. Origin, morphological variation, and uses of *Cucurbita ficifolia*, the mountain squash. *Cucurbitaceae 2006*, Asheville, North Carolina, USA: 326-332.
- Atkinson RG, Cipriani G, Whittaker DJ, Gardner RC., 1997. The allopolyploid origin of kiwifruit, *Actinidia deliciosa* (Actinidiaceae). *Plant Systematics and Evolution* 205(1-2), 111-124.
- Bayzid MS, Warnow T. 2013. Naive binning improves phylogenomic analyses. *Bioinformatics* 29(18), 2277-2284.
- Bemis WP., Nelson JM. 1963. Interspecific Hybridization within the Genus *Cucurbita* I, Fruit Set, Seed and Embryo Development. *Journal of Ariz. Acad. of Sci.* 2(3): 104-107.
- Bemis WP, Whitaker TW., 1969. The Xerophytic *Cucurbita* of Northwestern Mexico and Southwestern United States. *Madro* 20(2), 33-41.
- Caicedo AL, Schaal BA. 2004. Population structure and phylogeography of *Solanum pimpinellifolium* inferred from a nuclear gene. *Molecular Ecology* 13(7), 1871-1882.

608 Collins AG, Cartwright P, McFadden CS, Schierwater B. 2005. Phylogenetic context and basal
609 metazoan model systems. *Integrative and Comparative Biology* 45(4), 585-594.

610 Crowl AA, Visger CJ, Mansion G, Hand R, Wu H-H, Kamari G, Phitos D, Cellinese N. 2015.
611 Evolution and biogeography of the endemic *Roucela* complex (Campanulaceae:
612 *Campanula*) in the Eastern Mediterranean. *Ecology and Evolution* 5(22), 5329-5343.

613 Darriba, D., Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new
614 heuristics and parallel computing. *Nature Methods*. 9,772.

615 Decker DS. 1988. Origin(s), evolution, and systematics of *Cucurbita* (Cucurbitaceae). *Economic*
616 *Botany* 42(1), 4-15.

617 Decker DS, Wilson HD. 1987. Allozyme variation in the *Cucurbita pepo* complex - *Cucurbita*-
618 *pepo* var. *ovifera* vs *Cucurbita texana*. *Systematic Botany* 12(2), 263-273.

619 Decker-Walters D, Staub J, Chung S, Nakata E, Quemada H. 2002. Diversity in free-living
620 Populations of *Cucurbita pepo* (Cucurbitaceae) as assessed by random amplified
621 polymorphic DNA. *Systematic Botany* 27(1), 19-28.

622 Decker-Walters DS, Walters TW, Cowan CW, Smith BD. 1993. Isozymic characterization of
623 wild populations of *Cucurbita pepo*. *Journal of Ethnobiology* 13: 55-72.

624 Degnan JH, Rosenberg NA. 2009. Gene tree discordance, phylogenetic inference and the
625 multispecies coalescent. *Trends Ecol Evol* 24(6), 332-340.

626 Diez CM, Trujillo I, Martinez-Urdiroz N, Barranco D, Rallo L, Marfil P, Gaut BS. 2015. Olive
627 domestication and diversification in the Mediterranean Basin. *New Phytologist* 206(1),
628 436-447.

629 Doebley JF, Iltis HH. 1980. Taxonomy of *Zea* (gramineae) A subgeneric classification with key
630 to taxa. *American Journal of Botany* 67(6), 982-993.

631 Doyle JJ, Doyle JL 1987. A rapid DNA isolation procedure for small quantities of fresh leaf
632 tissue: *Phytochemical Bulletin*. 11-15.

633 Doyle JJ, Doyle JL, Brown AHD. 1990. A chloroplast-DNA phylogeny of the wild perennial
634 relatives of soybean (*Glycine* subgenus *Glycine*) - congruence with morphological and
635 crossing groups. *Evolution* 44(2), 371-389.

636 Driskell AC, Ane C, Burleigh JG, McMahon MM, O'Meara BC, Sanderson MJ. 2004. Prospects
637 for building the tree of life from large sequence databases. *Science* 306(5699), 1172-
638 1174.

639 Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high
640 throughput. *Nucleic Acids Research* 32(5), 1792-1797.

641 Edwards CE, Lefkowitz D, Soltis DE, Soltis PS. 2008. Phylogeny of *Conradina* and related
642 southeastern scrub mints (Lamiaceae) based on GapC gene sequences. *International*
643 *Journal of Plant Sciences* 169(4), 579-594.

644 Felsenstein J. 1973. Maximum likelihood and minimum-steps methods for estimating
645 evolutionary trees from data on discrete characters. *Systematic Zoology* 22(3), 240-249.

646 Ferriol M, Pico B, de Cordova PF, Nuez F. 2004. Molecular diversity of a germplasm collection
647 of squash (*Cucurbita moschata*) determined by SRAP and AFLP markers. *Crop*
648 *Science* 44(2), 653-664.

649 Gadagkar SR, Rosenberg MS, Kumar S. 2005. Inferring species phylogenies from multiple
650 genes: Concatenated sequence tree versus consensus gene tree. *Journal of Experimental*
651 *Zoology Part B-Molecular and Developmental Evolution* 304B(1), 64-74.

652 Gepts P. 1996. Origin and evolution of cultivated *Phaseolus* species. In: Pickersgill, B. & Lock
653 JM., eds. *Advances in Legume Systematics, Part 8; Legumes of economic importance*.
654 London, UK: Kew publishing, 65-74.

Gerard D, Gibbs HL, Kubatko L. 2011. Estimating hybridization in the presence of coalescence using phylogenetic intraspecific sampling. *Bmc Evolutionary Biology* 11:291

Gong L, Paris HS, Nee MH, Stift G, Pachner M, Vollmann J, Lelley T. 2012. Genetic relationships and evolution in *Cucurbita pepo* (pumpkin, squash, gourd) as revealed by simple sequence repeat polymorphisms. *Theoretical and Applied Genetics* 124(5), 875-891.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* 52(5), 696-704.

Guo J, Wang Y, Song C, Zhou J, Qui L, Huang H, Wang Y. 2010. A single origin and moderate bottleneck during domestication of soybean (*Glycine max*), Implications from microsatellites and nucleotide sequences. *Annals of Botany* 106(3), 505-514.

Heikal AH, Abdel-Razzak HS, Hafez EE. 2008. Assessment of genetic relationships among and within *Cucurbita* species using RAPD and ISSR markers. *Journal of Applied Sciences Research* (5), 515-525.

Heled J, Drummond AJ. 2010. Bayesian Inference of Species Trees from Multilocus Data. *Molecular Biology and Evolution* 27(3), 570-580.

Heun M, SchaferPregl R, Klawan D, Castagna R, Accerbi M, Borghi B, Salamini F. 1997. Site of einkorn wheat domestication identified by DNA fingerprinting. *Science* 278(5341), 1312-1314.

Hoffman DL, Soltis DE, Muehlbauer FJ, Ladizinsky G. 1986. Isozyme polymorphism in *Lens* (Leguminosae). *Systematic Botany* 11(3), 392-402.

Huang X, Han B. 2012. A crop of maize variants. *Nature Genetics* 44(7), 734-735.

Igea J, Juste J, Castresana J. 2010. Novel intron markers to study the phylogeny of closely related mammalian species. *Bmc Evolutionary Biology* 10:369

679 Jing R, Vershinin A, Grzebyta J, Shaw P, Smykal P, Marshall D, Ambrose MJ, Ellis THN,
680 Flavell AJ. 2010. The genetic diversity and evolution of field pea (*Pisum*) studied by
681 high throughput retrotransposon based insertion polymorphism (RBIP) marker
682 analysis. *Bmc Evolutionary Biology* 10:44

683 Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A,
684 Markowitz S, Duran C, *et al.*, 2012. Geneious Basic: An integrated and extendable
685 desktop software platform for the organization and analysis of sequence
686 data. *Bioinformatics* 28(12), 1647-1649.

687 Kellogg EA, Birchler JA. 1993. Linking phylogeny and genetics - *Zea-mays* as a tool for
688 phylogenetic studies. *Systematic Biology* 42(4), 415-439.

689 Kistler L, Newsom LA, Ryan TM, Clarke AC, Smith BD, Perry GH. 2015. Gourds and
690 squashes (*Cucurbita* spp.) adapted to megafaunal extinction and ecological anachronism
691 through domestication. *Proceedings of the National Academy of Sciences of the United*
692 *States of America* 112(49), 15107-15112.

693 Knowles LL, Klimov PB. 2011. Estimating phylogenetic relationships despite discordant gene
694 trees across loci: the species tree of a diverse species group of feather mites (*Acari*:
695 Proctophyllodidae). *Parasitology* 138(13), 1750-1759.

696 Knowles LLCFPdO. 2009. Estimating species trees: methods of phylogenetic analysis when
697 there is incongruence across genes. *Systematic Biology* 58(5), 463-467.

698 Kubatko LS, Carstens BC, Knowles LL. 2009. STEM: species tree estimation using maximum
699 likelihood for gene trees under coalescence. *Bioinformatics* 25(7), 971-973.

700 Leigh JW, Lapointe F-J, Lopez P, Baptiste E. 2011. Evaluating Phylogenetic Congruence in the
701 Post-Genomic Era. *Genome Biology and Evolution* 3: 571-587.

702 Li ZM, Zheng XM, Ge S. 2011. Genetic diversity and domestication history of African rice
703 (*Oryza glaberrima*) as inferred from multiple gene sequences. *Theoretical and Applied*
704 *Genetics* 123(1), 21-31.

705 Linder CR., Rieseberg LH. 2004. Reconstructing patterns of reticulate evolution in plants. *Amer.*
706 *Journal of Botany* 91(10): 1700-8.

707 Liu L, Pearl DK. 2007. Species Trees from Gene Trees: Reconstructing Bayesian Posterior
708 Distributions of a Species Phylogeny Using Estimated Gene Tree
709 Distributions. *Systematic Biology* 56(3), 504-514.

710 Liu L, Yu L, Kubatko L, Pearl DK, Edwards SV. 2009. Coalescent methods for estimating
711 phylogenetic trees. *Molecular Phylogenetics and Evolution* 53(1), 320-328.

712 Liu X-J, Li Y-J, Liang G-Y, Fang C, Liu D-C, Yang H, Zhao Y. 2010. Genetic diversity of
713 some accessions in *Cucurbita* by SRAP markers. *Sichuan Daxue Xuebao (Ziran*
714 *Kexueban)* 47(5), 1195-1200.

715 Luis Blanco-Pastor J, Vargas P, Pfeil BE. 2012. Coalescent Simulations Reveal Hybridization
716 and Incomplete Lineage Sorting in Mediterranean *Linaria*. *Plos One* 7(6).

717 Maddison WP. 1997. Gene trees in species trees. *Systematic Biology* 46(3), 523-536.

718 Maddison WP, Knowles LL. 2006. Inferring phylogeny despite incomplete lineage sorting.
719 *Systematic Biology* 55(1), 21-30.

720 Matsuoka Y, Vigouroux Y, Goodman MM, Sanchez GJ, Buckler E, Doebley J. 2002. A single
721 domestication for maize shown by multilocus microsatellite genotyping. *Proceedings of*
722 *the National Academy of Sciences of the United States of America* 99(9), 6080-6084.

723 Maureira-Butler IJ, Pfeil BE, Muangprom A, Osborn TC, Doyle JJ. 2008. The reticulate history
724 of *Medicago* (Fabaceae). *Systematic Biology* 57(3), 466-482.

725 Merrick L. C. 1990. Systematics and evolution of a domesticated squash, *Cucurbita*
726 *argyrosperma*, and its wild and weedy relatives. In: D. M. Bates, R. W. Robinson, and
727 C. Jeffrey, eds. *Biology and utilization of the Cucurbitaceae*. New York, USA: Cornell
728 University Press, 77–95.

729 Mayer MS, Soltis PS. 1994. Chloroplast DNA phylogeny of *Lens* (Leguminosae) - origin and
730 diversity of the cultivated lentil. *Theoretical and Applied Genetics* 87(7), 773-781.

731 Meyer RS, Karol KG, Little DP, Nee MH, Litt A. 2012. Phylogeographic relationships among
732 Asian eggplants and new perspectives on eggplant domestication. *Molecular*
733 *Phylogenetics and Evolution* 63(3), 685-701.

734 Mirarab S, Warnow T. 2015. ASTRAL-II: coalescent-based species tree estimation with many
735 hundreds of taxa and thousands of genes. *Bioinformatics* 31(12), 44-52.

736 Nee M. 1990. The domestication of *Cucurbita* (Cucurbitaceae). *Economic Botany* 44(3), 56-68.

737 Nesom GL 2011. New state records for *Citrullus*, *Cucumis*, and *Cucurbita* (Cucurbitaceae)
738 outside of cultivation in the USA. *Phytoneuron*. 1-7.

739 Olsen KM, Purugganan MD. 2002. Molecular evidence on the origin and evolution of glutinous
740 rice. *Genetics* 162(2), 941-950.

741 Olsen KM, Schaal BA. 1999. Evidence on the origin of cassava: Phylogeography of *Manihot*
742 *esculenta*. *Proceedings of the National Academy of Sciences of the United States of*
743 *America* 96(10), 5586-5591.

744 Paradis E, Claude J, Strimmer K. 2004. APE: Analyses of Phylogenetics and Evolution in R
745 language. *Bioinformatics* 20(2), 289-290.

746 Paris H, Yonash N, Portnoy V, Mozees-Daube N, Tzuri G, Katzir N. 2003. Assessment of
747 genetic relationships in *Cucurbita pepo* (Cucurbitaceae) using DNA
748 markers. *Theoretical and Applied Genetics* 106(6), 971-978.

749 Parks M, Cronn R, Liston A. 2009. Increasing phylogenetic resolution at low taxonomic levels
750 using massively parallel sequencing of chloroplast genomes. *Bmc Biology* 7:84.

751 Patel SK, R.T. Braun, E.L. 2013. Error in phylogenetic estimation for bushes in the tree of life:
752 *Phylogenet. Evol. Biol.* 1:110.

753 Penmetsa, VR., Carrasquilla-Garcia, N., Bergmann, E. M., Vance, L., Castro, B., Kassa, M. T.,
 754 Sarma, B. K., Datta, S., Farmer, A. D., Baek, J.-M., Coyne, C. J., Varshney, R. K., von
 755 Wettberg, E. J. B., Cook, D. R. 2016. Multiple post-domestication origins of kabuli
 756 chickpea through allelic variation in a diversification-associated transcription factor.
 757 *New Phytol* 211: 1440–1451.

758 Philippe H, Snell EA, Baptiste E, Lopez P, Holland PWH, Casane D. 2004. Phylogenomics of
 759 eukaryotes: Impact of missing data on large alignments. *Molecular Biology and*
 760 *Evolution* 21(9), 1740-1752.

761 Piperno DR, Pearsall DM 1998. *The Origins of Agriculture in the Lowland Neotropics*. San
 762 Diego: USA: Academic Press, 109-166.

763 Sanjur OI, Piperno DR, Andres TC, Wessel-Beaver L. 2002. Phylogenetic relationships among
 764 domesticated and wild species of *Cucurbita* (Cucurbitaceae) inferred from a
 765 mitochondrial gene: Implications for crop plant evolution and areas of
 766 origin. *Proceedings of the National Academy of Sciences of the United States of*
 767 *America* 99(1), 535-540.

768 Savage JA, Haines DF, Holbrook NM. 2015. The making of giant pumpkins: how selective
 769 breeding changed the phloem of *Cucurbita maxima* from source to sink. *Plant Cell and*
 770 *Environment* 38(8), 1543-1554.

771 Schaefer H, Heibl C, Renner SS. 2009. Gourds afloat: a dated phylogeny reveals an Asian origin
 772 of the gourd family (Cucurbitaceae) and numerous oversea dispersal events. *Proceedings*
 773 *of the Royal Society B-Biological Sciences* 276(1658), 843-851.

774 Schaefer H, Renner SS 2011. Cucurbitaceae. In: Kubitzki K ed. *Flowering Plants. Eudicots*
 775 *Sapindales, Cucurbitales, Myrtaceae*. New York, USA: Springer, 112-174.

776 Simmons MP, Gatesy J. 2015. Coalescence vs. concatenation: Sophisticated analyses vs. first
 777 principles applied to rooting the angiosperms. *Molecular Phylogenetics and*
 778 *Evolution* 91: 98-122.

779 Smith BD. 1997. The Initial Domestication of *Cucurbita pepo* in the Americas 10,000 Years
780 Ago. *Science* 276(5314), 932-934.

781 Smith SA, Moore MJ, Brown JW, Yang Y. 2015. Analysis of phylogenomic datasets reveals
782 conflict, concordance, and gene duplications with examples from animals and
783 plants. *Bmc Evolutionary Biology* 15:150.

784 Smýkal P, Kenicer G, Flavell AJ, Corander J, Kosterin O, Redden RJ, Ford R, Coyne CJ,
785 Maxted N, Ambrose MJ, *et al.*, 2011. Phylogeny, phylogeography and genetic diversity
786 of the *Pisum* genus. *Plant Genetic Resources* 9(1), 4-18.

787 Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with
788 thousands of taxa and mixed models. *Bioinformatics* 22(21), 2688-2690.

789 Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of
790 large phylogenies. *Bioinformatics* 30(9), 1312-1313.

791 Steane DA, Nicolle D, Sansaloni CP, Petroli CD, Carling J, Kilian A, Myburg AA, Grattapaglia
792 D, Vaillancourt RE. 2011. Population genetic analysis and phylogeny reconstruction in
793 *Eucalyptus* (Myrtaceae) using high-throughput, genome-wide genotyping. *Molecular*
794 *Phylogenetics and Evolution* 59(1), 206-224.

795

796 Sun Y, Moore MJ, Zhang S, Soltis PS, Soltis DE, Zhao T, Meng A, Li X, Li J, Wang H. 2016.
797 Phylogenomic and structural analyses of 18 complete plastomes across nearly all
798 families of early-diverging eudicots, including an angiosperm-wide analysis of IR gene
799 content evolution. *Molecular Phylogenetics and Evolution* 96: 93-101.

800

801 R Core Team 2014. *R: A language and environment for statistical computing*. *R Foundation*
802 *for Statistical Computing*. [WWW document] URL <http://www.R-project.org/> [accessed
803 1 June 2016].

804

805 Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. 2012.
806 Primer3--new capabilities and interfaces. *Nucleic Acid Research* 40(15), 115

807

808 Uribe-Convers S, Settles ML, Tank DC. 2016. A Phylogenomic Approach Based on PCR
809 Target Enrichment and High Throughput Sequencing: Resolving the Diversity within the
810 South American Species of *Bartsia* L. (Orobanchaceae). *Plos One* 11(2).

811 Walters TW, Decker-Walters DS. 1993. Systematics of the endangered okeechobee gourd
812 (*Cucurbita okeechobeensis* Cucurbitaceae). *Systematic Botany* 18(2), 175-187.

813 Ward DB, Minno MC. Rediscovery of the Endangered Okeechobee Gourd (*Cucurbita*
814 *okeechobeensis*) along the St. Johns River, Florida, Where Last Reported by William
815 Bartram in 1774. *Castanea* 67(2), 201-206.

816 Warnow T. Concatenation Analyses in the Presence of Incomplete Lineage Sorting. 2015. *PLOS*
817 *Currents Tree of Life*. Ed. 1 doi:
818 10.1371/currents.tol.8d41ac0f13d1abedf4c4a59f5d17b1f7.

819 Wessel-Beaver L. 2000. *Cucurbita argyrosperma* sets fruit in fields where *C. moschata* is only
820 pollen source. *Cucurbit Genetics Cooperative Report* 23: 62-63.

821 Whitaker TW., Bemis WP. 1964. Evolution in the genus *Cucurbita*. *Evolution* 18: 553-559.

822 Wiens JJ. 2006. Missing data and the design of phylogenetic analyses. 2006. *Journal of*
823 *Biomedical Informatics* 39(1), 34-42.

824 Wiens JJ, Kuczynski CA, Townsend T, Reeder TW, Mulcahy DG, Sites JW. 2010. Combining
825 Phylogenomics and Fossils in Higher-Level Squamate Reptile Phylogeny: Molecular
826 Data Change the Placement of Fossil Taxa. *Systematic Biology* 59(6), 674-688.

827 Wiens JJ, Moen DS. 2008. Missing data and the accuracy of Bayesian phylogenetics. *Journal of*
828 *Systematics and Evolution* 46(3), 307-314.

829 Wiens JJ, Morrill MC. 2011. Missing Data in Phylogenetic Analysis: Reconciling Results from
830 Simulations and Empirical Data. *Systematic Biology* 60(5), 719-731.

831 Wilkes HG. 1977. Hybridization of maize and teosinte in Mexico and Guatemala and
832 improvement of maize. *Economic Botany* 31(3), 254-293.

833 Xu, SZ. 2000. Phylogenetic analysis under reticulate evolution. *Mol Biol Evol* 17(6):897-907.

834 Zheng Y-H, Alverson AJ, Wang Q-F, Palmer JD. 2013. Chloroplast phylogeny of *Cucurbita*:
835 Evolution of the domesticated and wild species. *Journal of Systematics and*
836 *Evolution* 51(3), 326-334.

837 Zhenxiang Xi, Liang Liu, Charles CD. 2015. The Impact of Missing Data on Species Tree
838 Estimation. *Mol Biol Evol* 2016; 33 (3): 838-860.

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Table 1. <i>Cucurbita</i> species and their within-species resolution in the CA-ML tree				
Species	No. Individuals sampled/tips	Bifurcations	Percent Resolution	Example crop types
<i>Cucurbita pepo</i> ssp. <i>pepo</i> ^c , <i>C. pepo</i> ssp. <i>ovifera</i> var. <i>ovifera</i> ^c , <i>C. pepo</i> ssp. <i>ovifera</i> var. <i>texana</i> ^w , <i>C. pepo</i> ssp. <i>ovifera</i> var. <i>ozarkana</i> ^w , <i>C. pepo</i> ssp. <i>fraterna</i> ^w	27	12	48%	jack-o-lantern pumpkin, zucchini, summer squash, spaghetti squash; acorn squash, ornamental gourd
<i>Cucurbita maxima</i> ssp. <i>maxima</i> ^c , <i>C. maxima</i> ssp. <i>andreana</i> ^w	14	5	42%	giant pumpkin, hubbard squash, kabocha squash
<i>Cucurbita argyrosperma</i> ssp. <i>argyrosperma</i> ^c , <i>C. argyrosperma</i> ssp. <i>sororia</i> ^w	15	4	31%	cushaw pumpkin, Japanese pie pumpkin
<i>Cucurbita moschata</i> ^c	9	5	71%	butternut squash, Seminole pumpkin,

				canned pumpkin, crookneck squash
<i>Cucurbita ficifolia</i> ^c	2	1	-	figleaf gourd, malabar melon, Thai melon
<i>Cucurbita cordata</i> ^{w †}	2	-	-	-
<i>Cucurbita digitata</i> ^{w †}	2	-	-	-
<i>Cucurbita ecuadorensis</i> ^w	2	-	-	-
<i>Cucurbita foetidissima</i> ^{w †¹}	6	-	-	-
<i>Cucurbita lundelliana</i> w,1	4	-	-	-
<i>Cucurbita okeechobeensis</i> ssp. <i>okeechobeensis</i> ^w , <i>C. okeechobeensis</i> ssp. <i>martinezii</i> ^{w,1}	3	-	-	-
<i>Cucurbita palmata</i> w † ¹	2	-	-	-
<i>Cucurbita pedatifolia</i> w † ¹	3	-	-	-
<i>Cucurbita radicans</i> w †	0	-	-	-
^c crop taxon ^w wild taxon † Xerophytic perennial. Percent resolution calculated as nodes with > 50 % BS / possible nodes (two less than the number of tips) in the CA-ML tree. Species with – bifurcations are ¹ non-monophyletic or have only two accessions.				

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