ECOLOGY AND EVOLUTION OF SOUTHEASTERN UNITED STATES YUCCA SPECIES

by

JEREMY DANIEL RENTSCH

(Under the Direction of JIM LEEBENS-MACK)

ABSTRACT

The genus *Yucca* contains approximately 40 species with most diversity found in Mexico and the southwestern United States. The southeastern United States is home to three well-described yucca species: the fleshy-fruited *Y. aloifolia*, the capsular-fruited *Y. filamentosa*, and *Y. gloriosa* – with a fruit type that does not follow convention. *Yucca* species are perhaps best known for the obligate pollination mutualism they share with moths in the genera *Tegeticula* and *Parategeticula*. Such interactions are thought to be highly specialized, restricting gene flow between species and even make evolutionary reversions to generalist life history characterizes impossible. Here, we show that *Y. gloriosa* is an intersectional, homploid, hybrid species produced by the crossing of *Y. aloifolia* and *Y. filamentosa*. We go on to show that Y. aloifolia has escaped from the obligate pollination mutualism and is being pollinated diurnally by the introduced European honey bee, *Apis mellifera* – an observation that directly refutes the idea that highly specialized species interactions lead to evolutionary dead ends. Finally, we utilized high throughput sequencing a biotinylated probe set in order to sequence many genes of interest in *Y. aloifolia*, laying the ground work to better understand its introduction history and pattern of pollinator association.

INDEX WORDS: Yucca, hybrid speciation, population genetics, obligate mutualism

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I:

# INTRODUCTION AND LITERATURE REVIEW

## The *Yucca* Genus

The genus *Yucca* contains approximately 40 species with most diversity found in Mexico and the southwestern United States. The genus is divided into three major sections: *Chaenocarpa* with capsular-fruited yucca, *Clistocarpa* with spongy-fruited yucca and *Sarcocarpa* with fleshy-fruited species. The *Chaenocarpa* and *Sarcocarpa* are reciprocally monophyletic sections and contain most of the species within the genus (*1, 2*). The *Clistocarpa*, with an uncertain phylogenetic placement, contains only *Y. brevifolia* (Joshua tree) with two described varieties(*3, 4*). Capsular-fruited species are prevalent from South Dakota to Durango, Mexico and from the Atlantic coast to Nevada. Fleshy-fruited species are most often found in the high table land of Mexico and in the United States from the southern Rocky Mountains and western regions, reaching the Pacific coast in the southern part of California and at the extremity of lower California, with a single species (*Y. aloifolia* L.) being found along the southeastern Atlantic coast of the United States and islands to the east (*5*). *Yucca brevifolia*, of the *Clistocarpa*, is largely restricted to the Colorado Plateau (*3*). Yuccas occupy a wide range of habitats including chaparrals, shrub deserts, coastal dunes, grasslands, pine-oak woodlands, and even rainforests (*6-8*).

## Obligate Pollination Mutualism

*Yucca* species share a long-studied mutualistic relationship with pollinating yucca moths within the genera *Tegeticula* and *Parategeticula* (*5, 9*). Female yucca moths actively gather pollen from yucca anthers and vigorously insert the pollen into the yucca's cup shaped stigmatic surface after inserting eggs directly into the carpal of the flower. Developing moth larvae then feed on yucca

seeds. This mutualism is widely considered obligate, as yucca moths are thought to be the sole pollinators of yucca species and also require the yucca plant as a mating arena and larval food source. Under this model, the extinction of any one mutualist population would necessarily lead to the extinction of the other.

**Interspecific Gene Flow and Hybridization**

Interspecific hybridization is known to be an important evolutionary process contributing both to genetic variation within species and to the origin of new species, especially in plants (*10-14*). Hybrid species can arise as the result of the coupling (and doubling) of two parental species chromosomes (*15, 16*) or through the retention of parental chromosome numbers (so called 'homoploid hybrdization'). As Homoploid hybrids retain the chromosome count of their parents, they require additional divergence from parental lineages before the speciation process can be achieved. Many homoploid hybrid species (such as those found in the genera: *Iris*, *Helianthus,* and *Pinus*) thrive in a different habitat than their parents (*17-19*), in *Penstemon* we see pollinator divergence (*20*), and in *Hyobanche* we see the divergence of multiple ecological factors (*21*). It is also possible for hybrids to form in sympatry but only become reproductively isolated from parental species in allopatry as demonstrated in *Senecio* (*22*).

While hybrid speciation has not been documented in the *Yucca* genus, occasional hybridization (without subsequent reproductive isolation) has been documented between *Y. baccata* and *Y. schidigera* (*23, 24*), *Y. baccata* and *Y. torreyi* (*25*) and between *Y. brevifolia var. brevifolia* and *Y. brevifolia var. jaegeriana* (*4*). These hybridization events likely result from pollen transfer between a moth's typical host and a sympatric *Yucca* species that is typically pollinated by another moth species. Hybridization across distinct clades (i.e. capsular-fruited vs. fleshy-fruited) of the genus has been hypothesized by Lenz and Hanson (*26*) who suggested that the fleshy-fruited species *Y. baccata* and *Y. madrensis* may hybridize with the capsular-fruited *Y. elata* to produce intersectional hybrids, although

few individuals were described. *Yucca* species in the southeastern United States may provide the best opportunity to identify a hybrid species in the genus. The somewhat spongy fruit morphology of *Yucca gloriosa* deviates from the typical fleshy vs. capsular fruit seen throughout the rest of the genus. While this fruit type could have arisen through mutation of one of the traditional fruit types, it also may represent an intermediate morphological character that arose through hybridization between clades of the genus. Some (*27*) have hypothesized that *Y. gloriosa* may be of hybrid origin but may not be reproductively active. We investigate the potential hybrid origin of Yucca gloriosa as the result of a cross between the capsular-fruited *Y. filamentosa* and the fleshy-fruited *Y. aloifolia*.

**Exceptions to the Obligate Mutualism**

According to the 'law of the unspecialized' highly dependent species interactions are 'evolutionary dead ends', prone to extinction because reversion to more generalist interactions is thought to be unlikely (*28*). Cases of extreme specialization, such as those seen between obligate mutualists, are thought to be evolutionarily inescapable, inevitably leading to extinction rather than diversification of participating species. The pollination mutualism between *Yucca* species and yucca moths (*Tegeticula* and *Parategeticula*) are thought to be locked into such an obligate mutualism. Despite this assertion, it is estimated that there have been at least two shifts from pollination to parasitism within the yucca moth genus, *Tegeticula* (*29-31*). These 'cheater' moths, which coexist with pollinating species, are seed parasites that oviposits into the ovary of the yucca without pollinating the flower. A third potential outcome to obligate mutualist interactions is the shift to facultative mutualism (*32, 33*), where interacting species are not dependent on each other due the availability of alternative interaction partners. In this case, local or global extinction of one mutualist population does not necessarily lead to the extinction of other partners.

Generalist pollination has been used to explain erratic fruit sets throughout the *Yucca* genus. Addicott (*34*) noted that both *Y. baccata* and *Y. arizonica* produced significant fruit sets with little or no seed predation or larval infestation. A similar phenomenon was described by Dodd and Linhart (*35*) in *Yucca glauca*, and speculated that non-moth pollination was occurring. Lapping flies, in the genus *Pseudocalliope*, were observed frequently on *Y. glauca* flowers and were hypothesized to have pollinated flowers when fruits contained no signs of seed damage by moth larvae. C.V. Riley was perhaps the first to hypothesize that flies and small beetles may occasionally pollinate *Yucca* species as It was observed that these insects would occasionally dislodge pollen, which then made contact with the stigma with some frequency (*36*). Keeley et al. (*37*) hypothesized. Alternatively, egg or larval mortality, or yucca moth pollination without oviposition could also account for these observations – an explanation that does not require an escape from the obligate mutualism. Without proper exclusion treatments, however, it is impossible to conclude whether or not non-moth pollination is occurring in these species.

*Yucca aloifolia* has been documented as occasionally producing fruit outside of the range of its known pollinators (*Tegeticula yuccasella* and *T. cassandra*). Sparse or erratic fruit sets have been documented in Italy (*38*), New Caledonia (*39*), Australia (*40*) and Israel (*41, 42*). In the southeastern United States, *Y. aloifolia* is reported to be visited by *T. yuccasella* and *T. cassandra* (*38, 43, 44*), although fruits without oviposition scars have been documented (*45, 46*). It is possible that superficial oviposition just under the ovary's surface cuticle, as exhibited by *T. cassandra* (Pellmyr 1999), may have been missed in these studies. Alternatively fruit set in the absence of moth pollination may be the result of visitation by generalist pollinators, and thus evidence for an escape from this textbook example of obligate mutualism. C.V. Riley, however, suspected that the short style and open stigma of *Y. aloifolia* may facilitate self-pollination. To test this hypothesis, Trelease and Webber (*27*) enclosed a single inflorescence in a gauze bag to exclude pollinators but allow selfing. The plant produced no fruit.

**Population Biology of *Yucca* Species**

The life history characteristics (annual vs. perennial, selfing vs. outcrossing, geographic range, etc.) of a species largely influence that species' genetic diversity and genetic structure. *Yucca* species are long-lived and largely outcrossing species and may be expected to maintain relatively high levels of genetic diversity (*47*). Studies into the population genetics of *Y. filamentosa* (*48*) showed that the species maintained a level of genetic variation that was significantly higher than expected given the species' life history characters. This observation could be due to a number of factors including moth behavior, selective fruit abscission, and a history of gene duplication. At the same time, *Y. filamentosa* was shown to display surprisingly low levels of population genetic structure given its patchy distribution. This observation suggests a level of gene flow is occurring between populations that is sufficient to counteract the effects of genetic drift on population differentiation. *Yucca aloifolia* may have a particularly interesting story that can be told through the use of population genetics tools. This species is known to propagate clonally through both clonal extensions and severed plant tissue(*8*), which may result in high levels of population genetic structure. However, the species is known to be pollinated by both yucca moths and a generalist pollinator, which may facilitate gene flow between populations. Furthermore, the species fairly continuous distribution along sand dunes across the coast of the southeastern United States suggests that populations should be fairly well connected, maintain a high level of gene flow and a low level of between population differentiation.

While the southeastern United States may not represent the center of diversity of the *Yucca* genus, the species present boast an interesting evolutionary history and display unique present day ecological interactions.

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CHAPTER II

# HOMOPLOID HYBRID ORIGIN OF *YUCCA GLORIOSA*: INTERSECTIONAL HYBRID SPECIATION IN *YUCCA* (AGAVOIDEAE, ASPARAGACEAE)[1]

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**Abstract**

There is a growing appreciation for the importance of hybrid speciation in angiosperm evolution. Here, we show that *Yucca gloriosa* (Asparagaceae: Agavoideae) is the product of intersectional hybridization between *Y. aloifolia* and *Y. filamentosa*. These species, all named by Carl Linnaeus, exist in sympatry along the southeastern Atlantic coast of the United States. *Yucca gloriosa* was found to share a chloroplast haplotype with *Y. aloifolia* in all populations sampled. In contrast nuclear gene-based microsatellite markers in *Y. gloriosa* are shared with both parents. The hybrid origin of *Y. gloriosa* is supported by multilocus analyses of the nuclear microsatellite markers including principal coordinates analysis (PCO), maximum-likelihood hybrid index scoring (HINDEX) and Bayesian cluster analysis (STRUCTURE). The putative parental species share only one allele at a single locus, suggesting there is little to no introgressive gene flow occurring between these species and *Y. gloriosa*. At the same time, diagnostic markers are segregating in *Y. gloriosa* populations. Lack of variation in the chloroplast of *Y. aloifolia*, the putative maternal parent, makes it difficult to rule out multiple hybrid origins of *Y. gloriosa*, but allelic variation at nuclear loci can be explained by a single hybrid origin of *Y. gloriosa*. Overall, these data provide strong support for the homoploid hybrid origin of *Y. gloriosa*.

**Introduction**

Interspecific hybridization is known to be an important evolutionary process contributing both to genetic variation within species and to the origin of new species, especially in plants (*10-14*). Hybrid speciation may involve allopolyploidization followed by diploidization through fractionation (*15, 16*) or admixture and recombination of two parental genomes without change in ploidy (*17, 18, 49, 50*). The genomes of both allopolyploid and homoploid hybrid species are typically mosaics of their parental genomes (but see *51*), but whereas polyploid hybrid species acquire the full chromosomal sets from both parents, homoploid hybrids meld chromosomal segments from both parents while remaining diploid. The processes that give rise to polyploid versus homoploid hybrid species do not appear to be random as recent reviews have shown that the parents of homoploid hybrid species are typically more genetically similar to each other than the parents of allopolyploids (*52, 53*).

Chromosome doubling in polyploid hybrids creates an instant barrier to reproduction with the parental species (*54*). However, because homoploid hybrids retain the chromosome count of their parental species, barriers to reproduction with parental species may remain more porous and speciation may be less likely. Homoploid hybrid speciation is hypothesized to involve reproductive isolation between parent and hybrid populations due to resorting of chromosomal segments and traits from both parents to produce a unique constellation of traits in the hybrid species (*14, 55-57*). However, homoploid hybrid speciation may be driven by introgression or transgressive segregation of a single trait as has been shown in *Heliconius* butterflies (*58, 59*). Both cases support the hypothesis that a novel trait or suite of traits in the hybrid species can promote ecological isolation between hybrid and parental populations (*60-62*).

As predicted, most documented examples of homoploid hybrid speciation involve some form of ecological divergence between parental species and their hybrid progeny. Examples include: habitat

divergence in *Iris*, *Helianthus,* and *Pinus* (*17-19*), pollinator divergence in *Penstemon* (*20*), and the divergence of multiple ecological factors in the genus *Hyobanche* (*21*). It is also possible for hybrids to form in sympatry but only become reproductively isolated from parental species in allopatry as demonstrated in *Senecio* (*22*).

The genus *Yucca* contains approximately 40 species with most diversity found in Mexico and the southwestern United States. Two monophyletic sections include most of the species within the genu: *Chaenocarpa* with capsular-fruited yuccas and *Yucca* (syn, *Sarcocarpa*) with fleshy-fruited species. A third clade, *Clistocarpa*, includes only *Y. brevifolia* (Joshua tree) with two described varieties . All *Yucca* species share a fascinating mutualistic relationship with pollinating yucca moths within the genera *Tegeticula* and *Parategeticula..* Female yucca moths actively gather pollen from yucca anthers and insert the pollen into the yucca's cup shaped stigmatic surface after inserting eggs into the carpel or style of the flower. Developing moth larvae then feed on yucca seeds. The majority of seed-feeding insects involved in plant pollination mutualisms display high host specificity (*9, 63-65*). *Yucca* - yucca moth associations generally exhibit narrow specificity with 60% of pollinating moths visiting a single host (*42, 66*). The most significant departure from this pattern is the broad host range exhibited by the pollinating moth *Tegeticula yuccasella*, which utilizes seven host species (*67*).

It is thought that pollinator specificity may discourage interspecific hybridization through highly correlated plant and pollinator phenotypes. In the fig - fig wasp pollination mutualism, the wasp's ovipositor length is significantly correlated with the length of the fig's flora style (*68*). Similarly, unpublished data (Pellmyr and collaborators) from the yucca - yucca moth system suggests there is a significant correlation between the length of the yucca moth's ovipositor and the thickness of the yucca's carpel. A cross pollination event in which phenotypes do not match could lead to increased mortality for pollinator eggs and early instars. Nevertheless, hybridization has been documented

between *Y. baccata* and *Y. schidigera* (*23, 24*), *Y. baccata* and *Y. torreyi* (*25*) and between *Y. brevifolia var. brevifolia* and *Y. brevifolia var. jaegeriana* (*4*). These hybridization events likely result from pollen transfer between a moth's typical host and a sympatric *Yucca* species that is typically pollinated by another moth species. Although hybridization appears to be more common within distinct sections of the genus, it is certainly possible that the phenomenon is widespread, even occurring between plants in different sections. Morphological evidence from yuccas sampled in the Four Corners Region of the U.S.A. (Arizona, Colorado, New Mexico and Utah) suggests that the fleshy-fruited species *Y. baccata* and *Y. madrensis* may hybridize with the capsular-fruited *Y. elata* to produce intersectional hybrids, although few individuals were described (Lenz & Hanson 2001). Sympatric *Yucca* species pollinated by *Tegeticula yuccasella* in the southeastern U.S.A. may provide the best opportunity to detect and characterize intersectional hybridization within the genus.

Here, we test the hypothesis that *Y. gloriosa* is the product of intersectional hybridization between *Y. aloifolia* (section: *Yucca*) and *Y. filamentosa* (section: *Chaenocarpa*). These three diploid species (*69, 70*) occur sympatrically along the southeastern Atlantic coast of the United States (*8*) and share *T. yuccasella* as a pollinator (*71, 72*), although *Y. aloifolia* might also be pollinated by non-moth visitors as well (*38, 40*). *Yucca aloifolia* is thought to be a relatively recent addition to the flora of the southeastern United States possibly as a consequence of both human mediated dispersal (*73, 74*) and natural dispersal (*5*). Further, the species are known to partially overlap in their flowering phenology across much of their range (*75*). William Trelease suggested that *Y. gloriosa* exhibited a blend of *Y. aloifolia* and *Y. filamentosa* traits and hypothesized that *Y. gloriosa* was a hybrid likely limited to vegetative propagation (*5*). While hybrid species are not always morphologically intermediate, *Y. gloriosa* displays a fruit type that appears to be intermediate to the capsular and fleshy fruits of yuccas in sections *Chaenocarpa* and *Yucca*, respectively. In this study we use a combination of nuclear microsatellite data and chloroplast sequence data to address the following questions: (1) is *Y. gloriosa*

the product of intersectional hybridization within *Yucca*, (2) is there evidence for sexual reproduction within *Y. gloriosa* populations, (3) is there a signature of introgressive gene flow between *Y. gloriosa* and either parental species (*Y. aloifolia* or *Y. filamentosa*), and (4) are the marker data consistent with a single origin or multiple origins of the hybrid species *Y. gloriosa*.

**Materials and Methods**

*Plant material collection and DNA extraction*

Leaf material was collected from seven populations of *Y. aloifolia* (n = 32), six populations of *Y. filamentosa* (n = 29), and seven populations of *Y. gloriosa* (n = 35) primarily along the southeastern coast of United States (Figure 2.1). While these species are distributed across the southeastern United States, they are only found reliably in sympatry along the Atlantic coast. Approximately one gram of leaf material was harvested from each sample for DNA extraction. Material was flash frozen in liquid nitrogen until it could be stored in the lab at -80°C. Whole genomic DNA was extracted using a modified CTAB protocol (*76*). Several voucher specimens were collected from each population and deposited in the University of Georgia herbarium [GA].

*Chloroplast haplotype analysis*

Chloroplast markers were developed by aligning the *Yucca filamentosa* and *Hosta* chloroplast genomes (McKain et al. unpublished) and identifying the most variable regions between the two. The following markers were amplified and sequenced for six individuals per species in order to identify loci with interspecific polymorphisms: atpF-atpL, petA-psbJ, rpl20-rps12, tabE-F, trnT-trnL, ndhC-trnV, and ycf4-cemA. PCR reactions were performed in 20µl volumes containing 1.5 µl of template DNA (approximately 10ng), 17.0µl sterile distilled water, 2.5µl tricine taq buffer (0.37mM tricine, and 0.61mM KCL), 1.5µl of 25mM MgCl2, 0.5µl dNTP mixture (containing equal parts: 2mM dATPs, 2mM

dCTPs, 2mM dGTPs, and 2mM dTTPs), 1.0µl of 10.0µM reverse primer, 1.0µl of 10.0µM forward primer and one unit of taq polymerase. Cycling conditions were as follows: initial denaturation at 95°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 90 seconds, followed by a final extension at 72°C for 5 minutes. PCR products were purified by incubation with Exonuclease I and Shrimp Alkaline Phosphatase at 37°C for 15 minutes, followed by a 15 minute enzyme inactivation step at 70°C. PCR products were then sequenced in separate reactions for the forward and reverse primers using BigDye® Terminator v3.1 chemistry. Reactions conditions largely followed the manufacturer's protocols, however approximately one third of the suggested amount of BigDye® was used per reaction. Unincorporated ddNTPs were removed using Sephadex, a cross-linked dextran gel. Sanger sequencing was performed at the Georgia Genomics Facility (GGF) on an Applied Biosystems 3730xl 96-capillary DNA Analyzer.

*Microsatellite development and genotyping*

A transcriptome assembly for *Y. filamentosa* (OneKP consortium, unpublished data; http://www.onekp.com) was scanned for microsatellite repeats using MSATCOMMANDER (*77*). MSATCOMMANDER identifies simple repeats and uses Primer3 (*78*) to design flanking PCR primers. Primer pairs were tested for amplification in both hypothesized parental species. Three individuals per species were selected for initial genotyping in order to detect interspecific variation in microsatellite repeat number. Ultimately, 14 out of 55 screened loci were selected based on their polymorphic nature and ability to amplify reliably in all three species (Table 2.1).

A three primer PCR protocol was utilized to fluorescently label PCR products using a universal M13(-21) primer (*79*). Reactions were performed in 15µl volumes containing 1.5µl of template DNA (approximately 10ng), 7.5µl sterile distilled water, 3.6µl tricine taq buffer (containing 0.02mM MgCl2), 0.37mM tricine, and 0.61mM KCL), 0.06µl dNTP mixture (containing equal parts: 2mM

dATPs, 2mM dCTPs, 2mM dGTPs, and 2mM dTTPs), 0.4µl of 10.0µM reverse primer, 0.4µl of 10.0µM M13(-21) primer, 1.0µl of 1.0µM forward primer and one unit of taq polymerase. Thermocycle conditions followed a touchdown protocol as follows: initial denaturation 94°C for 5 min; 10 cycles of 94°C for 30 sec, 63°C for 30 sec with a 1°C drop each cycle, and 72°C for 30 sec; 27 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min; followed by a final extension at 72°C for 5 min. Products were diluted 1:15. A mixture of Rox dye-labeled size standard and formamide (in a 1:10 ratio) was added to each sample. Fragment analysis was performed on an Applied Biosystems 3730xl DNA Analyzer.

*Data Analysis*

Chloroplast sequence data were assembled and inspected using Sequencher® version 4.7. Nuclear microsatellite genotype data were visualized and scored using ABI's Peak Scanner™ software. The uncorrected p distance between *Y. aloifolia* and *Y. filamentosa* was calculated from a combined data set utilizing six samples per species and all seven sequenced chloroplast loci. Nucleotide alignments were made using MUSCLE (*80*) and the uncorrected p distance of the combined data set was calculated in Mesquite (*81*)

Multilocus nuclear microsatellite data were displayed graphically using principal coordinate analysis (PCO) as incorporated into GenAlEx version 6.41 (*82*). This analysis utilizes a covariance matrix based on genetic distance to plot individuals based on the variance among their multilocus genotypes.

The hypothesis that *Y. gloriosa* is a homoploid hybrid species was first tested through assessment of admixture using STRUCTURE (*83, 84*). STRUCTURE uses a Bayesian clustering algorithm to probabilistically assign the proportion of ancestry of unknown individuals into one or more source populations. In order to determine the appropriate number of clusters given the data, all individuals were included in initial analyses without a priori species designation. These data were analyzed for K values ranging from one to nine with five replicates per K. Each run had an initial burn-in period of

50,000 iterations, followed by 500,000 Markov chain Monte Carlo iterations. The ad hoc statistic ΔK (*85*), as calculated by STRUCTURE HARVESTER (*86*), was used to verify the separation of the parental species into distinct clusters.

Following the methods of James and Abbott (*22*), STRUCTURE was next used to approximate the proportion of the hybrid's nuclear genome that was inherited from each hypothesized parental species. Each parental species (*Y. aloifolia* and *Y. filamentosa*) was set as a distinct population, while the hybrid individuals (*Y. gloriosa*) were treated as having an unknown ancestry. In order to utilize the 'learning samples' function, USEPOPINFO was invoked, allowing for the data from individuals with a known ancestry to help inform the classification of individuals with an unknown ancestry. As before, each of five runs had an initial burn-in period of 50,000 iterations, followed by 500,000 Markov chain Monte Carlo iterations. All five runs were assessed for convergence.

The allelic composition of the putative hybrid's nuclear DNA was also investigated using HINDEX (*87*), a maximum-likelihood estimator of hybrid index scores. HINDEX uses codominant marker data to estimate the proportion of alleles that were inherited from each parental species. Each *Y. gloriosa* individual was given a hybrid index score ranging from 0 to 1, representing individuals that were more *Y. filamentosa*-like and more *Y. aloifolia*-like respectively. The likelihood function was determined by the frequency of each allele within the parental populations and by the unknown individual's genotype. For each multilocus genotype, the parent of origin was assigned for each locus using the approach of Gross *et al.* (*88*).

**Results**

*Chloroplast data*

Of the seven chloroplast loci (a total of 11.4 kilobases) screened, only ndhC-trnV and trnT-trnL were variable between *Y. aloifolia* and *Y. filamentosa*. At the ndhC-trnV locus, the *Y. aloifolia* haplotype differed from the *Y. filamentosa* haplotype by a transition, a transversion, a 22 based pair insertion / deletion, and a mononucleotide microsatellite repeat. At the trnT-trnL locus, the *Y. aloifolia* haplotype differed from the *Y. filamentosa* haplotype only by a mononucleotide microsatellite repeat. These genomic changes between parental species resulted in an uncorrected p distance of $1.776 \times 10^{-4}$. *Yucca aloifolia* and *Y. gloriosa* shared identical chloroplast haplotypes across all individuals and both loci.

*Nuclear data*

Of the 55 putative microsatellite amplifying primer pairs screened, 14 (25.4%) were selected after verifying that they amplified a single locus exhibiting polymorphisms between *Y. aloifolia* and *Y. filamentosa*. Sixteen primer pairs (32.7%) amplified multiple loci in at least one species, six (10.9%) primer pairs produced null alleles in *Y. aloifolia,* while the remaining 19 primer pairs (34.5%) were monomorphic between species. Based on data from the 14 suitable loci, *Y. aloifolia*, *Y. filamentosa*, and *Y. gloriosa* had an average of 1.1, 2.6, and 1.8 alleles per locus respectively. All *Y. aloifolia* samples were found have an identical multilocus genotype across all seven populations sampled.

The principal coordinate analysis (PCO) revealed three distinct clusters representing each of the species examined (Figure 2.2). Along the first principal coordinate, which explains 65.3% of the variation between individual multilocus genotypes, *Y. gloriosa* appears to be intermediate between both hypothesized parental species. The clear separation of species into distinct clusters provides evidence of

reproductive isolation between the parents and the putative hybrid. Backcrossed individuals would be expected to cluster much more closely to the parent with which they backcrossed.

Consistent with the hypothesis that *Y. gloriosa* is a product of intersectional hybridization between *Y. aloifolia* and *Y. filamentosa*, the methods of Evanoo et al. (*85*) identified two as the optimal number of clusters in the preliminary STRUCTURE analysis (Figure 2.3a). In this analysis, *Yucca aloifolia* and *Y. filamentosa* were placed in distinct clusters, with *Y. gloriosa* showing a pattern of mixed ancestry. The STRUCTURE analysis utilizing the USEPOPINFO flag indicated that alleles sampled in *Y. gloriosa* samples were shared with both parents with an average of 53% coming from *Y. aloifolia* (range: 43%-66%) and 47% from *Y. filamentosa* (range: 33%-57%)(Figure 3b). Using the maximum likelihood approach implemented in HINDEX, the mean hybrid index for all *Y. gloriosa* individuals was estimated to be 0.57 (S.E. ± 0.074), suggesting that the nuclear genome of *Y. gloriosa* is approximately 57% *Y. aloifolia*-like and 43% *Y. filamentosa*-like (Figure 2.4).

**Discussion**

When taken together, both the life history data and the genetic data clearly support the intersectional hybrid origin of *Y. gloriosa*. In agreement with the morphological distinctness of *Y. gloriosa* and its hypothesized parental species (*5*), the PCO plot reveals three distinct clusters representing *Y. aloifolia*, *Y. filamentosa*, and *Y. gloriosa*. Further, both Bayesian and maximum-likelihood methods confirm that the nuclear genome of *Y. gloriosa* is a mosaic of the hypothesized parental genomes. Based on data from two informative chloroplast loci, the plastid genome of *Y. gloriosa* was inherited from *Y. aloifolia*. Across all 14 nuclear loci examined, the parental species share only a single allele, likely as a retained ancestral polymorphism. This suggests that there is little to no introgression occurring between the hybrid and its parents. Additionally, sampled *Y. gloriosa* individuals display a wide range of genotypes at each locus including homozygosity for aloifolia-like or filamentosa-like

alleles. The segregation pattern for alleles in the hybrid suggests that *Y. gloriosa* individuals are interbreeding to produce later generation hybrids.

Of currently described homoploid hybrid species, the most common mechanism for isolating hybrid and parental populations seems to be habitat divergence (*62*). Ecological divergence may minimize both competition and interbreeding between hybrid and closely related parental populations. Transgressive segregation of parental traits may promote development of extreme traits in hybrid populations that allow them to thrive in new environments. For example, *Helianthus annuus* and *H. petiolaris* produced three hybrid species that exhibit divergent and extreme habitat preferences. Whereas *H. annuus* and *H. petiolaris* prefer mesic, clay-based soils and dry, sandy soils respectively, their progeny prefer active sand dunes (*H. anomalus*), xeric habitats (*H. deserticola*), and desert salt marshes (*H. paradoxus*) (*89*). In *Pinus*, *P. yunnanensis* and *P. tabulaeformis* hybridize to form *P. densata*, which inhabits extreme alpine environments. In contrast, the homoploid hybrid *Iris nelsonii* inhabits ecologically intermediate environments relative to its parental species. The hybrid *I. nelsonii* is found at intermediate water depths in cypress swamps, whereas *I. hexagona* thrives in open, deeper water and *I. fulva* inhabits shallower water in the understory.

Homoploid hybrid species rarely remain in local sympatry with its parental species. In 14 out of 19 examples reviewed by Gross and Rieseberg (*62*), habitat (vs. e.g. mating system) was the most important component of ecological divergence between hybrid and parental populations. Notable exceptions include the homoploid hybrid *Penstemon clevelandii,* which occurs in sympatry with its parental species, but is reproductively isolated due to a pollinator shift (*20*) and *Senecio eboracensis*, a tetraploid hybrid that is reproductively isolated from its tetraploid parent due in part to a shift in flowering phenology (*90*).

It has been posited that the creation of a 'hybrid habitat' through human-mediated or natural disturbance may promote the establishment of hybrid species (Anderson 1949). Dune habitats, where *Y. gloriosa* grows with *Y. aloifolia,* are dynamic with a high frequency of natural disturbance. Like *Y. aloifolia*, *Y. gloriosa* is able to propagate clonally through rhizomes and severed leaf tissue. This may contribute to the persistence of these species in disturbance-prone dune habitats. Both species (along with *Y. filamentosa*) also share the same moth pollinator, *Tegenticula yuccasella*. While all three species are known to flower simultaneously at some low frequency, their flowering times are largely non-overlapping, with *Y. filamentosa* flowering the earliest and *Y. gloriosa* flowering the latest on average (*27*). *Yucca gloriosa,* therefore*,* joins a small list of homoploid hybrid species that has persisted in sympatry with one or both of its parental taxa.

Recent reviews on hybrid speciation (*52, 53*) have found that the probability of polyploid (vs. homoploid) hybrid speciation increases with genomic divergence between parental species. At first glance, *Y. gloriosa* may seem to depart significantly from this pattern. The parental species in this hybridization event are in placed in reciprocally monophyletic sections of *Yucca* that have been separated by approximately 6.5 million years (*1*). Nonetheless, *Y. gloriosa* is homoploid hybrid species. An analysis of 11.4 kilobases of chloroplast sequence data show a strikingly low amount of sequence divergence between *Y. aloifolia* and *Y. filmentosa* (uncorrected p distance of $1.776 \times 10^{-4}$), suggesting that genetic distance is a more important impediment to homoploid hybrid speciation than phylogenetic (topological) distance. This paucity of genetic diversity within *Y. aloifolia* makes it impossible to determine with certainty whether *Y. gloriosa* is the result of a single or multiple hybridization events. Although *Y. gloriosa* displays only the *Y. aloifolia* chloroplast haplotype, the lack of intraspecific variation makes it impossible to rule out multiple origins of the hybrid with *Y. aloifolia* serving as the maternal parent in each event.

The hypothesized origin of *Y. gloriosa* may be promoting diversification in associated yucca moths through host race formation. Host races have been described for the flowering stalk feeding 'bogus' yucca moth species, *Prodoxus quinquepunctellus* (Svensson et al. 2005) and *P. decipiens* (*75*). Host race formation in *P. decipiens* occurred within the last 500 years following a host shift from *Y. filamentosa* to *Y. aloifolia* after the introduction of *Y. aloifolia* to the southeastern coast of the United States (*75*). Over a short period of time, host-specific *P. decipiens* populations have accumulated genetic, morphological, and phenological differences relative to each other (*75*). *Yucca gloriosa* represents another potentially even younger host for *P. decipiens*. Similarly, the divergence of *Y. brevifolia* into distinct subspecies is thought to have spurred the divergence of its pollinating yucca moth into species that display some degree of host specificity and reproductive isolation (*4, 9*). Although *T. yuccasella* (the pollinator of southeastern United States yucca species) tends to be more of a generalist than other pollinating yucca moths, certainly the potential for host race formation exists. **Conclusions**

Hybrid speciation involving polyploidy has long been recognized as an important phenomenon in plant evolution (*91*). Such events can create an instant barrier to reproduction with the parental species and may promote increased species and gene diversity. Further, it is becoming increasingly clear that all angiosperms contain a polyploidization event in their evolutionary history (*92-96*). The impact of homoploid hybridization on biodiversity is less certain because backcrossing with parental species is often possible, blurring species boundaries. Indeed, this form of hybrid speciation can be difficult to detect and a small (but growing) number of examples exist in the literature (*62*).

These data provide strong support for the hybrid origin of *Y. gloriosa* as the result of pollen dispersal from *Y. filamentosa* to the maternal parent, *Y. aloifolia*. *Yucca gloriosa* appears to be a later generation hybrid that is reproductively isolated from its parents, likely due to differences in flowering phenology. Although more data are needed to assess whether *Y. gloriosa* is the product of one or more

hybridzation events, the data provided highlight the significance of this species as being the first genetically characterized homoploid hybrid yucca species between the monophyletic sections of *Yucca* and *Chaenocarpa*.

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| | Microsatellite | Repeat Motif | Size Range |
|---|---|---|---|
| MSATYF01-Forward | CCGACTTCCACCGAACTTG | (CAG)^5 | 181-201 |
| MSATYF01-Reverse | AGACCCAGCGATGATGGAG | | |
| MSATYF03-Forward | TCAAAAGCCCCAAGAACCC | (CAT)^4 | 180-201 |
| MSATYF03-Reverse | CGATTCTCTGACCGGCGTG | | |
| MSATYF04-Forward | TCTTCCTCTGCCCAAAGCC | (CAA)^2 (CCT)^5 | 195-201 |
| MSATYF04-Reverse | TGCAGCTTCCTTGGAACAC | | |
| MSATYF12-Forward | AATGCAAGCCCTCCTCCTC | (CTT)^4 | 180-186 |
| MSATYF12-Reverse | GGGTTTTCCTTGGCACACG | | |
| MSATYF13-Forward | TTACCGAAGCCAGCTCTGC | (AG)^6 | 234-243 |
| MSATYF13-Reverse | GGAGTGAGAGAGGGAGTGG | | |
| MSATYF16-Forward | TGATTCCTGAACCCAGCCC | (CCG)^4 | 165-171 |
| MSATYF16-Reverse | GGGGTGATGGAGTAGGCAC | | |
| MSATYF28-Forward | CATGGCCACAGCCATTGAG | (AAG)^4 | 231-256 |
| MSATYF28-Reverse | CACAAATCGAGCTCCAGCG | | |
| MSATYF30-Forward | CCACCATTCCGTCACACTC | (CCG)^4 | 247-251 |
| MSATYF30-Reverse | CCATGCGGCGTTCTTGATG | | |
| MSATYF41-Forward | ACACTCCAGTCCTGCATCC | (TGA)^4 | 239-247 |
| MSATYF41-Reverse | AAGTGATCCAACATGAACATCC | | |
| MSATYF43-Forward | ACAGCAATAAGCAGGAGATAGG | (TTC)^5 | 261-279 |
| MSATYF43-Reverse | GGCCTTTTGGCTTCTGCTC | | |
| MSATYF44-Forward | TTCGAGCAGCCAGAGGAAC | (GCC)^4 | 257-266 |
| MSATYF44-Reverse | ACGCCAAGGAGAAGGACAG | | |
| MSATYF51-Forward | GTTCTCTGTCAAATTGGTTGCG | (CAT)^5 | 256-272 |
| MSATYF51-Reverse | TGCTGTGTGGTGACTTGTG | | |
| MSATYF52-Forward | TCTCAGTCTCGATGGACCC | (GCC)^4 | 175-181 |
| MSATYF52-Reverse | GGTCTTTGTCAGGAACGGC | | |
| MSATYF53-Forward | CGATCAACTGTGACATCCGC | (CTG)^5 | 322-328 |
| MSATYF53-Reverse | CTAGTCGTCCTGCACTCCC | | |

**Table 2.1:** Microsatellite loci found to be variable between *Y. aloifolia* and *Y. filamentosa.*

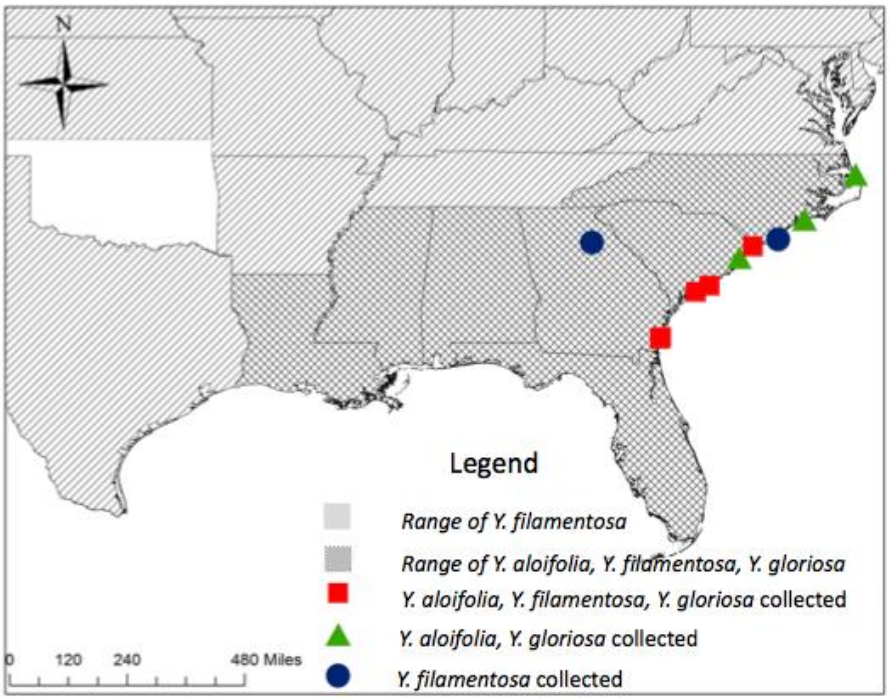



**Figure 2.1.** Location of field sites, species collected per site, and overall range of *Y. aloifolia*, *Y. filamentosa*, and *Y. gloriosa* in the United States.

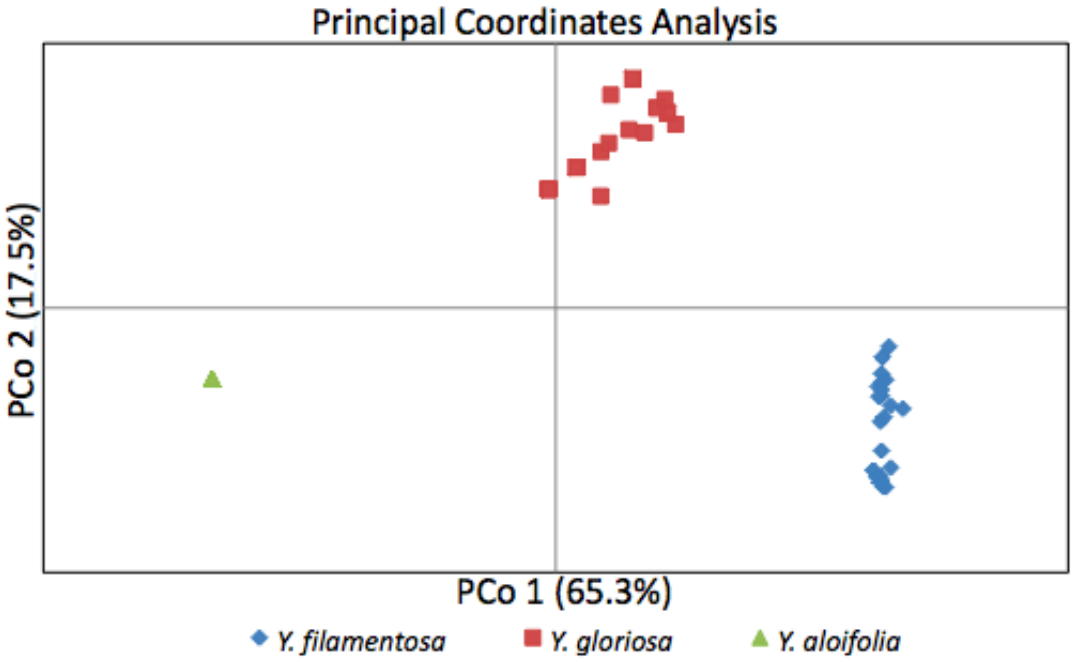
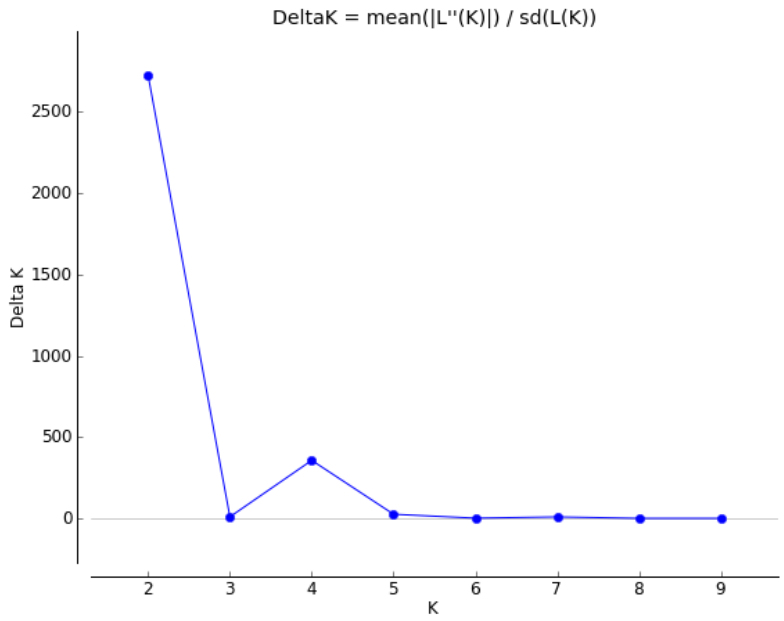



**Figure 2.2.** The first two axes of a principal coordinates analysis show distinct clusters of *Y. aloifolia*, *Y. gloriosa*, and *Y. filamentosa* individuals found along the southeastern coast of the United States.

(a)


DeltaK = mean(|L''(K)|) / sd(L(K))
Delta K
2500
2000
1500
1000
500
0
2
3
4
5
6
7
8
9
K

(b)

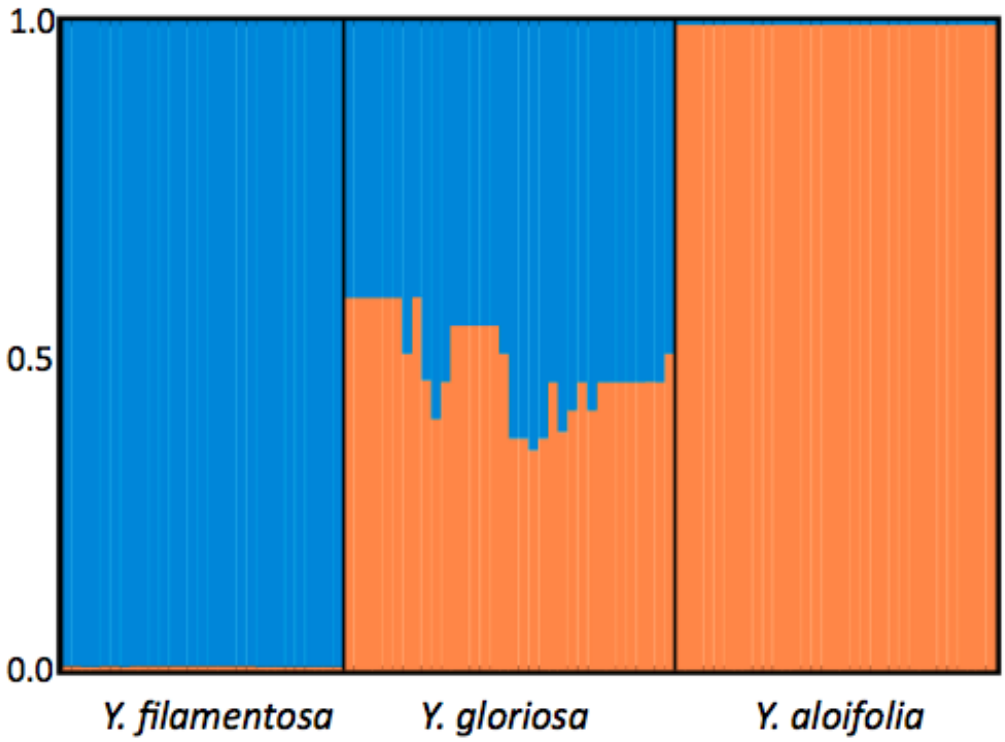

1.0
0.5
0.0
Y. filamentosa
Y. gloriosa
Y. aloifolia

**Figure 2.3.** STRUCTURE analysis of *Y. aloifolia*, *Y. filamentosa*, and *Y. gloriosa* multilocus genotypes. (a) Optimal number of clusters for the complete data set of nuclear microsatellite loci as calculated using the methods described by Evanoo *et al*. and displayed graphically using Structure Harvester (Earl and vonHoldt 2011). (b) Estimated proportion of *Y. aloifolia* (orange) and *Y. filamentosa* (blue) nuclear alleles found in all individuals sampled from the southeastern coast of the United States.

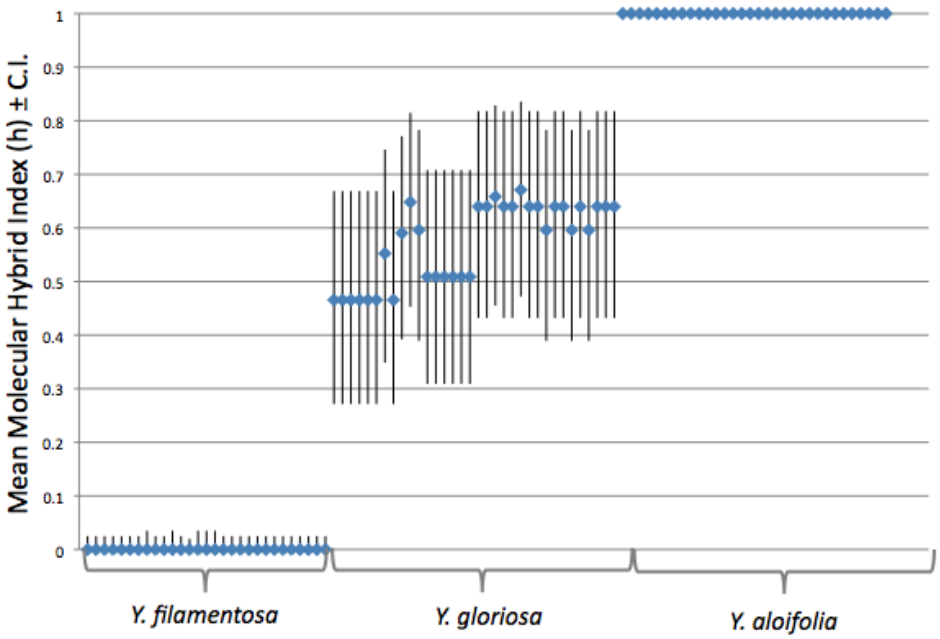



**Figure 2.4.** Maximum-likelihood estimates of molecular hybrid indices (± C.I.) based on 14 nuclear microsatellite loci for *Y. gloriosa* and its putative parents, *Y. filamentosa* and *Y. aloifolia*. The hybrid index ranges from a score of 0 to 1, where 0 is completely *Y. filamentosa*-like and 1 is completely *Y. aloifolia*-like

# CHAPTER III

## *YUCCA ALOIFOLIA* (ASPARAGACEAE) OPTS OUT OF AN OBLIGATE POLLINATION MUTUALISM.[2]

[2] Rentsch, J.D. and Leebens-Mack, J. Submitted to American Journal of Botany. 03/15/2013

**Abstract**

According to the 'law of the unspecialized' highly dependent species interactions are 'evolutionary dead ends', prone to extinction because reversion to more generalist interactions is thought to be unlikely (*28*). Cases of extreme specialization, such as those seen between obligate mutualists, are cast as evolutionarily inescapable, inevitably leading to extinction rather than diversification of participating species. The pollination mutualism between *Yucca* species and yucca moths (*Tegeticula* and *Parategeticula*) would seem to be locked into such an obligate mutualism. *Yucca aloifolia* populations, however, can produce large numbers of fruit lacking moth oviposition scars. Here, we present the results of pollinator exclusion studies performed on *Y. aloifolia* and a sympatric yucca species, *Y. filamentosa*. As expected, *Y. filamentosa* plants set fruit only when inflorescences were exposed to crepuscular and nocturnal yucca moths. In contrast, good fruit set was observed when pollinators were excluded from *Y. aloifolia* inflorescences from dusk to dawn, and no fruit set was observed when pollinators were excluded during the day. Follow-up observations, post-visit exclusion experiments, and fluorescent dye transfer experiments indicated that European honeybees (*Apis mellifera*) were passively yet effectively pollinating *Y. aloifolia* flowers. These results indicate that even highly specialized mutualisms may not be entirely obligate interactions nor evolutionary dead ends.

**Introduction**

Obligate mutualisms, where interacting species are vitally linked and exhibit mutual dependence, are fascinating and often cited products of co-evolution. Few obligate mutualisms garner as much attention as the interactions between species of the genus *Yucca* and their seed feeding pollinators, members of the genera *Tegeticula* and *Parategeticula*. This obligate pollination mutualism has been of great interest to biologists since George Engelmann first documented it in 1872. Riley (*43*) was the first to accurately characterize the highly specialized association between these species. Briefly, female moths gather pollen from yucca flower anthers. After moving to another flower within the same or a different inflorescence, the pollinating moth first oviposits into the floral ovary and then uses specialized mouth parts to deposit pollen on the yucca's bowl-shaped stigma. Yucca species are able to selectively abscise developing fruits, which discourages over-exploitation on behalf of the pollinating yucca moths (*97*). The moth larvae hatch approximately four to five days later to feed on the developing yucca seeds (*42*). Yucca moths are thought to be the sole pollinators of *Yucca* species and require the plant as a mating arena and larval food source. In this way, both the plant and the pollinator are reliant upon one another for sexual reproduction.

It has often been asserted that organisms participating in such obligate mutualisms are unlikely to experience reversions and are prone to extinction (e.g. *28, 33, 98, 99*) or shifts to parasitism (e.g. *32, 100-102*). It is estimated that there have been at least two shifts from pollination to parasitism within the yucca moth genus, *Tegeticula* (*29-31*). These 'cheater' moths coexist with pollinating species, but are purely seed parasites that oviposit into the ovary

of the yucca without pollinating the flower. A third potential outcome to obligate mutualist interactions is the shift to facultative mutualism (*32, 33*), where interacting species are not dependent on each other due the availability of alternative interaction partners. In this case, local or global extinction of one mutualist population does not necessarily lead to the extinction of other partners.

*Yucca aloifolia* has long been recognized for its perplexing ability to occasionally produce fruit outside of the range of its known pollinators (*Tegeticula yuccasella* and *T. cassandra*). Sparse or erratic fruit sets have been documented in Italy (*38*), New Caledonia (*39*), Australia (*40*) and Israel (*41, 42*). In the southeastern United States, *Y. aloifolia* is reported to be visited by *T. yuccasella* and *T. cassandra* (*38, 43, 44*), although fruits without oviposition scars have been documented (*45, 46*). It is possible that superficial oviposition just under the ovary's surface cuticle, as exhibited by *T. cassandra* (Pellmyr 1999), may have been missed in these studies. Alternatively, fruit set in the absence of moth pollination may be the result of visitation by generalist pollinators, and thus evidence for an escape from this textbook example of obligate mutualism. Riley, however, suspected that the short style and open stigma of *Y. aloifolia* may facilitate self-pollination. To test this hypothesis, Trelease and Webber (*27*) enclosed a single inflorescence in a gauze bag to exclude pollinators but allow selfing. The plant produced no fruit, leaving the mystery unsolved. Here, we investigate the reproductive ecology of *Y. aloifolia* in the southeastern United States by performing pollinator exclusion experiments, recording pollinator observations, observing fluorescent dye transfer, and describing resulting fruit sets. For comparison, identical experiments and observation were performed on *Y. filamentosa*, a sympatric yucca species that is known to be pollinated by *T. yuccasella* and *T.*

*cassandra*. The results of this study indicate that *Apis mellifera* is effectively pollinating populations of *Yucca aloifolia.* The European honey bee is a non-native, generalist pollinator that may visit a hundred or more plant species in any geographic location (*103-105*). While European honey bees prove to be effective pollinators outside of their native range (*106*), they may also serve as antagonists, occasionally acting as floral parasites (*107*) and out competing native bees for pollen and nectar resources (*108, 109*).

**Materials and Methods**

Study Species

The genus *Yucca* includes at least 40 species with most diversity being found in the southwestern Unites States and Mexico. The genus is divided into three major sections: the *Clistocarpa* (containing only *Y. brevifolia*), the *Chaenocarpa*, and the *Sarcocarpa*. *Chaenocarpa* contains capsular-fruited species and is distributed throughout the southern United States and northern Mexico, with *Y. filamentosa* native to the southeastern United States (*67*). *Yucca filamentosa* forms basal rosette of firm leaves that typically measure around half a meter long and 25 millimeters wide with thick, curly marginal threads. The inflorescences stand 1.5 – 4 meters high and consists of several hundred white (often tinged cream), pedunculate flowers (*5*). Subgenus *Sarcocarpa* contains fleshy-fruited species and is found primarily throughout eastern Mexico and the southwestern United States (*67*). *Yucca aloifolia* has the eastern-most species range within *Sarcocarpa* with populations in the southeastern United States, the Virgin Isles, and Jamaica (*5*). This species is described as a short, slender tree. The leaves are flat, thick

and rigid with denticulate margins. The inflorescence remains close to the leaves, appearing compactly panicled. The flowers are creamy, often tinged green or purple near the base (*5*).

## Study Sites

Pollinator activity was assessed in *Y. filamentosa* and *Y. aloifolia* populations within Clarke County, Georgia. The *Y. filamentosa* population (YF) was located on a highway roadside at 33.947 N, -83.409 W and included approximately 75 individuals, 55 of which appeared mature enough to flower regularly. The *Y. aloifolia* field site (YA), located on a strip of land between two parking lots (33.935 N, -83.461 W) was comprised of 95 ramets, all of which appeared mature enough to flower regularly. Both species can reproduce vegetatively and so the number of genetically distinct individuals was likely lower than the ramet census.

## Pollinator Exclusions

Throughout the months of April to July 2010 and 2011, we imposed three treatments on experimental inflorescences of *Y. aloifolia* and *Y. filamentosa* individuals: i) exclusion of diurnal pollinators, ii) exclusion of nocturnal pollinators, and iii) 24-hour exclusion. Inflorescences were bagged using a bridal veil mesh with sewn in drawstrings for closure. Six random inflorescences per species were manipulated per treatment per year. Treatments were imposed for the entirety of the inflorescences' flowering period. Diurnal pollinator exclusions began at dawn and lasted until one hour before dusk. To err on the side of excluding moth pollination, nocturnal exclusions began an hour before dusk and were removed at dawn. Controls were randomly selected inflorescences that received no treatment. For each inflorescence, the percentage of flowers that produced mature fruit was calculated by dividing the number of

ripened fruits by the number of initiated flowers as measured by the sum of fruit number and the number of remnant pedicels on the infructescence.

Assessment of Fruit Composition

Six ripened fruits per treatment (one per treated inflorescence, when available) were randomly selected each year for further observation. These fruits were dissected and the numbers of ovules, damaged seeds, otherwise aborted seeds and viable seeds were counted. The number of locular cavities per fruit was used a proxy for number of ovules. Seeds were considered predated if a significant portion of an otherwise viable seed was missing. Seeds were considered viable if they were mature and intact. Empty cavities within a locule were counted as unfertilized ovules or aborted seeds.

Visitation Observations and Post Visitation Exclusions

Observation of insect visitation was conducted on the population of *Y. aloifolia* throughout its flowering period, during the months of June and July 2011. One non-treated inflorescence was randomly selected each day for observation during two randomly selected hours between dawn and dusk. During this time, all insect visitors were documented regardless of their position on the plant. Further, in order to address pollinator efficiency, four Inflorescences received exclusion treatments as described above and were bagged for the entirety of their flowering period, except when direct insect visitation observations were being made. Each inflorescence was unbagged for intensive observation over separate time periods between dawn and dusk. During this time, all intrafloral visitors were recorded. At the end of

one hour, visitors were removed and the inflorescence was rebagged for the remained of its flowering period. When plants went to fruit, fruit set was calculated as described above.

### Fluorescent Pollen Analog Transfer

In July 2012, a powdered, fluorescent pollen analog (DayGlo Eco pigment, Day-Glo Color Corp.) was added to the stamens of select *Y. aloifolia* flowers in order to assess which intrafloral visitors may be passively collecting pollen. The analog was placed on stamens one hour after sunrise on days forecasted to be precipitation free. Intrafloral visitors were observed and collected after accessing flowers containing the pollen analog and placed under an ultraviolet light to qualitatively assess dye coverage and location.

### Statistical Analyses

A Student's t-test was used to make between species treatment comparisons. Assuming testing showed variation in number of fruits produced per species was normally distributed, a one-way analysis of variance (ANOVA) was used to compare different treatments within the same species. In the absence of normally distributed variation, a Kruskal-Wallis nonparametric test was performed. If means were found to be different for within species comparisons, a Tukey's range test was used to compare the means of each treatment to the means of every other treatment to detect which means were significantly different.

## Results

### Pollinator Exclusion

*Yucca aloifolia* and *Y. filamentosa* clearly differ in the timing of successful pollination events (Figure 1). The number of fruits produced in this experiment was found to be normally distributed, so a one-way ANOVA was utilized to test for differences among treatments within species. A significant difference was found between at least two groups for each species ($P < 0.001$, one-way ANOVA), so a Tukey's range test was used to make simultaneous comparisons among means. *Yucca filamentosa* produced its largest fruit sets (21%) when exposed to nocturnal pollinators, not significantly different from the 21.3% fruit set observed in the control. *Y. filamentosa* plants produced only a few fruits per thousand flowers when exposed to diurnal pollinators, significantly less than the control or diurnal exclusion treatment. In contrast, *Y. aloifolia* produced its largest fruit sets when exposed to diurnal pollinators (14.8%) and produced no fruit when exposed to nocturnal pollinators. Inflorescences of either species receiving the continuous exclusion treatment produced no fruit. Fruit set in the nocturnal exclusion treatment was not significantly different from zero. Significant differences were observed between species in almost every category, including: diurnal exclusion, nocturnal exclusion, and control fruit set. There was no significant difference in 24-hour exclusion treatments, as neither species produced fruit under this condition ($P > 0.05$).

### Seed Set and Predation

In treatments where fruit was produced, no significant differences were found between species or treatments for the number of ovules per fruit, or percent of ovules that were non-

viable ($P > 0.05$, one-way ANOVA). While no significant differences in the percentages of eaten or viable seeds per fruit were detected within species ($P > 0.05$, one-way ANOVA), significant differences for these measures did occur between species ($P < 0.001$, one-way ANOVA). As no within species fruit sets were statistically different, samples were pooled within species and compared to produce a summary of results for ovule fates (Table 3.1). It should be noted that the two fruits produced by *Y. filamentosa* in a nocturnal exclusion treatment (Figure 3.1) displayed levels of seed predation consistent with moth pollination in the control and diurnal exclusion groups, suggesting a low level of moth activity within the bagged inflorescences.

Visitation Observations and Post Visitation Exclusions

A number of insects were found interacting with *Y. aloifolia*, including yucca plant bugs (*Halticotoma valida*), black stink bugs (*Proxys punctulatus*), carpenter bees (*Xylocopa virginica*) and fire ants (*Solenopsis sp.*). However, the only insect found within *Yucca* flowers was the European honeybee (*Apis mellifera*), which displayed a complex behavior upon entering the yucca flower. Honeybees enter the flower, often disturbing stamens in the process. They then move to the base of the flower, where the petals attach to the carpel and circle the base of the flower, often climbing up and over the style and stigma. Often, honeybees then flew off to another flower on the same inflorescence and repeat the behavior or move to another inflorescence with a lower frequency. Further, inflorescences visited only by *A. mellifera* successfully produced fruit (Table 3.2).

Fluorescent Pollen Analog Transfer

*Apis mellifera* was found to collect the fluorescent pollen analog (Figure 3.2). While the dye was found distributed across the body of the bee, it seemed to be concentrated on the hind legs, suggesting that pollen is potentially being stored as a protein source.

**Discussion**

Seed-feeding yucca moths are generally described as the sole pollinators of plants in the genera *Yucca* and *Hesperoyucca* (e.g. *42*). A consequence of specialist moth mutualism is the predation of seeds by moth larvae, potentially resulting in a lower seed set versus a generalist, non-seed-feeding pollinator. One interesting observation, and line of evidence for non-moth pollination, in *Yucca aloifolia* is a complete lack of seed predation in the observed population. In this study, the moth pollination of *Y. filamentosa* resulted in 10.3% of seeds being predated by moth larvae. Other reports of larval seed predation in the *Yucca* genus vary significantly, although may not account for cheater moth seed predation. For example, Dodd and Linhart (*35*) estimated that approximately 67% seed predation as the result of the interaction between *Yucca glauca* and *Tegeticula yuccasella*, although this study was conducted before distinct cheater species were identified within the *T. yuccasella* complex (Pellmyr et al. 1998) . Wallen and Ludwig (*110*) found approximately 27% seed predation in *Y. baccata*. More similar to the results presented here, work done by John Addicott (*34*) investigating the reproductive ecology of eight species of *Yucca* found that seed predation was generally between 10.7% and 14.7% with significant variation occurring both between and within species.

Generalist pollination in the absence of obvious moth pollination has been invoked to explain erratic fruit sets throughout the *Yucca* genus. Addicott (*34*) noted that both *Y. baccata*

and *Y. arizonica* produced significant fruit sets with little or no seed predation or larval infestation. A similar phenomenon was described by Dodd and Linhart (*35*) in *Yucca glauca*, and speculated that non-moth pollination was occurring. Lapping flies, in the genus *Pseudocalliope*, were observed frequently on *Y. glauca* flowers and were hypothesized to have pollinated flowers when fruits contained no signs of seed damage by moth larvae. Riley was perhaps the first to hypothesize that flies and small beetles may occasionally pollinate *Yucca* species as it was observed that these insects would occasionally dislodge pollen, which then made contact with the stigma with some frequency (*36*). Keeley et al. (*37*) hypothesized that egg or larval mortality, or yucca moth pollination without oviposition could also account for these observations – an explanation that does not require an escape from the obligate mutualism. Without proper exclusion treatments, however, it is impossible to conclude whether or not non-moth pollination is occurring in these species.

Two years of experimental data presented here show that *Y. aloifolia* may not be locked into an obligate mutualism with yucca moths. While *Tegeticula yuccasella* has been documented as pollinating *Y. aloifolia*, the frequency of moth pollination across the range of *Y. aloifolia* is unknown. *Tegeticula yuccasella* pollinates a wide range of capsular-fruited *Yucca* species (*111, 112*), however divergent floral morphology and flowering time between the fleshy-fruited *Y. aloifolia* and capsular-fruited species may hinder effective pollination of *Y. aloifolia* by *T. yuccasella*. In particular, the stigmatic surface of *Y. aloifolia* is flat relative to cup-shaped stigmatic surface of other yuccas pollinated by *T. yuccasella* (*46*).Pollinator exclusion experiments document that at least some *Y. aloifolia* in the southeastern United States are pollinated by diurnal visitors rather than yucca moths (*T. yuccasella*). There is no known

mechanism for autogamous fertilization in *Yucca*. As expected, *Y. filamentosa* produced large fruit sets when exposed to nocturnal pollinators, presumably yucca moths, and an insignificant amount of fruit when exposed only to diurnal pollinators. The observed, rare fruit set observed for *Y. filamentosa* flowers that covered with exclusion bags at night when moths are active was apparently a consequence of one or more moths getting into the exclusion bag. This hypothesis is supported by the fact that these fruits displayed signs of seed predation by moth larvae. As with *Y. aloifolia*, *Y. filamentosa* produced no fruit when floral visitors were excluded around the clock.

Pollinator observations provide compelling support for the European honeybee (*Apis mellifera*) as the agent responsible or the diurnal pollination of *Y. aloifolia*. *Yucca aloifolia* sets fruit when *A. mellifera* is the only intrafloral visitor and honey bees clearly picked up fluorescent dye painted on *Y. aloifolia* anthers. These observations are consistent with the untested hypothesis of Galil (*41*), who suspected honeybees were pollinating *Y. aloifolia* in the Botanical Gardens of Tel Aviv University.

It is well known that local conditions such as foraging competition, flower abundance, and flower diversity impact the foraging behavior of bees (*113, 114*). The paucity of pollen resources available to bees in the southeastern United States during the hottest weeks of the year may explain why European honey bees are actively foraging pollen from a species that produces rather little pollen per flower. Additionally, this may explain why *A. mellifera* does not appear to visit *Y. filamentosa*, as this species tends to bloom when more pollen and nectar resources are available.

C.V. Riley (*46*) commented on the short style and open stigma of *Y. aloifolia,* suggesting that it might promote self-fertilization. While this study provides evidence against autogamous selfing in *Y. aloifolia*, it may be that the short style and open stigma of this plant does promote passive pollination by generalist pollinators such as honey bees.

While this work demonstrates that obligate mutualisms are not necessarily evolutionary dead ends, it is important to note that yucca moths have maintained a pollination mutualism with all extant *Yucca* species, most likely including *Y. aloifolia* populations, since the origin of the genus an estimated 15 million years ago (*1*). It is possible that both vegetative reproduction and facultative shifts between specialized and generalist pollinators may have buffered *Yucca* populations from extinction in the face of fluctuating pollinator populations and thus contributed to the long-term success of *Yucca* and yucca moth species. More empirical and theoretical research is needed to test whether such behavioral plasticity has been an important component in the origin and stability of ecological specialization in general (*115*) and so-called obligate mutualisms in particular.

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| Species | *N* | # Ovules | % Predated | % Viable | # Non-viable |
|---|---|---|---|---|---|
| ***Y. aloifolia*** | *12* | 247.4 ± 4.9 | 0 | 97.8 ± 1.1 | 2.1 ± 0.4 |
| ***Y. filamentosa*** | *12* | 238.5 ± 4.5 | 10.3 ± 2.5 | 87.7 ± 1.5 | 1.9 ± 0.1 |
| | | P > 0.05 | P < 0.001 | P < 0.001 | P > 0.05 |

Table 3.1: Descriptive statistics for fruit composition of *Y. aloifolia* (pollinated diurnally) and by *Y. filamentosa* (pollinated nocturnally). As data were distributed normally, a Student's t-test was used to assess between species differences in each category.

| Exclusion Treatment | Capsules | Flowers | Fruit Set (%) |
|---|---|---|---|
| **1** | 4 | 87 | 4.60 |
| **2** | 3 | 91 | 3.30 |
| **3** | 6 | 90 | 6.67 |
| **4** | 2 | 79 | 2.53 |
| | | | 4.27 ± 1.8 |

Table 3.2: Fruit set in *Y. aloifolia* produced as the result of exposure to diurnal pollinators for one hour. In each of four exclusion treatments, *Apis mellifera* was the only intrafloral visitor observed.

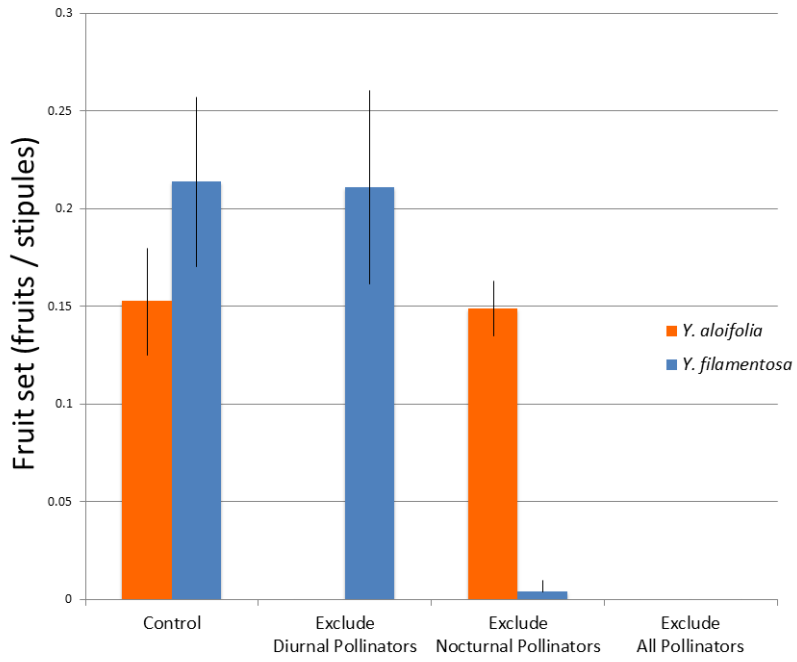


Figure 3.1: Mean and standard error of *Yucca aloifolia* and *Y. filamentosa* fruit sets under various exclusion treatments in 2010 and 2011. N = 12 individuals per species per treatment.

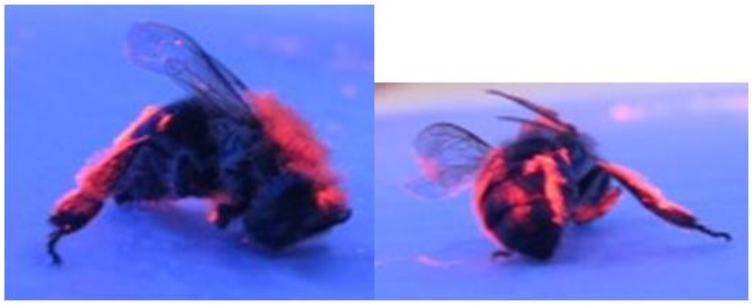

Figure 3.2: European honey bee captured after dislodging a fluorescent pollen analog from *Yucca aloifolia* anthers.

# CHAPTER IV

## POPULATION GENETICS OF YUCCA ALOIFOLIA (ASPARAGACEAE).[3]

[3] Rentsch, J.D. Heyduk, K, and Leebens-Mack, J. To be submitted to Molecular Ecology.

**Abstract**

*Yucca aloifolia* is a long-lived coastal species found distributed widely along the southeastern Unites States Atlantic coast. In addition to the south eastern U.S., *Y. aloifolia* is found in Jamaica, the U.S. Virgin Isles, and the eastern coast of Mexico. While the species is thought to be largely outcrossing with a fairly continuous distribution along the coast, it is also known to propagate clonally. Further, the species is known to be pollinated by both moth pollinators that are typical for the genus, and honey bee pollinators. All of these factors contribute to the way that genetic diversity is partitioned among and within populations. Previous work has shown that Y. aloifolia is also the maternal parent in a homoploid hybrid speciation event that produced Y. gloriosa. A lack of variation in chloroplast loci examined at the time made it impossible to infer the number of times the speciation event may have taken place. Here, we use target enrichment through use of a biotinylated probe set in order to assess SNP variation across seven field sites of *Y. aloifolia* in the southeastern United States. Using these data, we show that *Y. aloifolia* can be divided into at least two distinct populations, those found on Bear Island, North Carolina and those found elsewhere along the coast. We have also identified chloroplast SNP variation that will allow us to further characterize the hybrid speciation event in which *Y. aloifolia* was the maternal parent.

**Introduction**

*Yucca aloifolia* is a species with a convoluted and uncertain biogeographic history. While it is found distributed across the southeastern United States, Jamaica, the U.S. Virgin Isles, and the eastern coast of Mexico, William Trelease described *Y. aloifolia* as having "no known geographic origin" (*5*). The uncertainty concerning *Y. aloifolia*'s geographic origin can be attributed to its current distribution when compared to other fleshy-fruited yucca species coupled with the potential for long distance dispersal and human association. Aside from *Y. aloifolia* fleshy-fruited *Yucca* species (sect Yucca formerly Sarcocarpa Engelm.) are distributed from the Yucatan to northwestern Mexico and the American Southwest, reaching the Pacific coast in Baja and southern California. *Yucca aloifolia* is the only fleshy-fruited species represented in the southeastern United States.

A species' life history characteristics influence its pattern of genetic diversity within and among populations. As a long-lived, largely outcrossing species, *Yucca aloifolia* may be expected to maintain relatively high levels of genetic diversity (*47*). Furthermore, the species' fairly continuous distribution along sand dunes across the coast of the southeastern United States suggests that populations are genetically well connected through high levels of gene flow and thus little among-population genetic differentiation is expected.

However, the phylogeographic history of *Y. aloifolia* and its mixed pollination system (Chapter 2) may have resulted in more genetic structure than expected. Like all yuccas, *Y. aloifolia* participates in the remarkable yucca / yucca moth mutualism (*38, 43, 44*). All members of the *Yucca* genus are known to associate with mutualistic moth pollinators in the genera *Tegeticula* and *Parategeticula*. Moths are often regarded as the sole pollinators of *Yucca* species, and moths require the plants as a mating arena and larval food source. The highly specialized pollination mutualism between these species is likely to influence pollen movement, and subsequent gene flow. Interestingly, although *Yucca* species are self-compatible, a mating systems analysis in *Y. filamentosa* has shown lower rates of self-pollination than

expected by moth behavior – suggesting *Yucca* species may selectively abscise fruits containing predominantly self-fertilized ovules(*116*). Interestingly, *Y. aloifolia* has been shown to be diurnally pollinated by the European honey bee (*Apis mellifera*). While other studies have found that the European honey bee may pollinate poorly when compared to native pollinators (*117*), evidence collected in Athens, GA suggests that *Y. aloifolia* flowers pollinated by *A. mellifera* achieve fruit set at rates nearly equal to those seen in moth pollinated flowers of other *Yucca* species (Chapter 2). Further, this species propagates vegetatively exceptionally well from both clonal extension and the regeneration from rootstock, stems and leaves (*8*)! *Yucca aloifolia* is often found in very tightly associated clumps of what are suspected to be clones. At the same time, the extent of seed movement in this species is unknown and in the absence of a rodent vector seeds may be solely distributed by gravity. These life history characteristics make it difficult to accurately predict the spatial pattern of genetic diversity for *Y. aloifolia*.

*Yucca aloifolia* was recently shown to be the maternal parent in a homoploid hybrid speciation event that produced *Y. gloriosa* (*118*). Remarkably, no variation was identified in 10 nuclear microsatellite loci and two chloroplast marker loci sampled in 28 individuals sampled across seven *Y .aloifolia* populations (Chapter 1). The observed lack of variation in the plastid and nuclear loci is consistent with the hypothesis that the species is highly clonal and maybe be genetically depauperate in the southeastern United States. In any case, we were unable to assess whether *Y. gloriosa* was the product of a single or multiple hybridizations given the lack of observed genetic variation in the putative maternal parent, *Y. aloifolia*.

In order to further investigate the low levels of genetic variation we had seen in *Y .aloifolia*, we assessed nucleotide sequence variation at many nuclear and plastid loci sampled from seven *Y. aloifolia* populations distributed along the Atlantic coast of the southeastern United States (Figure 1). Enrichment of target genes in DNA templates was achieved using a biotinylated exon probe set that was

designed to address a variety of questions concerning the evolution of genes and genomes throughout the history of the Agavoideae. Target-enriched DNA templates were sequenced and assemblies of single-copy target genes were analyzed in order to assess within and among population sequence variation in natural or naturalized *Y. aloifolia* populations.

**Materials and Methods**

Study species

*Yucca aloifolia* often grow in large, impenetrable cluster of ramets with short trunks, pointed leaf blades and impressive panicles when they are flowering. The pointed leaf blades are flat, thick, rigid, and denticulate on the margins. The inflorescence is a compact panicle, with white flowers that may be tinged green towards the base. Unlike most other species of yucca, the style is quite short with a relatively flat stigmatic surface. *Yucca aloifolia* is distributed across the southeastern United States, Jamaica, the U.S. Virgin Isles, and Mexico, but its native range is unknown owing to a history of human mediated and long-distance dispersal.

Plant material collection and DNA extraction

Fresh leaf material was collected from seven field sites of *Y. aloifolia* (n = 28) along the southeastern coast of the United States (Figure 4.1). One gram of leaf material was harvested from each sample for DNA extraction. Material was flash frozen in the field in liquid nitrogen and subsequently stored in the lab at -80°C. Whole genomic DNA was extracted using a modified CTAB protocol (*119*). Voucher specimens were collected from each population and deposited in the University of Georgia herbarium [GA].

Genomic library preparation and probe hybridization

Approximately 1.1µg of DNA was sheared per sample. Libraries were produced using a modified Illumina DNA-Seq genomic library preparation protocol. The barcoded genomic DNA libraries were then

heat-denatured and hybridized to RNA baits for 36 hours. RNA baits were designed to hybridize to exons of nuclear genes and the large-single copy region of the chloroplast genome. After hybridization, targeted fragments were pulled out of solution using streptavidin-coated magnetic beads. Non-targeted DNA was washed off and targeted DNA was released by chemical degradation of RNA baits. The captured DNA fragments include the target exons and adjacent non-coding intron sequences. Barcoded, target-enriched libraries for each sample were pooled and send to the BGI Americas lab in Davis, CA for paired-end sequenced on an Illumina HiSeq v. 2500.

Illumina read cleaning

Illumina sequence pools were obtained from the sequencing lab as a fastq file and separated into sample-specific fastq files based on their barcode sequences. Sequences were then cleaned by trimming nucleotides at the 3' ends with Phred scores of less than 20 (to a minimum length of 40 nucleotides). Next, reads were discarded if they had a Phred score of less than 20 across more than 80% of their remaining length. Finally, cleaned reads were scanned for internal or end ligation of Illumina adapter sequences. External adapters were trimmed and reads with internal adaptors were removed.

Sequence assembly

The *Inchworm* and *Chrysalis* and *Butterfly* modules within the Trinity *de novo* assembler software package(*120*) were used to produce genome assemblies from each sequenced library. *Inchworm* utilizes a greedy k-mer based approach to recover a single representative among sequence variants that share the same k-mer. *Chrysalis* then clusters Inchworm contigs and generates de Bruijn graphs for each cluster, enumerating all possible linear reconstructions of contig overlaps. Butterfly then reports full-length sequences that may represent alternate forms of a gene. The R script RSEM (*121*) was then used to remove putative isoforms that were not supported by greater than 1% of reads mapping to a given component (i.e. putative locus). In order to remove contigs that are likely

sequence errors, CAP3 (*122*) was then used to collapse contigs within assemblies exhibiting less than 5% sequence divergence. Collapsed alleles, were reconstructed from the CAP3 consensus sequences after screening for multi-copy genes (see below).

Gene selection

A database of putatively low or single copy exons was constructed using a list of genes that have been found to be retained in single copy across 10 sequenced land plant genomes (dePamphils lab unpublished,(*123*). A BLAST (*124*) search was performed with each assembly against the low-copy exon database. BLAST results were assessed for evidence of recent gene duplication as indicated by multiple high quality matches Loci with evidence of recent duplication were removed from further consideration in order to avoid confusion between allelic (orthologous) and paralogous gene sequences. For each locus, exons that were not joined in the assembly process were concatenated into gene sequence scaffolds and multiple sequence alignments were produced for each gene using MUSCLE(*80*). A final set of genes was compiled by selecting only genes that were present in at least 18 of 24 samples and which had an average pairwise distance of less than 10%. Distances of greater than 10% most often represented poor assembly quality. Selected genes exhibited an average divergence of 4% (SE = 0.65%) between sample pairs and were represented in an average of 21 (SE = 1.1) of 24 samples.

Recovering alleles

In order to recover alleles that may have been collapsed in the assembly process, we utilized SAMtools mpileup (*125*) to map reads back to a reference sequence for each locus. Reads were mapped with the local alignment option with the short-read aligner, Bowtie (*126*). For each sample, all sequence variants relative to the reference were called and filtered for quality scores less than 20 (based on Illumina Phred scores), in order to avoid calling sequencing errors as SNP

variation. For each individual, the location of each SNP was recorded and the sample was scored for homo or heterozygosity at the SNP locus. Data for all samples were compiled for each locus.

Identification of populations

A Principal Coordinates Analysis (PCoA) executed in GenAlEx v.6.5 (*82*) was used to visually assess patterns of genetic diversity among individuals and populations. PCoAs locate the major axes of variation within a multivariate data set and plots them, allowing for the assessment of spatial patterns of genetic variation based on clustering. This was used to estimate the number of populations present in the dataset for further analyses. After distinct clusters were defined as populations, remaining individuals with no clear clustering pattern were separated into geographic divisions and both Fst and the estimated number of migrants per generation (Nm) were calculated in order justify leaving the remaining individuals as a single population.

Genetic diversity

For each population of *Y. aloifolia*, we described the number of unique genotypes (G), proportion of unique genotypes (G/N), expected heterozygosity (He), observed heterozygosity (Ho) proportion of polymorphic loci (PPL) and average number of alleles per locus (A). We then calculated Fst and Nm among populations, as described above.

Chloroplast analysis

Plastid assemblies were performed using YASRA (*127*), a *de novo* short-read assembler. Chloroplast genomes were aligned using the multiple sequence aligner, MAFFT (*128*). The large single copy region of the plastid genome was selected for further analysis by aligning individual plastid genomes to the annotated large single copy region of *Yucca schidigera.* A maximum parsimony consensus tree was produced in order to assess the distribution of chloroplast haplotypes geographically.

## Results

### Sequencing and assembly statistics

Of 28 total samples sequenced, 24 had an average raw read count of 3.47 million. The remaining four samples had less than 5000 reads and were excluded from further analyses. Table 4.1 provides a summary of sequencing and assembly statics including average raw reads per library, number of contigs after Trinity assembly, number of contigs after CAP3 (contigs CAP3), number of contigs after a BLAST query against single / low copy genes (SC BH), number of contigs after duplicates were removed (RD), total number of genes after exon concatenation (Genes), and number of genes selected for analyses (Final genes). A total of 817 SNPs were detected across the 99 loci ultimately selected for analysis (average = 8.2 SNPs per gene). Table 4.2 summarizes both the location and quality of SNP loci. As expected, more SNPs are found in intronic than exonic regions.

### Identification of genetically distinct populations

A PCoA was performed on 24 Y. aloifolia individuals collected from seven field sites. The genetic distance matrix was produced using SNP data from the 99 genes selected for analyses. The PCoA revealed the distinct clustering of *Y. aloifolia* individuals sampled on Bear Island, NC (Figure 4.2). These individuals are clearly separated on the X-axis, which explains 70.31% of the variation within the matrix. The remaining individuals showed no clear clustering by collection site, so were grouped into populations by broad geographic location and Fst and Nm were calculated between southern (GA) and northern (SC + Carolina Beach State Park) field sites. The Fst between the southern and northern populations was found to be quite low (Fst = 0.045, SE = .008). These results suggest the coastal populations are part of a single population, with the exception of Bear Island, NC – which is genetically distinct.

Genetic diversity

Population genetics statistics are summarized in Table 4.3. While the Coastal population and Bear Island population are quite similar in terms of their observed heterozygosity, number of alleles per locus, and proportion of polymorphic loci, they appear to be genetically distinct with an Fst of 0.19 (SE = .01), and an estimated number of migrants per generation of only 0.25 (SE = 0.02).

Chloroplast Analysis

A total of 83,669 base pairs were aligned from the single copy region of the chloroplast of the 24 individuals in this analysis. A total of 56 parsimony informative sites were found and each individual displays a unique chloroplast haplotype. A Maximum Parsimony Consensus tree shows little structure among chloroplast haplotypes (Figure 4.3) when compared to nuclear loci.

**Discussion**

Gene capture methods utilized in this manuscript have proven useful for uniquely differentiating all *Y. aloifolia* individuals sampled. While heterozygosity averaged across all loci was only around 16% (15% for the Coastal population and 18% for the Bear Island, NC population), the percentage of polymorphic loci was quite high (50% on average), resulting in a unique multilocus genotype for each individual.

The nuclear SNP data presented here clearly justify the separation of southeastern U.S. *Yucca aloifolia* individuals into two distinct populations; those found on Bear Island, North Carolina and those found elsewhere on the coast (including individuals from Georgia, South Carolina, and North Carolina). The Fst between these populations is 0.19, which is considered moderate genetic differentiation. The Fst between individuals found in Georgia and those found in South Carolina and North Carolina (excluding Bear Island, NC), on the other hand, was found to be 0.045, which is considered low differentiation. This suggests that either there is a high degree of connectivity

between the Coastal population field sites, or that they have been separated such a short amount of time that the effects of genetic drift have not acted to significantly differentiate populations.

It is estimated that there are approximately 0.25 migrants per generation between the Coastal population and Bear Island. Wright's (*129*) work on the idealized infinite-islands model implied that an effective number of migrants per generation of only 0.5 is sufficient to counteract the effects of genet drift and keep populations genetically homogenous. In practice, this number is typically around one migrant per population (*130, 131*). An effective number of migrants per population of 0.25, as seen between the Coastal population and Bear Island population, is likely low enough such that genetic drift is a significant factor differentiating these populations. Given that fleshy-fruited *Yucca* species are rare in the southeastern United States, these data may imply several things: either the species has been introduced to the United States on least two occasions (either via human mediated dispersal or long distance dispersal), or populations have been separated for a sufficient amount of time for genetic drift to make them quite distinct.

Work performed previously (*118*) has shown a remarkable lack of copy number variation among *Y. aloifolia* individuals across 10 nuclear microsatellite loci developed from the *Y. filamentosa* transcriptome. This observation coupled with *Y. aloifolia'*s ability to propagate clonally through extensions and the regeneration of severed leaves (*8*) implied that the species could be largely clonal throughout the southeastern United States. The amount of data analyzed here (817 SNPs across 99 genes) far exceeds the 10 nuclear microsatellite loci used previously and has shown that each individual sampled has a unique multilocus genotype. The PCoA shows that samples collected in the Coastal population in close proximity to each other (e.g. within the same field site) do not necessarily cluster together based on pairwise genetic distance, implying there is actually little clonality in this locale when compared to sexual reproduction.

While chloroplast SNP variation was found to be quite low (nucleotide diversity index of 0.00612), variation was identified that will likely be able to further characterize the hybrid speciation event that took place between *Y. aloifolia* and *Y. filamentosa*. *Yucca aloifolia* has previously been described as the maternal parent in the homoploid hybrid speciation event that produced *Y. gloriosa*, but a complete lack of plastid variation within *Y. aloifolia* at the loci examined previously made it impossible to infer the minimum number of speciation events. Amplifying these loci in *Y. gloriosa* will likely result in one of two patterns: all sampled *Y. gloriosa* individuals will display a single chloroplast haplotype (implying a single hybrid speciation event or multiple events within the same population) or *Y. gloriosa* individuals contain various chloroplast haplotypes among individuals (implying multiple hybrid speciation events).

This work has added a great deal to what we know about *Y. aloifolia* in the southeastern Unites States. The identification of several distinct populations allows us to infer multiple introduction events, or a single introduction event with significant separation of populations after introduction. Given that *Y. aloifolia* is thought to be a relatively recent addition to the flora of the southeastern United States, it seems more likely that founders from each current day population evolved independently and were subsequently introduced to the southeastern U.S. This work makes studying this species on an international scale a very attractive prospect as source populations could be identified in order to form a more complete phylogeographic story.

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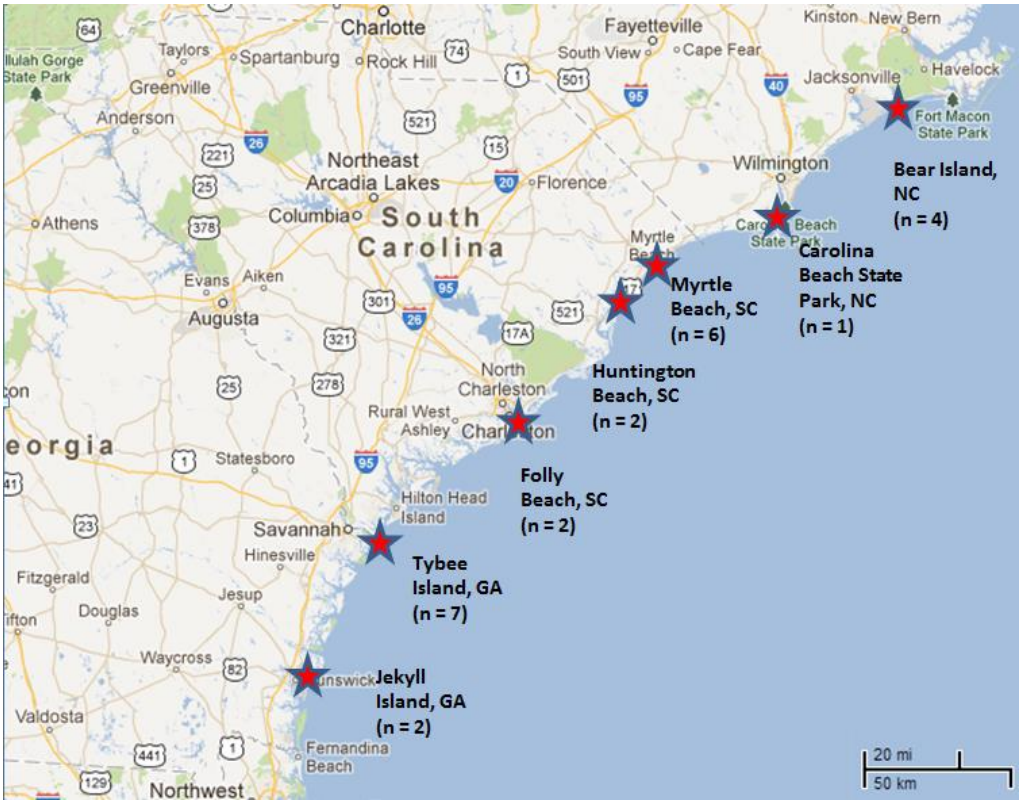


Figure 4.1: *Yucca aloifolia* collection sites with the number of individuals sequenced per field site.

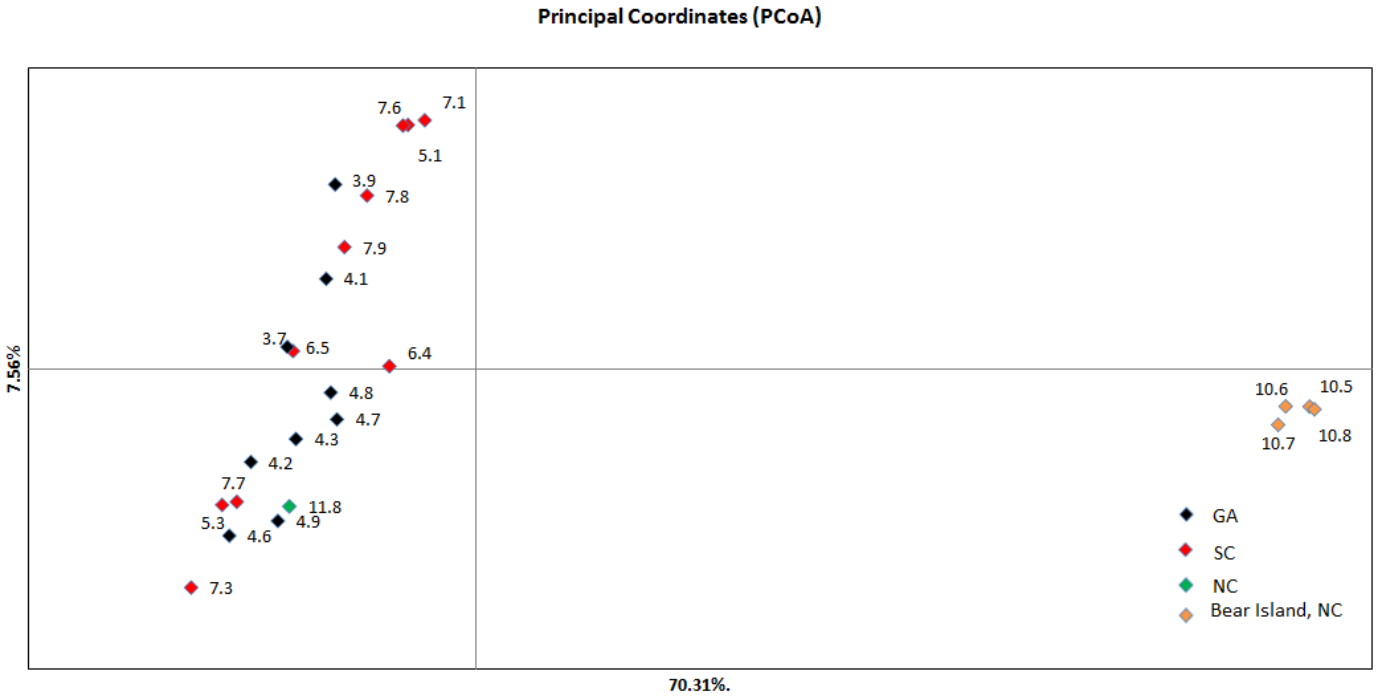

Figure 4.2: Principal Coordinates Analysis (PCoA) based on a genetic distance matrix generated with SNP data from 99 genes for 24 *Y. aloifolia* individuals. The PCoA reveals distinct clustering of the Bear Island, NC individuals along the X-axis, which represents 70.31% of the variation within the distance matrix. A clear pattern is not seen among the remaining field sites.

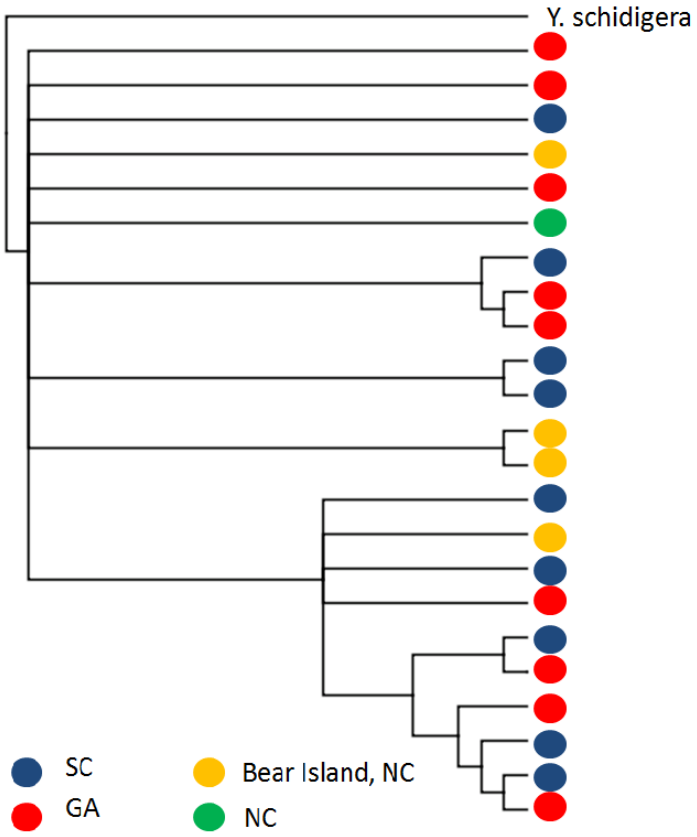


Figure 4.3: Maximum Parsimony Consensus tree produced from an alignment of 83,669 base pairs of the chloroplast genome from the large single copy region.

| | Average | SD |
|---|---|---|
| Raw reads (million) | 3.47 | 1.9 |
| Trinity contigs | 14282 | 6893 |
| Contigs (CAP3) | 13238 | 6472 |
| Contigs (SC, BH) | 2247 | 308 |
| Contigs (RD) | 966 | 30 |
| Genes | 752 | 22 |
| Final genes | 99 | n/a |

Table 4.1: Summary of sequencing and assembly statistics. Number of contigs after Trinity assembly (Trinity contigs), number of contigs after CAP3 (contigs (CAP3)), number of contigs after a BLAST query against single / low copy genes (Contigs (SC BH)), number of contigs after duplicates were removed (Contigs (RD)), total number of genes after exon concatenation (Genes), and number of genes selected for analyses (Final genes)

| | Exons | Introns |
|---|---|---|
| Total number SNPs | 352 | 465 |
| Coverage (average) | 18.5 | 16.2 |
| Missing data (average %) | 9% | 14.50% |
| SNPs 10x coverage+ | 256 | 316 |

Table 4.2: Summary of SNP location and quality

.

| | Coastal | Bear Island |
|---|---|---|
| PPL | 51.43% | 49.68% |
| A | 1.43 (0.21) | 1.32 (0.13) |
| Ho | 0.155 (0.006) | 0.185 (0.012) |
| He | 0.159 (0.004) | 0.173 (0.009) |
| G | 20 | 4 |
| G/N | 1 | 1 |

Table 4.3: Population genetic statistics calculated for the Coastal population of *Y. aloifolia* (n = 20) and the Bear Island population of *Y. aloifolia* (n = 4).

# Chapter V

## CONCLUSION AND DISCUSSION

The work completed in this dissertation has highlighted the interesting evolutionary and ecological history of *Yucca* species in the southeastern United States. Data provided here convincingly shows that *Yucca gloriosa* is a homoploid hybrid species formed between *Y. aloifolia* and *Y. filamentosa*. When taken together, both the life history data and the genetic data clearly support the intersectional hybrid origin of *Y. gloriosa*. In agreement with the morphological distinctness of *Y. gloriosa* and its hypothesized parental species (*5*), analyses provided here show a distinct clustering of individuals representing *Y. aloifolia*, *Y. filamentosa*, and *Y. gloriosa*. Further, both Bayesian and maximum-likelihood methods confirmed that the nuclear genome of *Y. gloriosa* is a mosaic of the hypothesized parental genomes. Based on data from two informative chloroplast loci, the plastid genome of *Y. gloriosa* was inherited from *Y. aloifolia*. Across all 14 nuclear loci examined, the parental species share only a single allele, likely as a retained ancestral polymorphism. This suggests that there is little to no introgression occurring between the hybrid and its parents. Additionally, sampled *Y. gloriosa* individuals display a wide range of genotypes at each locus including homozygosity for aloifolia-like or filamentosa-like alleles. The segregation pattern for alleles in the hybrid suggests that *Y. gloriosa* individuals are interbreeding to produce later generation hybrids. Further work on this system could involve examining how physiological traits are segregating in the hybrid. For example, it is known that *Y. aloifolia* utilizes the CAM photosynthetic pathway, while *Y. filamentosa* utilizes the $C_3$ pathway. Further, it is know that *Y. gloriosa* is able to shift between pathways, possibility owing to its hybrid origin. Researching the mechanisms behind this shift would be of great interest to biologists in general.

The results of his work challenges the idea that highly specialized species interactions are evolutionary dead-ends. Cases of extreme specialization, such as those seen between obligate mutualists, are cast as evolutionarily inescapable, inevitably leading to extinction rather than diversification of participating species. Work provided here has shown that the European honey bee (*Apis mellifera*) is successfully pollinating *Y. aloifolia*, an observation that refutes the idea of evolutionary inescapability. Generalist pollination in the absence of obvious moth pollination has been invoked to explain erratic fruit sets throughout the *Yucca* genus. Addicott (*34*) noted that both *Y. baccata* and *Y. arizonica* produced significant fruit sets with little or no seed predation or larval infestation. A similar phenomenon was described by in *Yucca glauca*, and Dodd and Linhart (*35*) speculated that non-moth pollination was occurring. Lapping flies, in the genus *Pseudocalliope*, were observed frequently on *Y. glauca* flowers and were hypothesized to have pollinated flowers when fruits contained no signs of seed damage by moth larvae. Riley was perhaps the first to hypothesize that flies and small beetles may occasionally pollinate *Yucca* species as it was observed that these insects would occasionally dislodge pollen, which then made contact with the stigma with some frequency (*36*). Keeley et al. (*37*) hypothesized that egg or larval mortality, or yucca moth pollination without oviposition could also account for these observations – an explanation that does not require an escape from the obligate mutualism. These explanations lacked proper exclusion treatments, however, so it was impossible to conclude whether or not non-moth pollination was occurring in these species. Pollinator observations presented in this dissertation provide compelling support for the European honeybee (*Apis mellifera*) as the agent responsible or the diurnal pollination of *Y. aloifolia*. *Yucca aloifolia* sets fruit when *A. mellifera* is the only intrafloral visitor and honey bees clearly picked up fluorescent dye painted on *Y. aloifolia* anthers. These observations are consistent with the untested hypothesis of Galil (*41*), who suspected honeybees were pollinating *Y. aloifolia* in the Botanical Gardens of Tel Aviv University.

The work completed here on the population genetics of *Y. aloifolia* synergizes well with the rest of the dissertation. Utilizing target enrichment methods we were able to assay the existing genetic variation within southeastern U.S. *Y. aloifolia* individuals. We found that two distinct populations of the species exist in the southeastern U.S., those found on Bear Island, NC and those found elsewhere along the coast. The implication of this is that Y. aloifolia has likely been introduced to the United States on at least two separate occasions. This observation makes studying *Y. aloifolia* abroad a very attractive perspective, as it is now possible to identify source populations and tell an interesting phylogeographic history, while simultaneously studying the pollination biology of the species in various locations. SNP loci located in the chloroplast will also make it possible to tell a more complete story on the hybrid origin of *Yucca gloriosa*. A lack of variation among *Y. aloifolia* individuals at a number of chloroplast loci made it impossible to infer the number of hybrid speciation events that produced *Y. gloriosa*. These new data will help us tell a more complete story and further characterize and generalize the process of hybrid speciation.

Overall, Yucca species of the southeastern United States have lent themselves nicely to a broad array of interesting evolutionary and ecological questions.

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