ASSESSING THE IMPACTS OF GENE FLOW BETWEEN ENDEMIC HAWAIIAN COTTON, *Gossypium tomentosum*, AND TWO COMMERCIAL COTTON SPECIES

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ABSTRACT

To determine the ease with which Hawaiian cotton, *Gossypium tomentosum,* hybridizes with two introduced commercial cotton species, *G. hirsutum* and *G. barbadense,* reciprocal crosses were carried out in a controlled setting. To assess the impacts of gene flow on fitness and fecundity, F_1 hybrids were grown in a common garden plot along with parental species. Results show that there are no strong hybridization barriers between *Gossypium tomentosum* and either commercial cotton species. In hand pollination trials, *G. tomentosum* is more prone to accept foreign pollen than either commercial species, with higher rates of hybridization. The same hand pollination trial showed that *G. tomentosum* hybridizes more freely with *G. hirsutum* than with *G. barbadense.* All F_1 hybrids show significantly greater fitness and fecundity than conspecific F_1 *G. tomentosum* suggesting that natural F_1 hybrids could outcompete native cotton populations for habitat and resources should they become established.

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CHAPTER 1. INTRODUCTION

INTRODUCTION

The Hawaiian archipelago is the most isolated island group in the world, separated by over 800 km to other islands and over 3,000 km to the next major land mass (McDonald *et al.*, 1986). Due to this isolation and 1–5 million years of evolution (McDonald *et al.*, 1986), the Hawaiian Islands are home to one of the highest levels (~90%) of plant endemism in the world (Wagner *et al.*, 1999). This high endemism rate makes any biodiversity loss in the islands even more poignant (Gagné, 1988). Currently, 319 native Hawaiian plant species, accounting for over 25% of the native flora, are threatened or endangered, accounting for 40% of all federally listed plant species (USFWS 2012).

Island populations of some plant species have a much higher risk of extinction than continental populations (Frankham, 1988; Rieseberg, 1991; Levin *et al.*, 1996) and greater susceptibility to hybridization because of their small numbers and their frequent proximity to numerically larger congeners (Carlquist, 1974; Crawford *et al.*, 1987). Another factor that increases hybridization rates in insular populations is that they often have poorly differentiated floral architecture, thus facilitating interspecific cross-pollination (Carlquist, 1974). Plants in insular populations can also feature morphological characteristics that promote out-crossing, such as stigmatic surfaces exerted above the anthers (Fryxell, 1979). Long flowering seasons in many tropical islands may also promote opportunity for hybridization (Carlquist, 1974). Insular species are also particularly vulnerable to hybridization due to gametic wastage, reduced seed-set, and the

production of ill-fit progeny on the one hand, and the swamping effect of gene flow on the other (Levin *et al.*, 1996). These effects are not likely to be counterbalanced by immigration from other conspecific populations due to isolation and remoteness.

One species that is at risk of alien-native hybridization is Hawaiian cotton or *ma'o* (*Gossypium tomentosum*) with two introduced agriculturally important cotton species Pima cotton (*G. barbadense*) and Upland cotton (*G. hirsutum*) (Fryxell, 1979; Ellstrand *et al.*, 1999; Daehler and Carino, 2001; Münster and Wieczorek, 2007; Pleasants and Wendel, 2010). This long lived, woody perennial species occurs in arid, lowland and coastal sites between sea level and 120 m on all the main Hawaiian Islands, except the Islands of Hawai'i and Kaua'i (Wagner *et al.*, 1999) though it has recently been reintroduced to Kaua'i (Krakos *et al.*, 2010). Both introduced cotton species are now sporadically naturalized; Pima cotton occurs on all the main islands except Kaho'olawe, and Upland cotton is present on O'ahu (Wagner *et al.*, 1999) and East Maui (Wagner *et al.*, 2012). In addition, transgenic varieties of both commercial species may potentially be grown by the seed industry in Hawai'i making the threat of crop-to-wild gene flow for this species even more poignant.

NATURAL INTERSPECIFIC HYBRIDIZATION

Conservation Impacts of Natural Interspecific Hybridization

Arnold (1997) defines natural hybridization as "*involving successful matings in nature between individuals from two populations, or groups of populations, which are distinguishable on the basis of one or more heritable characters.*" Hybridization can

have profound effects on a species' ability to adapt and survive with outcomes of hybridization ranging from new species formation to species extinction (Ellstrand, 2003b). When related species occur in mixed populations, it is not uncommon to find hybrids amongst them (Ellstrand, 2003b). One example of this involves the California black oak (*Quercus kelloggii*) and the interior live oak (*Quercus wislizenii)*. F_1 hybrids (named the oracle oak, *Q.* x *morehus*) are found where the two species occur sympatrically (Nason *et al.*, 1992). In addition to proximity, populations size influences hybridization rates with gene flow expected to dominate from large to small populations (Ellstrand and Elam, 1993; Vila *et al.*, 2000). For example, little gene flow was observed between small wild radish (*Raphanus sativus*) populations in close proximity to one another while high rates of gene flow were observed from large distant populations (Ellstrand *et al.*, 1989). Such interspecific hybridization can be detrimental to rare plant species both demographically and genetically.

Demographically, hybridization can disrupt the ability of a population to persist, especially if native populations are small. For example, hybrid seed production (sterile or fertile) is produced at the expense of conspecific seed therefore reducing a species' ability for population maintenance and growth (Felber, 1991; Levin *et al.*, 1996; Daehler and Carino, 2001). Resulting hybrids also compete for limited resources (Levin *et al.*, 1996; Daehler and Carino, 2001), this is especially hazardous if hybrids exhibit heterosis or hybrid vigor or are better adapted to niche spaces as with *Spartina* x *townsendii* on the British Isles, see below (Thompson, 1991). Hybrids between native and introduced species may outcompete native parental species if they combine the adaptability to the

local environment of the native species with the alien's weedy growth habits (Daehler and Carino, 2001). Furthermore, hybrids may increase herbivore or pathogen pressure to the native population as they are often more susceptible to exploitation than their parental species (Whitham *et al.*, 1994; Levin *et al.*, 1996).

Hybridization can lead to a loss of genetic diversity through introgression and genetic assimilation (Rieseberg, 1991; Ellstrand, 1992; Levin *et al.*, 1996; Vila *et al.*, 2000; Haygood *et al.*, 2003). Introgression occurs when the genes of one species permanently become part of another species' genome as a result of an initial hybridization event followed by repeated backcrossing events to one of the parental species (Anderson and de Vicente, 2010). This pollutes a native species' gene pool, degrading its genetic purity and potentially affecting its ability to survive. Genetic assimilation occurs after extensive hybridization and introgression, resulting in a loss of unique alleles and the genetic identity of the native species as it is absorbed by the introduced species (Rieseberg, 1991; Ellstrand, 1992; Daehler and Carino, 2001). Small populations are at a greater risk of genetic assimilation as they have greater potential to become swamped with genes from the numerically larger alien congener (Ellstrand, 1992; Ellstrand and Elam, 1993; Levin *et al.*, 1996). In addition, the risk of genetic assimilation increases if hybrids are more fit than the parental species, as they are more likely to survive and backcross (Ellstrand and Elam, 1993; Daehler and Carino, 2001). Conversely, outbreeding depression, caused by the mixing of two distinct gene pools, can result in a loss of local adaptability and fitness (Ellstrand, 1992; Rhymer and Simberloff, 1996). Symptoms of outbreeding depression include reduced seed set, reduced fertility (including sterility) and increased abortion

rates (Ellstrand and Elam, 1993). For example, pollen stainability, an indication of pollen fertility, for natural hybrids of *Rubus hawaiensis* × *R. rosifolius* were sterile while both parents had mean stainability between 60 and 70% (Randell et al., 2004).

Speciation Through Hybridization

Despite its potential negative effects (e.g., outbreeding depression, resource competition, genetic assimilation), interspecific hybridization has played an important role in angiosperm speciation (Arnold, 1997; Soltis and Soltis, 2009). Speciation events by natural hybridization has been well documented, both ancient (Beasley, 1940; Wendel and Cronn, 2003) and modern (Thompson, 1991). It is estimated that up to 70% of all plant species can be traced back to a hybridization event (Grant, 1981). Several of the world's most important crop plant varieties were derived through hybridization, including wheat, cotton, and the sweet potato (Ellstrand, 2003b).

Hybrid speciation continues to happen, largely spurred by international exchange of plant material. Abbott (1992) listed ten plant taxa that have evolved in the past few hundred years as a result of interspecific hybridization between native and alien species. One example is that of the salt marsh grass, *Spartina anglica,* of the British Isles (Thompson, 1991). The allopolyploid *S. anglica* is a result from a cross between the accidentally introduced North American *S. alterniflora* ($2n = 62$) and the British Isle native *S. maritima* ($2n = 60$) (Thompson, 1991). Because of poor chromosome pairing, the initial hybridization event resulted in the sexually sterile, yet vegetatively viable, *S.* × *townsendii* ($2n = 61$). After little more than ten years, chromosome doubling resulted in

the fully fertile *S. anglica* (2*n* =122) which has become a major weed of the British Isles, outcompeting the native species and currently occupies over 10,000 acres along the British coastline (Thompson, 1991).

Given the tendency for angiosperms to hybridize, it is often difficult to determine species boundaries (Rieseberg and Willis, 2007). Carl Linnaeus assumed species were reproductively isolated yet recognized that species had potential to hybridize (Arnold, 1997). More recently, several concepts have been presented defining species and interspecific interactions including: the biological species concept (BSC), the recognition species concept (RSC), the cohesion species concept (CSC), and the phylogenetic species concept (PSC) (Arnold, 1997). For plants, the morphology-based species concept has long been used by taxonomists to delineate species by grouping morphologically similar individuals into unique assemblages (Soltis and Soltis, 2009). Apart from the morphology-based species concept, the BSC has played a major role in describing plant species (Agapow *et al.*, 2004; Soltis and Soltis, 2009). The BSC defines a species as "*groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups"* (Arnold, 1997). Allopatry is proposed to be the main driver of speciation and if hybridization is to occur, the resulting fertile progeny are to be classified as subspecies or semispecies (Arnold, 1997). Advances in molecular phylogentics over the past few decades has led to increasing popularity of the PSC (Agapow *et al.*, 2004; Soltis and Soltis, 2009) which defines a species as "the least inclusive taxon recognized in a formal phylogenetic classification… within which there is a parental pattern of ancestry and descent" (Soltis and Soltis, 2009). Based on these

species interpretations, the BSC often "lumps" species together based on their sexual compatibility while the PSC "splits" species apart based on monophyletic origins (Agapow *et al.*, 2004). The phylogenetic species concept is used for the duration of this paper and study to define the study species relationship amongst each other.

HYBRIDIZATION IN THE HAWAIIAN ISLANDS

Conservation Impacts of Alien- Native Hybridization in the Hawaiian Islands

The Hawaiian flora is especially susceptible to hybridization due to the tendency for island endemics to have few or no pre- or post-zygotic reproductive barriers (Carlquist, 1974; Ganders and Nagata, 1984; Crawford *et al.*, 1987; Levin *et al.*, 1996) and be prone to outcross (Carlquist, 1974). In addition, poorly differentiated floral architecture and a year round flowering season increase Hawaiian species' vulnerability to hybridization (Carlquist, 1974). Yet natural interspecific hybridization events appear to be relatively rare, highlighting the significance of geological and ecological separation in preventing potentially deleterious gene flow (Carlquist, 1974; Ganders and Nagata, 1984; Crawford *et al.*, 1987). The breakdown of geographical and/or ecological barriers by human disturbance and habitat fragmentation greatly increases the risk of gene flow between native and alien species (Rieseberg, 1991; Levin *et al.*, 1996; Vila *et al.*, 2000).

Hybridization becomes a threat for island endemics as alien species are introduced. Daehler and Carino (2001) found 59 genera and 176 endemic Hawaiian species to be at risk for hybridization with their naturalized alien congeners. Species were considered to be at risk if the alien congener had naturalized in a similar habitat and at a similar

elevation as the native species range. If all alien species were considered (naturalized and non-naturalized horticultural or crop species), an additional 27 genera and 82 endemic Hawaiian species were considered to be at risk (Daehler and Carino, 2001).

Three cases of spontaneous native-alien hybrids have been well documented in the Hawaiian Islands. Hybrids between native *Rubus hawaiensis* and the naturalized *R. rosifolius* were found on Maui where the two species occurred sympatrically (Randell *et al.*, 2004). Hybrids were confirmed using both morphological and polymorphic DNA markers (Randell *et al.*, 2004). Similarly, on Moloka‘i, a presumed allopolyploid hybrid between the native *Portulaca lutea* and the alien *P. oleracea* was identified using chromosome counts and leaf morphology comparisons (Kim and Carr, 1990). The final example of native-alien interspecific hybridization occurred between the endemic *Gossypium tomentosum* and alien *G. barbadense* on West O‘ahu (Stephens, 1964). Hybrids were confirmed by extensive leaf and floral morphological comparisons (Stephens, 1964). Herbarium specimens collected in 1980 and deposited at the University of Hawai‘i’s Joseph F. Rock herbarium (HAW) verify Stephen’s (1964) observations. These confirmed cases, along with numerous putative native-alien hybrids reviewed by Daehler and Carino (2001) highlight the risk of gene flow when alien species have sympatric distribution with their native congeners.

Because of its extreme isolation from any continental landmass, natural (i.e., non-human mediated) immigrant events of plant species to the Hawaiian Islands are rare (Gillett, 1972; Loope and Mueller-Dombois, 1989). A presumed 291 angiosperm colonists gave

rise to Hawai'i's unique flora (Sakai *et al.*, 1995) often as a single seed or propagule (Carr *et al.*, 1986). Given the paucity of initial colonizers and the tendency for successful colonizers to be self-compatible (Baker, 1955), initial colonizing populations were inherently marked by inbreeding depression and limited genetic resources (Gillett, 1972). Hybridization (outcrossing) was likely one of the mechanisms that enabled founding species to overcome founder effects such as genetic bottlenecks (Gillett, 1972; Carlquist, 1974). In a review of the flora of the Hawaiian Islands, Ellstrand *et al.* (1996) found 169 cases of spontaneous interspecific hybridization within 37 genera. Although ancient hybridization among endemic flora likely influenced the species diversity of present day Hawaii (≈956 native flowering species(Wagner *et al.*, 1999)), present day hybridization between native and alien species may prove detrimental to their survival (Randell *et al.*, 2004). In fact, Ellstrand (1992) states "*interspecific gene flow is perhaps the greatest gene flow hazard in plant conservation genetics."*

Populations of rare, endangered, and threatened species are typically small and geographically isolated (Ellstrand, 1992). This population dynamic is typical for insular species (Rieseberg, 1991; Levin *et al.*, 1996) and certainly true for the native Hawaiian flowering flora with 89% of species endemic to the Hawaiian Islands (Wagner *et al.*, 1999), often occurring in small, scattered populations (Crawford 1987). Insular species are at a greater risk of genetic assimilation when they come into contact with numerically larger numbers of, or reproductively more successful species (Rieseberg, 1991; Levin *et al.*, 1996). Small population size and isolation also result in less frequent conspecific immigration events which could help counterbalance the effects of alien-native gene flow

(Levin *et al.*, 1996). One such example is that of the Catalina mahogany, *Cercocarpus traskiae*, endemic to Santa Catalina Island, off the coast of Southern California (Rieseberg *et al.*, 1989). *Cercocarpus traskiae* is confined to a single canyon and is comprised of seven adult individuals. Allozyme analysis showed that hybridization between *C. traskiae* and the more abundant *C. betuloides* had occurred in two of the seven remaining *C. traskiae* adults, putting the species at serious risk of genetic assimilation by its more abundant congener (Rieseberg *et al.*, 1989).

Another, perhaps more poignant example, is that of *Gossypium darwinii* of the Galapagos Islands. (Wendel and Percy, 1990) used allozyme markers to detect significant introgression between *G. hirsutum* and *G. darwinii*. Interestingly, the introgression of *G. hirsutum* alleles to *G. darwinii* is thought to have been facilitated through gene flow from *G. barbadense* which contained introgressed *G. hirsutum* alleles (Wendel and Percy, 1990). Nonetheless the gene pool of *G. darwinii* has been contaminated to the point where a pure *G. darwinii* genotype cannot be determined (Wendel and Percy, 1990), and the ecological consequences this may have for an island endemic is not yet known. Hawaiian plant species, especially those limited to small scattered populations, could suffer similar fates as human disturbances continue to decrease endemic population sizes, increase the distance between endemic populations, and introduce cross-compatible alien species.

CROP-TO-WILD GENE FLOW

Risks of Crop-to-Wild Gene Flow

Ecological barriers often limit hybridization and gene flow, the incorporation of genes from one population into the gene pool of another. Human disturbances, such as agriculture and urban sprawl often reduce or remove these protective barriers (Levin *et al.*, 1996; Vila *et al.*, 2000). Landscape ornamentals, agricultural and horticultural crops, and naturalized alien species are all potential sources of gene flow (Levin *et al.*, 1996; Vila *et al.*, 2000). Over the past two decades, largely spurred by the advent of genetic engineering (GE) of crops, crop-to-wild plant gene flow has received significant attention (Klinger *et al.*, 1992; Raybould and Gray, 1993; Klinger and Ellstrand, 1994; Arriola and Ellstrand, 1996; Ellstrand *et al.*, 1999; Rieseberg *et al.*, 1999; Burke *et al.*, 2002; Arnaud *et al.*, 2003; Ellstrand, 2003a; Jenczewski *et al.*, 2003; Anderson and de Vicente, 2010; Vacher *et al.*, 2011). Evidence for spontaneous hybridization between 12 of the world's 13 most important food crops and their wild relatives has been well documented (Ellstrand *et al.*, 1999). Gene flow between crops and their wild relatives happens regularly, whether the crops are transgenic or conventional (i.e., non-transgenic) (Ellstrand, 2003a) and has played an important role in crop development (Harlan, 1992).

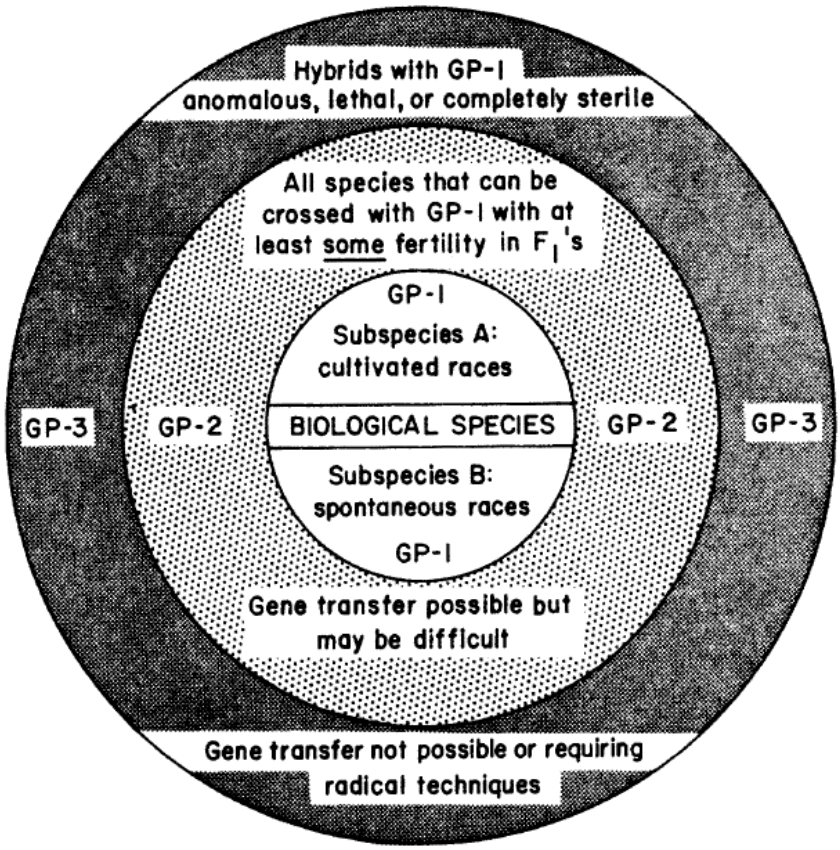
Crop-to-wild gene flow also presents significant ecological risks. Because crop plant varieties have been bred with specific traits such as increased vigor and yield, impacts of crop-to-wild gene flow may be different than that of other introduced species. For example, maladaptive traits such as male sterility, dwarfing or lack of seed dormancy or dispersibility may lead to a selective disadvantage for hybrids while increased pest

resistance may prove advantageous to hybrids, increasing their likelihood for persistence (Ellstrand and Hoffman, 1990; Ellstrand, 2003b). Impacts of crop-to-wild gene flow include increased weediness of wild species (Tiedje *et al.*, 1989; Snow and Palma, 1997; Ellstrand *et al.*, 1999; Ellstrand, 2003a), loss of genetic diversity of wild, sexually compatible species (Gepts and Papa, 2003; Haygood *et al.*, 2003) with possible genetic assimilation and species extinction (Levin *et al.*, 1996; Ellstrand, 2003a; Haygood *et al.*, 2003).

Crop-to-wild gene flow is primarily pollen mediated (Ellstrand and Hoffman, 1990), occurring when pollen from one species successfully fertilizes the egg of another. Gene flow can occur in both directions (i.e., crop-to-wild and wild-to-crop), however given the tendency for modern crop populations to be large concentrated monocultures, wild populations are more likely to be exposed to crop pollen than visa versa (Kwit *et al.*, 2011). For crop genes (transgenic or conventional) to be transferred to wild populations via pollen mediated gene flow, crop plants and their wild relatives need to: 1) be sympatric, 2) have similar pollination biology, 3) share pollinator vectors, 4) overlap in flowering time, 5) be cross-compatible (i.e., capable of producing viable offspring), and 6) persist in the environment (Jenczewski *et al.*, 2003; Kwit *et al.*, 2011) (See. 1). In short, if a crop and wild relative co-exist, the frequency of gene flow under natural conditions will strongly depend both on their relatedness and on the phenology and pattern of pollen dispersal (Lavigne *et al.*, 2002).

The degree of relatedness for crop plants is often correlated to the crop's "gene pools", as described by Harlan and de Wet (1971) (See Fig. 1.1). Gene pools are divided into three categories: primary, secondary, and tertiary with ease of gene flow decreasing from primary to tertiary. Species lying within the primary gene pool of each other easily cross and produce generally fertile offspring as a result of good chromosome pairing and normal gene segregation. Species belonging to the secondary gene pool can cross with members of the primary gene pool with difficulty. Offspring are often sterile and with low fitness as a result of poor chromosome pairing. Species belonging to the tertiary gene pool cannot cross with members of the primary gene pool without radical techniques such as embryo rescue, chromosome doubling or using other species as "bridges." The primary gene pool encompasses the biological species, which includes cultivated races and their "spontaneous" wild/weedy relatives (Harlan and de Wet, 1971); wild species falling within the primary gene pool of a crop plant are at the highest risk of gene flow (Anderson and de Vicente, 2010).

FIGURE 1.1. SCHEMATIC DIAGRAM SHOWING GENE POOLS.



Taken from Harlan and de Wet (1971).

Crop-to-Wild Gene Flow in the Hawaiian Islands

Since humans first arrived to the Hawaiian Islands, and especially after European contact, hundreds of agricultural species have been introduced (Crawford, 1937). Münster and Wieczorek (2007) analyzed phylogenetic relationships and species distributions to assess the potential for gene flow between crops currently being grown in or with potential to be grown in the Hawaiian Islands and their native congeners. They found potential for gene flow to be present, albeit low, for species in three tribes: *Heliantheae, Solaneae,* and *Phaseoleae*. However, potential for gene flow in the *Gossypieae* tribe was found to be

high, particularly between native Hawaiian cotton, *Gossypium tomentosum* and commercial cotton species, *G. hirsutum* and *G. barbadense.*

Snow and Palma (1997) designated crops as high-risk species for gene flow to wild relatives if they occur as free-living populations or if they hybridize easily with wild relatives. In the Hawaiian Islands, commercial cotton species (*G. hirsutum* and *G.* barbadense) meet both criteria; being capable of sporadically naturalizing (Wagner *et al.*, 1999; Wagner *et al.*, 2012) and known to be interfertile with the native Hawaiian cotton (*G. tomentosum*) (Stephens, 1964; DeJoode and Wendel, 1992; Percival *et al.*, 1999). Natural hybridization between Hawaiian cotton and a commercial cotton species (*G. barbadense*) was first documented in 1950 by Dr. Otto Degener at Nānākuli on O‘ahu (Hutchinson, 1959) and later confirmed by morphological observations by Stephens (1964). To help better manage the risk to native species, Münster and Wieczorek (2007) advocated the need for small-scale cotton pollination studies to help quantify the risk of hybridization.

STUDY SPECIES

Genus Overview

The cotton genus, *Gossypium* (Malvaceae), is a monophyletic group composed of 50 species of shrubs and small trees (Brubaker *et al.*, 1999; Wendel *et al.*, 2010). *Gossypium* diverged from its sister clade (*Kokia* and *Gossypioides*) between 12.5 MYBP (Seelanan *et al.*, 1997) and 33MYBP (Wendel and Albert, 1992). The genus has a pantropical distribution with three primary centers of diversification: Australia,

northeastern Africa plus the southern part of the Arabian peninsula, and Mesoamerica (Brubaker *et al.*, 1999; Wendel *et al.*, 2010). Speciation was likely promoted because of the tendency for the genus to have long distance oceanic dispersal and subsequent geographic isolation (Fryxell, 1979). Based on morphological and cytological observations, Fryxell (1979) grouped the species into eight diploid genomic groups (A–G and K) and one allotetraploid group (AD). The New World allotetraploid AD genome, the group in which Hawaiian, Upland and Pima cotton are placed, is a monophyletic lineage of five species that arose through the hybridization of the New World diploid D genome (*G. raimondii*) and an Old World diploid A genome (*G. herbaceum*) (Beasley, 1940; Wendel, 1989; Wendel *et al.*, 2010)

Aided by the ability for seeds to withstand long distance ocean dispersal (Stephens, 1958, 1966) and rapid evolution over the past one to two million years (Senchina *et al.*, 2003), the AD is distributed from the Caribbean Islands to the remote archipelagos of Hawaii and the Galapagos (Wendel *et al.*, 2010). Early radiation of the monophyletic AD genome created three lineages (Wendel *et al.*, 2010). *Gossypium mustelinum* of northeast Brazil is the sole representative of one of the earliest allotetraploid radiations (DeJoode and Wendel, 1992; Wendel *et al.*, 2010). The other two lineages each contain one domesticated species and one island endemic. The domesticated *G. barbadense* of northwest South America is phylogenetically a sister species to *G. darwinii,* endemic to the Galapagos Islands while the domesticated *G. hirsutum* lineage of Mesoamerica is a sister species to the Hawaiian endemic, *G. tomentosum* (DeJoode and Wendel, 1992; Wendel *et al.*, 2010).

Gossypium in Agriculture

Of the 50 *Gossypium* species, four have been domesticated for fiber production (Fryxell, 1979; Brubaker *et al.*, 1999; Wendel *et al.*, 2010). These include the Old World diploid species, 2*n*=2*x*=26, *G. arboretum* and *G. herbaceum* and the New World allotetraploid species, 2*n*=4*x*=52, *G. hirsutum* and *G. barbadense* (Fryxell, 1979; Brubaker *et al.*, 1999; Wendel *et al.*, 2010). All four cultivated species were domesticated independently with archaeological evidence dating cultivation of both New and Old World species to over 4000 years BP (Wendel *et al.*, 2010). Historically, the Old World diploid cotton cultivars accounted for the known world's cotton supply, with cultivation centered in India and stretching to China in the East and Ethiopia in the West (Fryxell, 1979). Today, the world's cotton production is primarily dependent upon the agriculturally superior New World allotetraploids (Wendel *et al.*, 2010).

According to the United States Department of Agriculture (USDA), for the 2010–2011 growing season 35,829,000 hectares produced 123,041,000 (480-lb) bales of cotton worldwide (FAS USDA 2012). China, India, and the United States are the world's top cotton producing countries accounting for over 61% of total production (FAS USDA 2012). The majority of the world's cotton supply comes from *Gossypium hirsutum* (Upland) varieties (>90%) with the remaining production comprised of *G. barbadense* (Pima or Sea Island)*, G. arboreum,* and to a lesser extent *G. herbaceum* (Wendel *et al.*, 2010). Worldwide, and particularly in the United States, farmers are increasingly relying on genetically engineered (GE) cotton varieties. In 2009, the most recent year on record, 49% of global cotton production was GE (GMO Compass 2010). In 2011, 90% of Upland

cotton planted in the United States was GE with 17% insect resistant (Bt), 15% herbicide tolerant, and 58% stacked (i.e., containing both insect resistant and herbicide tolerance traits) (ERS USDA 2012).

Cotton in the Hawaiian Islands

The Hawaiian Islands are home to one endemic cotton species, *Gossypium tomentosum,* and two alien species, which have sporadically naturalized: *G. hirsutum and G. barbadense.*

Alien Cotton History in the Hawaiian Islands

Both *Gossypium hirsutum* and *G. barbadense* were introduced to the Hawaiian Islands during the 1800's, with production peaking during the American Civil War (Crawford, 1937). Interest in growing cotton in Hawai‘i reemerged at the turn of the 20th century, with hopes to cater to the emerging Asian market (Crawford, 1937). Cotton (most likely predominately *G. barbadense*) was planted on the leeward sides of Kaua‘i, O‘ahu, and Hawai‘i, from sea level to 240 m elevation in areas that received 50–150 cm of annual rainfall (Crawford, 1937). Insect pressure, especially that of the pink bollworm (*Pectinophora gossypiella*), eventually put an end to any hope of profitable cotton production in the state with the industry all but disappearing by the time the USDA implemented quarantine restrictions in 1913 on counties harboring the pink boll worm (Crawford, 1937).

There is currently no large-scale commercial cotton production in the state of Hawai‘i and production of GE cotton is banned in the state. However, seed companies regularly obtain EPA permits to use the Hawaiian Islands as winter nursery sites, primarily for GE (herbicide tolerant or insect resistant (Bt)) Upland cotton and potentially Pima cotton (ISB, 2012). Both species, particularly Pima cotton, are also grown as landscape ornamentals in the state of Hawai‘i (Staples and Herbst, 2005)

Regulation of Cotton in the Hawaiian Islands

To mitigate the potential for interspecific gene flow from transgenic varieties, transgenic cotton production is prohibited in the state of Hawai‘i with the exception of United States Environmental Protection Agency (EPA) permitted test plots and breeding nurseries (EPA, 2005). Seed companies regularly grow permitted transgenic cotton (herbicide resist and Bt) in the state; the last issued test permit, 2008-2009, allowed for production on 2,725 acres (ISB, 2012). Permitted test plots cannot be planted within three miles of wild *G. tomentosum* populations and must be surrounded by 24 border rows of a “suitable pollinator trap” (EPA, 2005). Neither commercial (non-transgenic) species are regulated in the horticulture trade and can be purchased from local landscape nurseries (Danielle Frohlich, pers. com.).

Hawaiian Cotton:* Gossypium tomentosum *Nutt. ex Seem.

Description

Gossypium tomentosum is a long lived perennial shrub capable of persisting for several decades (Pleasants and Wendel, 2010). Shrubs are 0.5–1.5 m tall [up to 3 meters when

growing as an understory plant (Stephens, 1964)] with tomentose 3–5 lobed leaves that are slightly wider (3–10 cm) than long (Wagner *et al.*, 1999). The flowers are yellow, have deeply dentate bracts that are born solitary or few in short cymes, and lack extrafloral and functioning nectaries (Meyer and Meyer, 1961). The fruit is 3-celled, ovoid capsule, 1.2–1.8 cm long, and produces 2–4 seeds per cell which are covered in strongly adherent undifferentiated reddish brown fiber (Stephens, 1964; Wagner *et al.*, 1999).

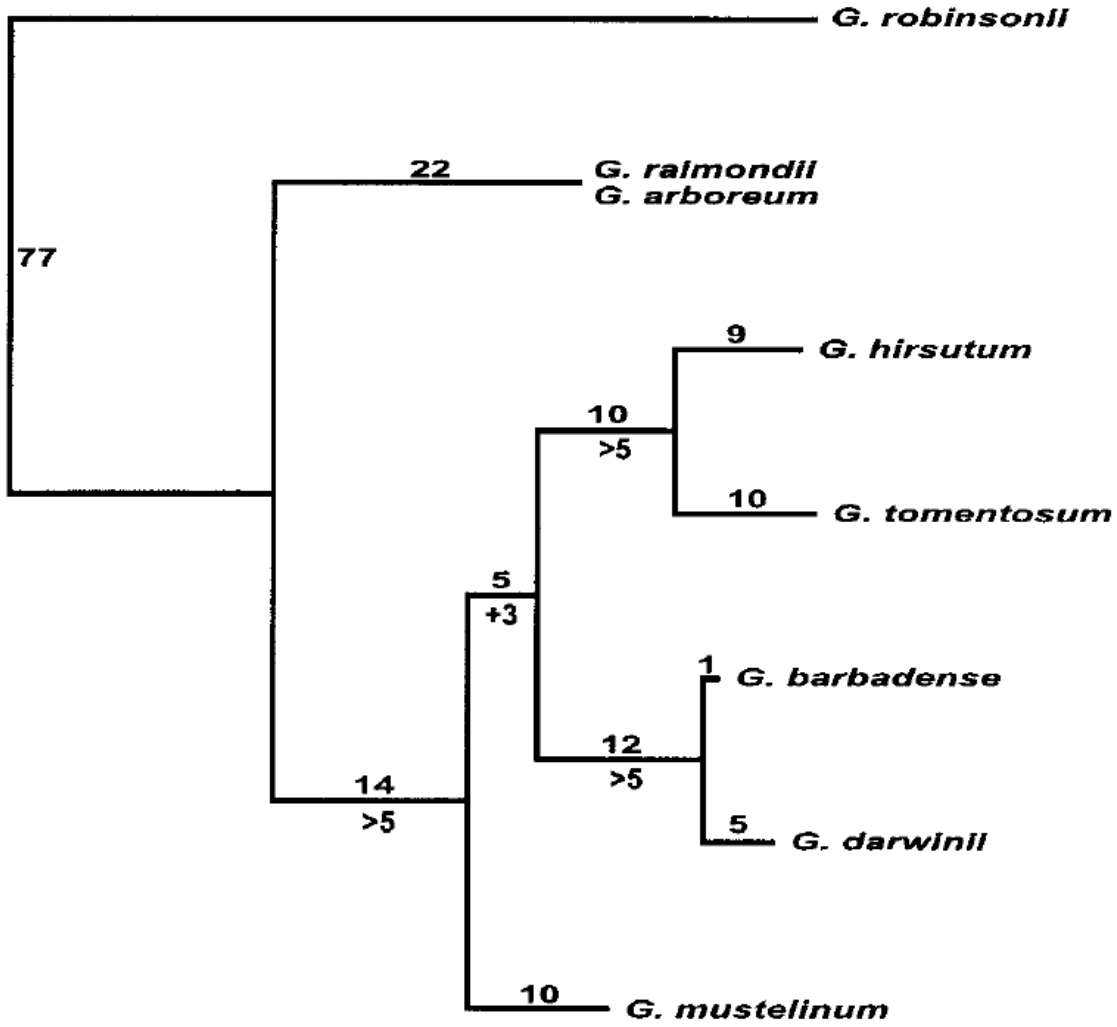
Habitat and Distribution

Seeds of *Gossypium spp*. are buoyant (Stephens, 1958) and tolerant of seawater immersion, remaining viable for up to two months when immersed in seawater (Stephens, 1966). For this reason, many species, including *G. tomentosum,* inhabit coastal and insular habitats. Allopolyploid seeds of *Gossypium* from the Neotropics arrived in the Hawaiian Islands via ocean currents no sooner than 1–2 MYA (Senchina *et al.*, 2003; Wendel and Cronn, 2003; Hawkins *et al.*, 2005). From the initial colonizer, aided by the extreme isolation of the Hawaiian Islands, *G. tomentosum* diverged as a unique species (DeJoode and Wendel, 1992). Currently, this species occurs in arid, lowland and coastal sites between sea level and 120 m on all the main Hawaiian Islands, except the Islands of Hawai‘i and Kaua‘i (Wagner *et al.*, 1999) though it known to be planted on Kaua‘i (Krakos *et al.*, 2010).

Phylogeny

Due to its distinct morphological characteristics, the relationship of *G. tomentosum* to the other allotetraploid species has been uncertain (DeJoode and Wendel, 1992). Morphological studies have suggested close relationships to both *G. hirsutum* (similar floral and capsule characteristics) and *G. barbadense* (similar bracteole characters) (Stephens, 1967). Based on data on flavonoid variability, Parks et al. (1979 from DeJoode and Wendel 1992), suggested that *G. tomentosum* is most similar to *G. barbadense* and *G. mustelinum*. Multivalent frequencies in interspecific hybrids between *G. tomentosum* and *G. hirsutum* and *G. barbadense* (Gerstel and Sarvella, 1956), suggested that *G. tomentosum* was cytogenetically similar to and equidistant from the two other species. DeJoode and Wendel (1992), using ribosomal DNA (rDNA) and chloroplast DNA (cpDNA) restriction site variation, placed *G. tomentosum* as sister species to *G. hirsutum* and sister clade to the *G. barbadense*-*G. darwinii* clade while *G. mustelinum* was found to be sister to both clades. These findings have been verified by other studies looking at internal transcribed spacer (ITS) regions (Wendel *et al.*, 1995; Seelanan *et al.*, 1997), nuclear-encoded alcohol dehydrogenase (*Adh*) genes (Small *et al.*, 1998) (See Fig. 1.2), and by microsatellite (SSR) markers (Lacape *et al.*, 2007).

FIGURE 1.2. DENDROGRAM SHOWING PHYLOGENETIC RELATIONSHIPS AMONGST ALLOTETRAPLOID COTTON SPECIES.



Single most-parsimonious tree (length = 43, CI = 0.91, RI = 0.91) from analysis of combined AdhC data showing relationships among the New World Allotetraploid cottons and their ancestral parentage. Branch lengths are shown above and decay values below each branch. Branch lengths are shown above and decay values below each branch. Taken from Small et al. (1998).

Genetic Diversity

Like most island endemics (Crawford *et al.*, 1987), *G. tomentosum*, possesses limited genetic diversity. DeJoode and Wendel (1992) found, through allozyme analysis, little panmictic heterozygosity (Ht) (Ht= 0.0333) at 50 loci between 30 accessions representing

populations from each island where *G. tomentosum* occurs. Furthermore, no patterns of geographic differentiation were observed. Hawkins *et al.* (2005) used AFLP markers and found similar results. Of the 351 AFLP markers tested, only 6.3% were polymorphic with a mean Ht value of 0.012±0.004. Again, no clear geographic distinctions could be made. Using AMOVA, Hawkins *et al.* (2005) found only 13.2% of variation occurring among populations with 86.8% of variation residing within populations. These low levels of genetic diversity support the hypothesis of a relatively recent, 1–2 MYA, single individual colonizing event, with limited gene flow between established populations (Hawkins *et al.*, 2005).

Reproduction and Pollination Biology

Unlike the tetraploid commercial cotton species that are facultative self-pollinators (Oosterhuis and Jernstedt, 1999), *G. tomentosum,* due to it's characteristic exerted style, is a facultative outcrosser (Pleasants and Wendel, 2010). Historically, *G. tomentosum* was considered to be moth pollinated. This observation was backed by morphological evidence: the exerted style (which should inhibit self-pollination), lack of petal spots, glossy yellow petals, and the ability for flowers to remain accessible to pollinators into the night (Fryxell, 1979). Little empirical evidence was presented to support these pollinator claims. Recent field observations by Pleasants and Wendel (2010) and Krakos *et al.* (2010) have shed light on the reproductive and pollination biology of *G. tomentosum.* Krakos et al. (2010) studied the floral biology of two small, reintroduced populations of *G. tomentosum* at the National Tropical Botanical Gardens (NTBG) on

Kaua‘i. Pleasants and Wendel (2010) observed three natural populations on the islands of O‘ahu, Maui, and Kaho‘olawe (see below).

Flowering Phenology and Floral Receptivity

G. tomentosum can take up to two years to produce flowers when planted from seed (Culliney and Koebele, 1999). Pleasants and Wendel (2010) observed open flowers from March through August while Stephens (1964) observed flowering throughout the year, with a temporary cessation in flowering from March through late April. Stephens (1964) notes that flowering herbarium specimens deposited at the Bishop Museum (BISH) were collected from every month with exception of December and that plants observed in the field exhibited inconsistent flowering, with some plants in flower and fruit while other remained vegetative. This observation was first recorded by Sinclair (1885) who describes *G. tomentosum* as flowering "*more or less, all the year round; therefore, both seed and flowers are found simultaneously*." Lack of rainfall is the likely cause of little or no flower production; flowering peaks during times of heavy rainfall (Stephens, 1964; Pleasants and Wendel, 2010).

Krakos et al. (2010) found flowers for pollination to be receptive for one day, between the hours of 7am and 8pm, with the corolla collapsing around the stigma at sundown. Similarly, Pleasants and Wendel (2010) observed flowers opening in the morning with anthers dehiscing 15–30 minutes thereafter and flowers beginning to senesce by late afternoon.

Selfing verses Outcrossing

Despite producing flowers which are fully self-compatible, (Stephens, 1964; Fryxell, 1979), Krakos et al. (2010) observed that *G. tomentosum* primarily outcrosses. When comparing pollen tube formation between self-pollinated and cross pollinated flowers, it was shown that pollen from another individual produced a more vigorous pollen tube; reaching the ovary faster than that of a self-pollinated flower (Krakos *et al.*, 2010). In addition, the extended styles of *G. tomentosum* limited self-pollination without the aid of a pollen vector. However, all *G. tomentosum* populations observed by Pleasants and Wendel (2010) produced a small percentage (10%) of flowers exhibiting a failsafe self-pollination mechanism of non-exerted or bent styles that sometimes made direct contact with stamens. Of flowers observed by Krakos *et al.* (2010), 7.2% ± 5.2% exhibited this failsafe self-pollination mechanism.

Floral Attractants, Visitors, and Pollinators

Stephens (1964) concluded, that despite having a floral nectary anatomically present, floral nectar production was either absent or reduced in *G. tomentosum.* Similarly, Krakos *et al.* (2010) found that flowers produced no floral nectar or scent. Because of the paucity of nectar production or other pollinator rewards, it is likely that insects visiting flowers are feeding on or gathering pollen (Stephens, 1964; Krakos *et al.*, 2010).

Pleasants and Wendel (2010) found an array of non-native insect pollinators visiting the flowers of *G. tomentosum* at each population observed. Large carpenter bees (*Xylocopa*

sonorina), small carpenter bees (*Ceratina* sp.)*,* and honey bees (*Apis mellifera*) were seen visiting the flowers and depositing *G. tomentosum* pollen on receptive stigmas (Pleasants and Wendel, 2010). At NTBG on Kaua‘i, Krakos et al. (2010) observed that the small Nitidulid beetle, *Aethina concolor,* was the primary pollinator, apparently feeding on pollen (Krakos *et al.*, 2010). These beetles appeared to primarily self-pollinate the flowers they foraged on, but were also observed flying between flowers loaded with pollen and therefore potentially outcrossing (Krakos *et al.*, 2010). In addition to beetles, honeybees were found to visit the *G. tomentosum* flowers at this site.

Cultural and Economic Importance

Gossypium tomentosum has long been valued for its cultural and economic importance. There are two Hawaiian names for *Gossypium tomentosum*: the more common *ma‘o* meaning “green” and *huluhulu* meaning “hairy” (Stephens, 1964). Flowers of the plant were traditionally and continue to be used to create a yellow dye while leaves are used to create a delicate green dye, both used to dye tapa cloth (Krauss, 1993)

More recently *G. tomentosum* has been recognized for its agronomical value. Hawaiian cotton is considered to be part of the primary gene pool, as described by Harlan and de Wet (1971), to both *G. hirsutum* and *G. barbadense* (Percival *et al.*, 1999; Anderson and de Vicente, 2010). Crosses in the primary gene pool generally produce fertile hybrids with good chromosome pairing and normal gene segregation and transfer (Harlan and de Wet, 1971). Because of this relationship and certain agronomically important characteristics (e.g., absence of extra-floral nectaries, heat resistance), *G. tomentosum* has

been used in several breeding programs (Meyer and Meyer, 1961; Meredith *et al.*, 1973; Meyer and Meredith, 1978; Saha *et al.*, 2006; Ahuja *et al.*, 2009). Most notably, Meyer and Meyer (1961) successfully transferred the nectariless trait from *G. tomentosum* to *G. hirsutum*. This trait was shown to reduced insect pressure from tarnished plant bugs (*Lygus lineolaris*), fleahoppers (*Pseudatomoscelis seriatus*) and bollworms (*Helicoverpa zea* and *H.* virescens) which subsequently reduces boll rot (Meredith *et al.*, 1973).

Alien Cotton Species in Hawai‘i: Upland, Gossypium hirsutum L. and Pima, G. barbadense L.

Of the two alien cotton species present in the Hawaiian Islands, *G. hirsutum* is the most economically important; therefore this species is the focus of the majority of experiments and observations made on commercial cotton species. Data are presented for *G. barbadense* when available.

Plant Descriptions

Gossypium hirsutum is a shrub or annual subshrub 1–2 m tall with stellate pubescent, 3–5 lobed leaves that are usually wider (8–15 cm) than long (Wagner *et al.*, 1999). Flowers are born solitary or in sympodial inflorescences and have cream-colored to yellow petals (Wagner *et al.*, 1999). The fruits are 3–5 celled, ovoid capsules that are 2.5–3.5 cm long with each cell containing 5–11 seeds that are covered in copious white fiber (Wagner *et al.*, 1999).

Gossypium barbadense is a shrub or annual subshrub 1–2 (–3) m tall with glabrous to sparsely stellate pubescent, 3–7 lobed leaves that are slightly wider (5–15 cm) than long (Wagner *et al.*, 1999). Flowers are born solitary or in sympodial inflorescences and have bright yellow petals often with a basal maroon spot (Wagner *et al.*, 1999). The fruits are 3-celled, narrowly ovoid capsules that are 3–5 cm long with several seeds per cell that are covered in copious white fiber (Wagner *et al.*, 1999). Both species produce extrafloral nectaries subtending the bracts and a nectar gland found basally on the lower midvein surface of the leaves (Wagner *et al.*, 1999)

Distribution in Hawai‘i

In the Hawaiian Islands, *G. hirsutum* has naturalized in Hale‘iwa, O‘ahu (Wagner *et al.*, 1999), on East Maui (Wagner *et al.*, 2012) and perhaps elsewhere in the islands (Wagner *et al.*, 1999). Because of its economic prowess over other cultivated cotton species, *G. hirsutum* is most likely the cotton species being grown by seed companies in the islands. *Gossypium barbadense* is naturalized in dry and disturbed areas, 10–200 m in elevation, on all the main islands with the exception of Kaho‘olawe and Maui (Wagner *et al.*, 1999).

Centers of Diversity, Domestication and Distribution Worldwide

Gossypium hirsutum has two centers of diversity, southern Mexico and the Caribbean (Wendel *et al.*, 1992). Domestication most likely occurred on the Yucatan peninsula 4000–5000 years BP (Wendel *et al.*, 2010). Wild populations of *G. hirsutum*, though uncommon and geographically widely dispersed, grow on beach strands or are restricted

to small islands, occurring from drier areas of Central and Northern South America to the Caribbean and the South Pacific (Stephens, 1963; Lee, 1984).

Gossypium barbadense has a center of diversity in Northwestern South America (Lee, 1984; Percy and Wendel, 1990). Archeological remains date domestication in central Peru to around 5500 years BP (Wendel *et al.*, 2010). Today, *G. barbadense* grows wild on the coasts of Peru, while marginally cultivated forms grow throughout the northern parts of South America, the southern parts of Central America, and the Caribbean (Lee, 1984).

Genetic Diversity

Hutchinson (1951) separated *G. hirsutum* into seven races, with the hypothesis that each race arose from human manipulation and subsequent geographical isolation. Wendel *et al.* (1992), using allozyme analysis, showed 34 distinct geographical regions (compared to Hutchinson's (1951) seven races), reflecting influence by humans (both pre and post European contact) and geographic isolation. The most commonly cultivated variety, early maturing Upland cotton, is comprised of four categories (Acala, Delta, Plains and Eastern) of the race *latifolium* which is naturally distributed throughout Mexico, El Salvador and Nicaragua with a center of diversity in Guatemala (Brubaker *et al.*, 1999). When analyzed for allozyme variation at 50 loci, Wendel *et al.* (1992) found little genetic diversity in Upland cultivars (including all four categories) with mean panmictic heterozygosity (Ht=0.056) less than half that of the species' mean heterozygosity value (Ht=0.163).

Despite it's large geographic distribution, *G. barbadense* shows little genetic diversity. Using allozyme analysis at 59 loci of 153 plants, Percy and Wendel (1990), found mean panmictic heterozygosity to be 0.06. Despite this, there is at least one recognized variety, *G. barbadense* var. *braziliense,* which has distinct kidney-shaped seeds (Fryxell, 1979).

Reproduction and Pollination Biology

Both commercial cotton species are facultative self-pollinators and opportunistic outcrossers when insect pollinators are present (Oosterhuis and Jernstedt, 1999). Cotton flowers have five showy petals and five much reduced united sepals. Fused filaments form a staminal column about the long style, which protrudes to expose 3–5 stigmas (Free, 1970). The superior ovary has 3–5 united carpels, each containing several ovules (Free, 1970).

Flowering Phenology and Flower Receptivity

Plants begin flowering from apical buds 4–5 weeks after planting, and continue for several weeks (Oosterhuis and Jernstedt, 1999). Stephens (1964) notes that in its native range, *G. hirsutum* exhibits a seasonal flowering period: vegetative during the long days of the wet season while flowering and setting seed during the short days of the dry season. Stephens (1964) observed *G. barbadense* flowering synchronous with *G. tomentosum* on O'ahu. Both species grow as perennials in the Hawaiian Islands and if cultivated can potentially flower throughout the year (Crawford, 1937).

Flowers of *G. hirsutum* open near dawn and remain open for a single day (Free, 1970; Oosterhuis and Jernstedt, 1999). Anthers dehisce; shedding pollen shortly after petals open though this may be delayed by 2–3 hours by high humidity or cool temperatures (Oosterhuis and Jernstedt, 1999). Stigmas are receptive while the flower is shedding pollen (Free, 1970). Pollen remains viable for up to 16 hours though a marked decline in viability is observed after 8 hours (93.4% viable at 8 hours vs. 30.7% viable at 16 hours) (Richards *et al.*, 2005). Exposure to water, (i.e., heavy rain) causes cotton pollen to burst and thus become sterile (Burke, 2002).

Gossypium barbadense flowers open shortly after dawn remaining open for a single day (Van Deynze *et al.*, 2011). Anthers dehisce just before petals open or shortly after (Free, 1970) with pollen remaining viable for at least eight hours (Barrow, 1981). Stigmas are receptive until early afternoon loosing receptivity as the petals close (Van Deynze *et al.*, 2011).

Floral Visitors and Pollinators

Cotton pollen grains are large, heavy, and sticky and are therefore not effectively transported by wind (Fryxell, 1979). In the absence of pollinators, self-pollination occurs shortly after anthesis (Mamood *et al.*, 1990; Oosterhuis and Jernstedt, 1999). Numerous studies show that bee species, including melissodes (*Melissodes* spp.) (McGregor *et al.*, 1955; McGregor, 1976; Van Deynze *et al.*, 2005), bumblebees (*Bombus* spp.) (McGregor, 1976; Umbeck *et al.*, 1991), carpenter bees (*Xylocopa varipuncta*) (Waller *et al.*, 1985b; Keasar, 2010), and honeybees (*Apis mellifera*) (McGregor *et al.*, 1955;

McGregor, 1976; Vaissiere *et al.*, 1984; Waller *et al.*, 1985a; Waller *et al.*, 1985b; Mamood *et al.*, 1990; Umbeck *et al.*, 1991; Heuberger *et al.*, 2010) to be the primary pollinators of commercial cotton. Honeybees are considered to be the most important pollinator due to their abundance (Free 1970). Proximity to pollinators, such as bee hives, results in an increase in pollination (McGregor *et al.*, 1955; Vaissiere *et al.*, 1984; Waller *et al.*, 1985b; Mamood *et al.*, 1990) and outcrossing rates (McGregor *et al.*, 1955; Van Deynze *et al.*, 2005; Heuberger *et al.*, 2010).

GENE FLOW POTENTIAL BETWEEN COMMERCIAL COTTON SPECIES AND HAWAIIAN COTTON

The potential risk of gene flow between *Gossypium tomentosum* with *G. hirsutum* or *G. barbadense* in the Hawaiian Islands is high (Fryxell, 1979; Ellstrand *et al.*, 1999; Daehler and Carino, 2001; Münster and Wieczorek, 2007; Anderson and de Vicente, 2010) due to the following factors:

Known Interfertility Between the Species

Gossypium tomentosum belongs to the primary gene pool of both commercial species (Percival *et al.*, 1999; Anderson and de Vicente, 2010). Because of this, Hawaiian cotton and the two alien cotton species are fully interfertile (Hutchinson, 1959; DeJoode and Wendel, 1992) being successfully used in commercial breeding programs (Meyer and Meyer, 1961; Meredith *et al.*, 1973; Meyer and Meredith, 1978; Saha *et al.*, 2006).

Shared Insect Pollinators

Carpenter bees (*Xylocopa sp.*) and honeybees (*Apis mellifera*) are known pollinators of all three cotton species in Hawaii (Umbeck *et al.*, 1991; Keasar, 2010; Pleasants and Wendel, 2010). These bee species are known to fly several kilometers to forage; up to 12 km for honey bees (Seeley, 1985) and six km for carpenter bees (Pasquet *et al.*, 2008) potentially moving pollen across vast distances.

Shared Habitat

All three species share similar lowland, coastal habitats (Wagner et al. 1999), increasing the chances that Pima and Upland cotton may colonize areas where Hawaiian cotton currently exist. In addition, agricultural zones in the state of Hawai‘i are often within pollinator foraging distances of native cotton populations (See species distribution maps, conclusion chapter).

Overlap in Flowering Phenology

All three species are capable of flowering during the same periods of the year (Stephens 1964; Lehman, pers obs.). In addition, all three species have flowers that are receptive during the day, for one day, opening in the morning and closing by late afternoon (Free, 1970; Oosterhuis and Jernstedt, 1999; Krakos *et al.*, 2010; Pleasants and Wendel, 2010). *Gossypium tomentosum,* though fully self-compatible, favors outcrossing (Krakos *et al.*, 2010) and this increases the odds for gene flow.

History of Sporadic Gossypium Hybridization in the Hawaiian Islands

Early reports of hybridization between *G. barbadense* and *G. tomentosum*, first observed at Nānākuli by Dr. Otto Degener in 1950 (Hutchinson 1959) and later described by Stephens (1964), provided detailed morphological and geographical descriptions of two hybrid cotton populations at Nānākuli on the Wai‘anae Coast of O‘ahu. More recently, Kenneth Nagata (then affiliated with Lyon Arboretum) collected herbarium vouchers of *G. barbadense* ×*G. tomentosum* hybrids in 1980, close to one of the sites described by Stephens (1964). Using microsatellite markers of extracted nuclear DNA, these vouchers were confirmed to be hybrids (See Chapter 2).

QUANTIFYING THE RISK

The risk for gene flow between commercial cotton species and native Hawaiian cotton species is apparent yet not quantified. One way to quantify the risk of gene flow between two species is through controlled artificial hybridization (i.e., hand pollination) (Ellstrand and Hoffman, 1990; Armstrong *et al.*, 2005; Anderson and de Vicente, 2010). If artificial hybridization is relatively easy, then it can be assumed that gene flow is possible given favorable circumstances, thus warranting further research on the likelihood of natural gene flow and the impacts if may have on native Hawaiian cotton (Armstrong *et al.*, 2005). On the other hand, if artificial hybridization is difficult or requires technology such as embryo rescue or chromosome doubling, it can be assumed that spontaneous interspecific gene flow is unlikely in the wild (Armstrong *et al.*, 2005; Anderson and de

Vicente, 2010). If artificial hybrids are formed, evaluation under controlled field conditions can help assess their potential ecological impacts (Tiedje *et al.*, 1989; Ellstrand and Hoffman, 1990; Johnston *et al.*, 2006).

For this thesis, to determine the ease with which *G. tomentosum* hybridizes with *G. hirsutum* and *G. barbadense,* reciprocal crosses were carried out in a controlled setting. To assess the impacts of gene flow on fitness and fecundity, F_1 hybrids were grown in a common garden plot along with parental species. To determine if natural hybrids remain extant at Nānākuli, morphological field surveys were undertaken and DNA was collected from herbarium specimens deposited at the Joseph F. Rock Herbarium (HAW). These specimens along with samples collected from field surveys were analyzed with microsatellite markers to detect for the presence of hybrid alleles.

CHAPTER 2. AN ASSESSMENT OF THE PERSISTENCE OH HYBRIDS BETWEEN ALIEN PIMA COTTON, *Gossypium barbadense,* (MALVACEAE) AND ENDEMIC HAWAIIAN COTTON, *G. tomentosum* IN HAWAII

INTRODUCTION

The Hawaiian Islands are regarded as one of the most biologically invaded archipelagos on Earth (Loope and Mueller-Dombois, 1989; Daehler *et al.*, 2004). Over one thousand alien plant species have naturalized, exceeding the total native angiosperm flora (Wagner et al. 1999). Invasive plant species are detrimental to native ecosystem processes, altering the nutrient cycling, hydrology and disturbance regimes that are critical to the life cycles of native floras (Vitousek *et al.*, 1997). Furthermore, alien and native congener plant species may hybridize (Levin et al. 1996, Vila et al. 2000, Daehler and Carino 2001). Alien-native hybridization may be detrimental to native species. Ecologically, this may occur when hybrid progeny directly displace native species by competing for resources (e.g., water, nutrients, sunlight, pollinators) and thereby inhibit population growth (Levin et al. 1996, Vila et al. 2000). Genetically, this also occurs when hybridization erodes the genetic integrity of native species, potentially resulting in a loss of local adaptability and an increased risk of genetic assimilation (Rieseberg 1991, Ellstrand 1992, Levin et al. 1996).

Oceanic floras, such as in Hawai‘i, are particularly vulnerable to alien-native hybridization, due to factors such as: the susceptibility of island ecosystems to alien plant invasion (Loope and Mueller-Dombois 1989), the tendency for island endemics to have

few or no pre- or post-zygotic reproductive barriers (Carlquist 1974, Ganders and Nagata 1984, Crawford et al. 1987, Levin et al. 1996), and be prone to outcross (Carlquist 1974). As a result, multiple cases of alien-native plant hybridization have been documented in Hawai‘i (Stephens 1964, Kim and Carr 1990, Randell et al. 2004). The potential for further hybridization events in Hawai'i is high; Daehler and Carino (2001) list 59 genera, containing 176 native plant species, at risk of hybridizing with non-native congeners.

One species that is at risk of alien-native hybridization is Hawaiian cotton or *ma'o*, *Gossypium tomentosum* (Daehler and Carino 2001, Münster and Wieczorek 2007). This long-lived perennial shrub occurs in arid, lowland and coastal sites between sea level and 120 meters on all the main Hawaiian Islands, except the islands of Hawai‘i and Kauai, though it has recently been re-introduced to Kauai (Wagner et al. 1999). Two agriculturally important cotton species, Pima cotton (*G. barbadense*) and Upland cotton (*G. hirsutum*) have been introduced to the islands and are now sporadically naturalized; Pima cotton on all the main islands except Kaho'olawe, and Upland cotton on O'ahu (Wagner et al. 1999) and East Maui (Wagner et al. 2012). Both Pima and Upland cotton were introduced to Hawai‘i during the 1800's owing to a commercial boom during the American Civil War (Crawford 1937). There is currently no commercial cotton production in the State of Hawai‘i. However, seed production companies regularly use Hawai‘i as winter nursery sites, primarily for transgenic (herbicide tolerant or insect resistant) Upland cotton and potentially for Pima cotton (ISB 2012). Both species are also grown as ornamentals (Staples and Herbst 2005).

Hawaiian cotton and both alien cotton species are closely related allopollyploids with a shared ancestral genome (Wendel et al. 2010). In addition, the three species have shared habitat preferences (Wagner et al. 1999) and insect pollinators (Krakos et al. 2010, Pleasants and Wendel 2010) and are known to be inter-fertile (Meyer and Meyer 1961, Stephens 1964, DeJoode and Wendel 1992). All three species are fully self-compatible; both alien cotton species are facultative self-pollinators and opportunistic outcrossers (Oosterhuis and Jernstedt, 1999) while Hawaiian cotton primarily outcrosses (Krakos et al. 2010). For these reasons, Daehler and Carino (2001) suggested that alien-native cotton species are at high risk of hybridization and spread in the Hawaiian Islands.

Early reports of hybridization between *G. barbadense* and *G. tomentosum*, first observed at Nānākuli by Dr. Otto Degener in 1950 (Hutchinson 1959) and later described by Stephens (1964), provided detailed morphological and geographical descriptions of two hybrid cotton populations at Nānākuli on the Wai‘anae Coast of O‘ahu. More recently, Kenneth Nagata (then affiliated with Lyon Arboretum), collected herbarium vouchers of *G. barbadense* × *G. tomentosum* hybrids in 1980 close to one of the sites described by Stephens (1964) (See Fig 2.1). These putative hybrids indicate that either the populations documented by Stephens (1964) persisted for several decades or that subsequent hybridization events took place during the intervening decades.

The primary objective of the current study was to assess whether the initial hybrid cotton populations documented by Stephens (1964) and later purportedly confirmed by Nagata still exist at Nānākuli. Using microsatellite DNA markers, the study also aimed to verify

if Nagata's collections were indeed hybrids. A secondary objective, if hybrids were identified, was to assess the level of their spread and to measure the gene flow that had potentially taken place between populations of Pima and Hawaiian cotton.

MATERIALS AND METHODS

Study Sites

Between December 2010 and November 2011, six populations of *G. tomentosum* and one population of *G. barbadense* were surveyed at, and near to, Nā nākuli on the Waianae coast of Oahu, (See Table 2.1 and Fig. 2.1). The populations were located using herbarium records at the B. P. Bishop Museum (BISH) and the Joseph F. Rock Herbarium of the University of Hawaii, (HAW), as well as by field surveys. Special emphasis was placed on areas described by Stephens (1964) and Nagata's 1980 voucher collection sites to determine if populations of hybrids between *G. barbadense* and *G. tomentosum* still exist.

TABLE 2.1. NĀNĀKULI, OAHU *G. tomentosum* × *G. barbadense* HYBRID MORPHOLOGICAL SURVEY DATES, LEAF SAMPLES COLLECTED AND SSR SCREENING RESULTS- HISTORIC AND PRESENT DAY.

Population Name	Morphological Observation	Leaf Samples Collected	SSR Marker Confirmed Hybrids
Stephens, 1964, Waste Lot	1964	N/A	N/A
Stephens, 1964, Piliokahe Beach	1964	N/A	N/A
Nagata, 1980	March 31, 1980	2*	**
	July 10, 1980	3*	1***
Morden, 1999, Tracks Beach	July 15, 1999	24	0
Morden, 1999, Koolina	July 15, 1999	4	0
N. Power Plant	December 15, 2010	0	
	March 31, 2011	0	
	April 16, 2011	0	
	July 19, 2011	0	
	November 6, 2011	5	0
S. Power Plant	December 15, 2010	0	
	March 31, 2011	0	
	April 16, 2011	13	0
	July 19, 2011	0	
	November 6, 2011	0	
Train Tracks	July 19, 2011	2	0
	November 6, 2011	6	0
Lualualei Naval Base Rd., Dump	December 15, 2010	0	
	March 31, 2011	0	
	April 16, 2011	0	
	July 19, 2011	0	
	November 6, 2011	21	0
Lualualei Naval Base Rd. (G. barbadense)	December 15, 2010	0	
	March 31, 2011	0	
	April 16, 2011	0	
	July 19, 2011	0	
	November 6, 2011	3	0
Maili	July 19, 2011	0	
	November 6, 2011	4	0

*Herbarium Records. ** No successful DNA extraction. *** One successful DNA extraction.

Morphological Screening

Morphological screening for putative hybrids was undertaken using descriptive information provided in Stephens (1964) and Nagata's herbarium specimens as well as characteristics represented by artificial interspecific F_1 hybrids grown at the Magoon Research Facility, UH Manoa (A. Lehman unpublished data). Populations were surveyed by walking along transects spaced approximately 20 m apart running parallel to the roadways at each site. Successive transects were established until no more plants were encountered or natural (e.g., rocky outcroppings) or manmade features (e.g., roadways, residential developments) were encountered.

Plants encountered within five meters of a transect were visually assessed for the presence or absence of the following characteristics: tomentum, leaf nectary, bright yellow petals, maroon petal spots, both short and long lint fiber and light brown lint. Morphological data was recorded for plants selected for molecular analysis (See Table 2.2). Between the two species, tomentum and bright yellow petals are unique to *G. tomentosum,* leaf nectary and petal spots are unique to *G. barbadense* while the presence of both short and long light brown lint fibers is a common characteristic of hybrids between the two species (Stephens 1964, Saha et al. 2006). Hybrids were expected to express at least one characteristic common to both parental types (either intermediately or fully expressed).

To ensure that plants of both cotton species were observed in both vegetative and reproductive phases of growth, populations were repeatedly visited (up to five times) during the course of one year. In addition, those plants expressing possible hybrid characteristics, i.e., reduced leaf pubescence in a *G. tomentosum* population, were tagged and revisited multiple times to account for possible environmental or developmental effects on phenotypic expression. All plants tagged, regardless of if they were later determined to be morphologically conspecific, were further analyzed with molecular markers.

TABLE 2.2. SUMMARIZED MORPHOLOGICAL GPS SURVEY POINTS AND SCREENING RESULTS.

One representative sample, per population, is displayed; voucher specimens deposited at HAW contain exact GPS locations for each collection.

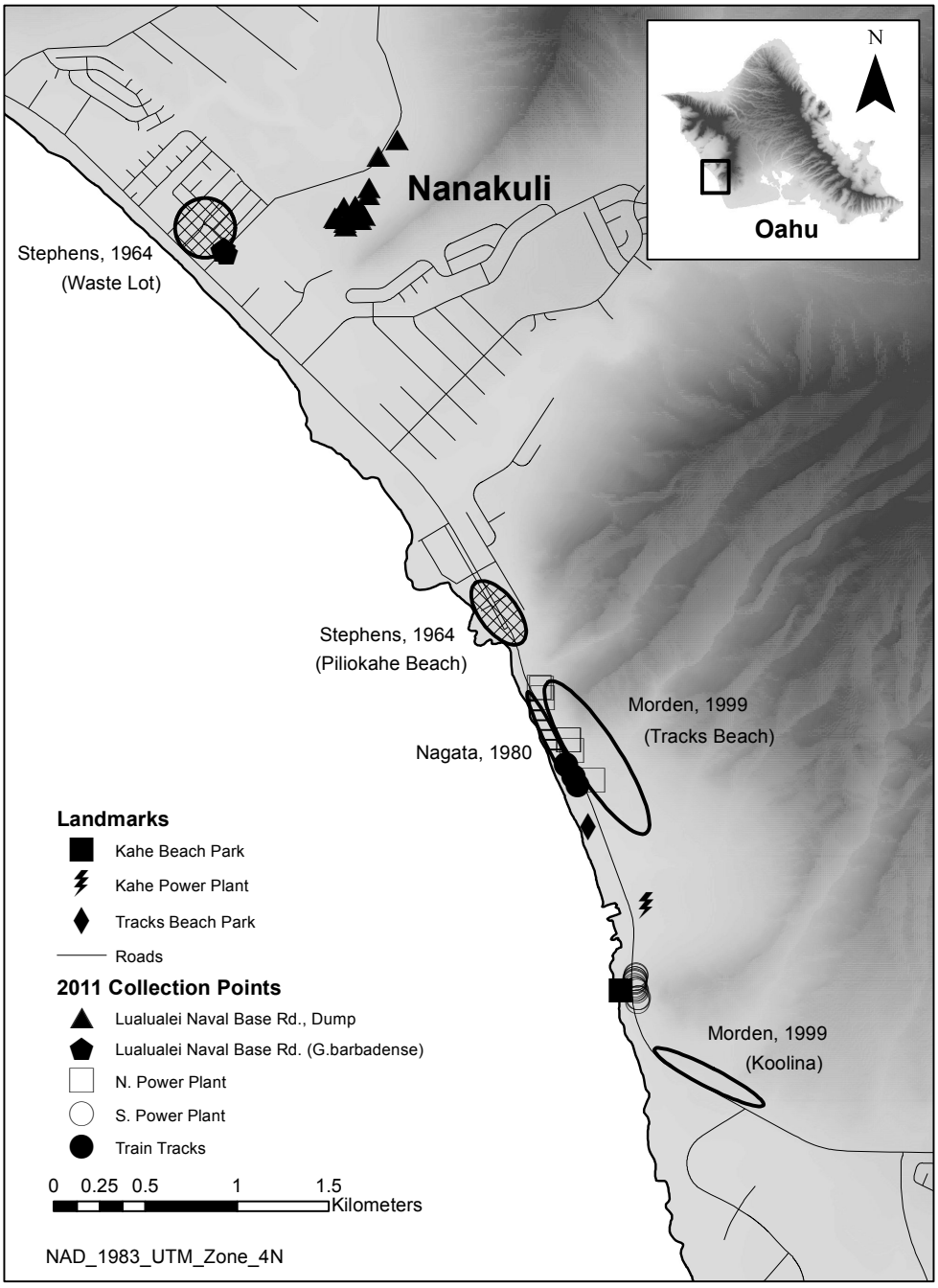
Location	Collection #	n	Species	Lat	Long	Elevation	H	Ne	Y	R	F	B
Train Tracks	85	5	*Gossypium tomentosum*	21.36265	-158.132750	131	+	-	+	-	-	-
N. Power Plant	88	7	*Gossypium tomentosum*	21.367067	-158.134500	171	+	-	+	-	-	-
Lualualei Naval Base Rd.	95	3	*Gossypium barbadense*	21.388533	-158.150833	96	-	+	+/-	+	-	-
Lualualei Naval Base Rd., Dump	98	21	*Gossypium tomentosum*	21.393283	-158.142833	116	+	-	+	-	-	-
Maile	120	4	*Gossypium tomentosum*	21.402917	-158.175500	92	+	-	+	-	-	-
S. Power Plant	1	15	*Gossypium tomentosum*	21.352883	-158.129500	82	+	-	+	-	-	-

H= tomentum, Ne= leaf nectary, Y= bright yellow petal, R= maroon petal spot, F= presence of both long and short "fuzz" fibers, B= light brown lint. (+)= characteristic observed while (-)= lacking characteristic.

Molecular Screening

While conducting morphological screening, leaf samples were collected at random along transects for molecular analysis from all five native *G. tomentosum* populations (n=52) and the one alien *G. barbadense* population (n=3) known in the region (See Table 2.1). Samples were collected by placing ten leaf punches per leaf, per plant, into labeled sterile 1.5 ml test tubes and kept on ice until DNA was extracted. Due to their close proximity (0.5 km) to the naturalized *G. barbadense* population, a larger number of plants at the southwest extreme of the Lualualei Navel Base Rd. Dump population were sampled. Due to substantial sampling by Morden in 1999 (n=24), which were included in our molecular analysis, relatively few samples were collected from the N. Power Plant population during the 2011 survey (n=5). In addition, all plants tagged during morphological surveys were sampled. GPS locations at each collection point were taken using a Garmin 60CSx (See Table 2.2). Voucher specimens were collected from all plants used in molecular screening and deposited in the Joseph F. Rock Herbarium (HAW), (See Table 2.2 for collection numbers). All *G. tomentosum* populations were within 0.25 kilometers of major roadways and within one kilometer of residential areas where *G. barbadense* is most likely to occur as a landscape ornamental.

FIGURE 2.1. 2010-2011 SURVEY POINTS AND HISTORIC COLLECTION POINTS NEAR NĀNĀKULI, OAHU.



1999 and 2011 collection points are from *G. tomentosum* populations unless otherwise noted. Each collection point represents an individual plant assayed for hybrid markers (phenotypic and morphological). Putative hybrids were found at the Stephens 1964 sites ("Waste Lot" and Piliokahe Beach") and Nagata 1980 site.

DNA Extraction and Microsatellite Amplification

DNA was extracted from all fresh leaf samples (n=55), as well as from dried leaf samples from each of Nagata's 1980 herbarium specimens (n = 5), following the methods described below. In addition, DNA samples (n=28) of putative *Gossypium tomentosum* hybrids collected by Morden in 1999 (extracted following procedures in Morden *et al.* (1996)) from sites at and near to N$\bar{a}$n$\bar{a}$kuli were analyzed.

Leaf material (approximately 100 mg wet weight) was disrupted using TissueLyser (QIAGEN® Industries) by adding lysis buffer and operating the TissueLyser for 2 min at 30 Hz (2x). DNA was subsequently extracted using either Macherey-Nagel's NucleoSpin 96 Plant II Kit (MACHERY-NAGEL®) or Qiagen DNeasy Plant Mini Kit (QIAGEN® Industries). Eluted DNA was diluted to acquire concentrations between 5 and 20 ng/µl for optimal PCR amplification.

Five microsatellite (SSR) markers were selected from the Cotton Marker Database (Blenda *et al.*, 2006) and five from Gardunia (2006) for use based on prescreened marker data. Individual PCR reactions contained 0.1µg bovine serum albumin (BSA), 1x PCR buffer (NEW ENGLAND BIOLABS®), 0.2 mM deoxynucleotides (dNTPs), 2.0 mM $MgCl_2$, and 0.4 ul of Taq polymerase. BSA was added to the individual PCR reactions following difficulty in amplifying samples as suggested by Horne et al. (2004). Individual PCRs were performed in a thermocycler (BIORAD) using a touchdown PCR cycle: initial denaturation at 94 °C for 15 min, denaturation at 94 °C for 60 s, annealing at 55 °C

for 60 s, elongation at 72 °C for 2 min, decreasing 1 °C/cycle for 9 cycles followed by 30 cycles of denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s, elongation at 72 °C for 2 min, and a final elongation at 72 °C for 7 min. Three of the most informative microsatellite markers (See Table 2.3) were selected for the hybrid confirmation study based on successful individual PCR amplification and the variation in size of PCR products. Markers were selected for their capacity of distinguishing between *G. hirsutum, G. barbadense* and *G. tomentosum* as well as their F_1 hybrid progeny (Lehman unpublished data).

A multiplex PCR reaction was designed using the three microsatellite markers. The composition for each multiplex PCR was performed in a total reaction volume of 10 µl (5µl of 2x Multiplex PCR mix (Qiagen ®), 0.2 µl of each primer (10 pmole), 1 µl Q-solution, 6 µl of molecular biology grade water, 1.4 µl of eluted DNA. Multiplex PCRs were performed in a thermocycler (BIORAD) under the following conditions: initial denaturation at 95 °C for 15 min, 32 cycles of denaturation at 94 °C for 30 s, annealing at 58.7 °C for 90 s, elongation at 60 °C for 60 s, and final elongation at 60 °C for 30 min. Following multiplex PCR, samples were run on a 2% gel stained with GelRed™ (BIOTUM) nucleic acid stain, for 100 minutes at 110 volts. Gels were visualized using the BioDoc-It (UVP) imaging system. In total, 88 individuals collected from the Nānākuli area were screened using molecular markers.

TABLE 2.3. PRIMERS AND MICROSATELLITE LOCI FOR GOSSYPIUM SPECIES (Blenda *et al.*, 2006; Gardunia, 2006).

Name	Primer sequence (5'-3')	Repeat Motif	Size (bp) *G. tom*	Size (bp) *G. hir*	Size (bp) *G. barb*
BNL1034	F- TTGCTTTCAATGGAAAACCC R- CGTCGCAAAGTTGAGAATCA	$(CT)_{16}$	96, 206, 253	118, 206, 224, 254	118, 208, 244, 259, 279
DPL0019	F- ATTCACGAATCTCGTGGTGTATC R- GTTATGATCAAGTCGGGTGGTTAT	$(CT)_{17}$			
DPL0094	F- CCCAAACCACATTCATTTCG R- AGATGTCTGTGATGAGTTTGGAGA	$(AG)_{29}$			

**G. tom* = *G. tomentosum*, *G. hir* = *G. hirsutum*, *G. barb* = *G. barbadense*.

Molecular Analysis of Herbarium Vouchers

Due to the degraded nature and limited supply of the 1980 herbarium voucher leaf material (HAW Voucher numbers 5134, 5135, 6071, 6070, 6069), DNA extraction protocols were modified to increase effectiveness. Juvenile leaf tissue (100 mg dry weight) samples were ground to a course powder in a 1.5 ml test tube with a pestle, using liquid nitrogen. Lysis buffer (700 µl) was added and samples were kept in the dark at room temperature for one week with tubes inverted twice daily to mix the buffer solution with leaf material. Normal extraction protocols were followed after the extended lysis buffer soak. To increase band clarity during electrophoresis, 0.1 µl BSA was added per 10 µl PCR multiplex mix. To more accurately quantify base pairs at each banding site, results from herbarium specimens, along with DNA samples from all three potential parents (*G. tomentosum, G, barbadense* and *G. hirsutum*) were bioanalyzed using the Agilent 2100 Bioanalyzer.

RESULTS

Morphological Assessment

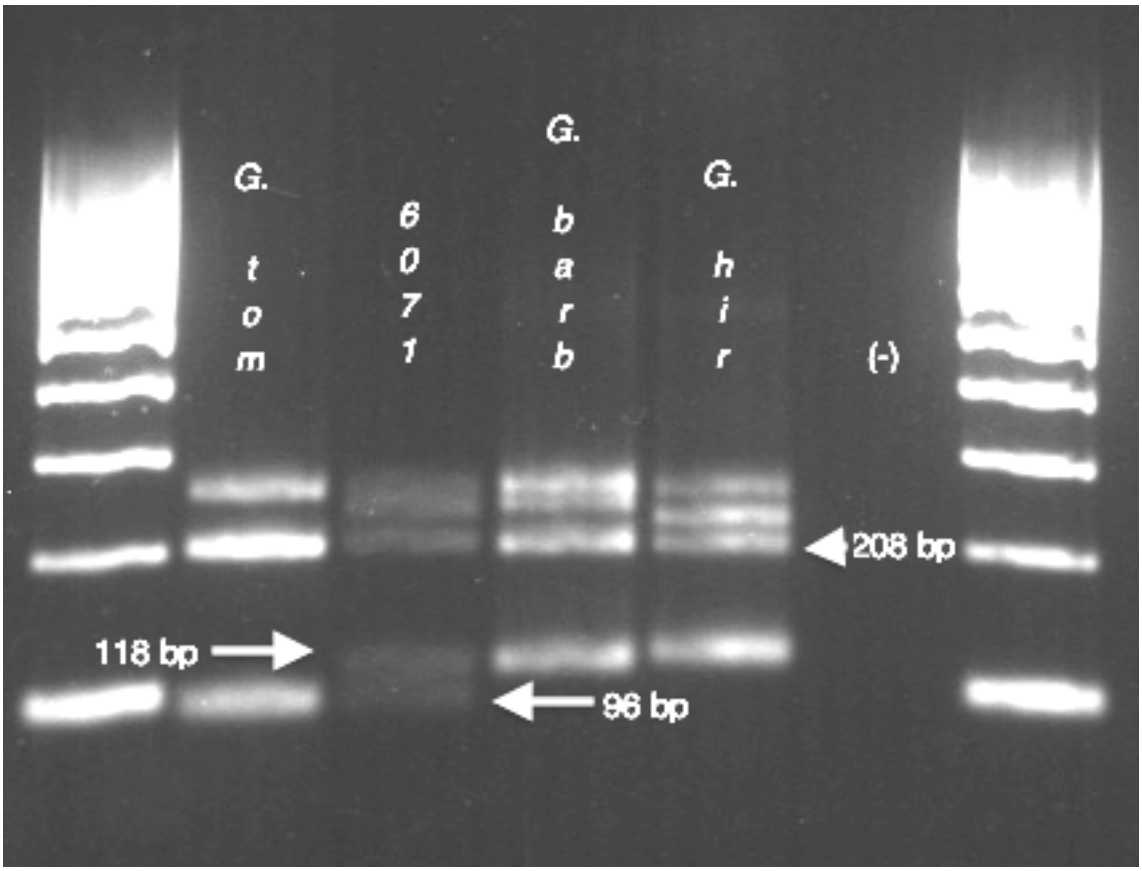
Despite several surveys undertaken throughout the growing season, based on morphology, no hybrids were identified in either the Hawaiian or Pima cotton populations that matched 1) Stephens' (1964) hybrid description, 2) herbarium vouchers of cotton hybrids at HAW, or 3) artificial F_1 hybrids being grown concurrently at the UH University greenhouses. No plants of *G. tomentosum* (n>100) and *G. barbadense* (n=9) expressed intermediate or abnormal traits when compared to morphological descriptions of each respective species provided in Stephens (1964) and Wagner et al. (1999). Plants tagged as potential morphological hybrids (all from Hawaiian cotton populations) expressed reduced leaf tomentum, a trait strongly correlated to local environmental conditions (Stephens 1964). Stephens (1964) observed that plants of Hawaiian cotton that grew in exposed areas had dense leaf tomentum while those that grew in shade had reduced tomentum. Of the plants tagged as potential morphological hybrids in our study, all either grew in the understory of *Prosopis pallida* or *Leucaena leucocephala* stands or amidst dense swords of *Cenchrus ciliaris*.

Molecular Assessment

None of the leaf samples collected in 2011 of either *G. barbadense* or *G. tomentosum* were from F_1 hybrid plants (i.e., possessing microsatellite banding patterns indicative of hybrid parentage). Samples collected by Cliff Morden in 1999 also lacked hybridization banding patterns. Of the five herbarium vouchers collected by Nagata in 1980, DNA was successfully extracted in adequate amounts from one specimen, *K. Nagata 2068* (HAW

6071). This specimen produced gel electrophoresis banding patterns consistent with a hybridization event between *G. tomentosum* and a commercial cotton species, though the exact commercial species, as well as the hybrid filial generation, cannot be determined (See Fig. 2.2). Bioanalyzer results of *K. Nagata 2068* (HAW 6071) consistently showed a band at 96 base pairs (bp), unique to Hawaiian cotton, and a band at 118 bp, unique to both polyploid commercial cotton species. The upper banding region showed banding at 208 bp, consistent with all three species, and at roughly 236 bp, intermediate between *G. hirsutum* (+/- 224 bp) and *G. barbadense* (+/- 244 bp).

FIGURE 2.2. GEL ELECTROPHORESIS SHOWING BANDING PATTERNS OF POTENTIAL PARENT SPECIES AND HYBRID PROGENY.



Banding patterns of HAW 6071 (*Nagata 2068*) compared to potential parent species. *G. tom* = *G. tomentosum*, *G. barb* = *G. barbadense*, *G. hir* = *G. hirsutum*. (-)= negative control. Using a 100 base pair ladder (far left and far right).

DISCUSSION

The results of our field surveys indicate that the two hybrid populations of *G. barbadense* × *G. tomentosum* that were recorded by Stephens (1964) at Nānākuli likely no longer exist, despite the persistence of *G. barbadense* close to where the hybrids were initially recorded. Because of the limited number of microsatellites utilized in this study, later generations of hybrids could be missed and thus introgression cannot be fully ruled out

based on molecular evidence alone. However, the combination of the results of our molecular analysis and detailed morphological field observations (Table 2.2) strongly suggests that all individuals of both cotton species sampled are not hybrids. Using these methods in concert has been effective in screening artificial F_1 hybrids of these cotton species grown at UH Manoa (Lehman unpublished data).

Molecular marker confirmation of Nagata's 1980 voucher (HAW 6071) proves that hybrids did exist in the area as late as 1980, suggesting either the persistence of the original population or further hybridization events since Stephens' 1964 survey. We cannot be fully certain that these specimens were collected from the original populations that Stephens (1964) described. If the vouchers are representatives of the original populations, it suggests that hybrids and/or their descendants are capable of persisting for at least several decades. This is a realistic assumption, as *G. tomentosum* is a long-lived woody shrub, capable of persisting for several decades (Pleasants and Wendel, 2010).

The lack of evidence of current hybridization may be a product of human development rather than the ability of these cotton hybrids to persist and spread. The areas immediately surrounding the sites where hybrids were initially recorded by Stephens (1964) have been greatly modified in the subsequent four decades by development, fire, and likely also by alien plant species and the activities undertaken to manage them (A. Lehman pers. obs.). For example, the waste lot adjoining a Texaco Service Station described in Stephens (1964) no longer exists and although an abandoned area nearby contains *G. barbadense*, we cannot be certain if these are descendants from the original

Pima cotton population that Stephens described. In addition, Nagata's 1980 collection site, in "tall grass west of railroad tracks" is periodically mechanically cleared of vegetation by the Hawai'i Department of Transportation, with the few remaining *G. tomentosum* plants exhibiting stunted growth (A. Lehman pers. obs.). However, through morphological and molecular analysis, we do know that these plants are not hybrids.

The potential risk of future hybridization events between Hawaiian cotton and Pima or Upland cotton at this (in the case of Pima cotton) and other sites in Hawai'i is still relatively high (Fryxell 1979, Ellstrand et al. 1999, Daehler and Carino 2001, Münster and Wieczorek 2007, Pleasants and Wendel 2010). First, both Hawaiian cotton and the two alien cotton species are interfertile and produce fertile progeny (Meyer and Meyer 1961, Meyer and Meredith 1978, DeJoode and Wendel 1992). Second, all three species share similar lowland, coastal habitats (Wagner et al. 1999) increasing the chances that Pima and Upland cotton may colonize areas where Hawaiian cotton currently exist. Third, all three species are capable of flowering during the same periods of the year (Stephens 1964, A. Lehman pers. obs.). Fourth, Pleasants and Wendel (2010) found that the flowers of Hawaiian cotton populations on O'ahu, Maui and Kaho'olawe were visited by large carpenter bees (*Xylocopa* sp.) and honeybees (*Apis mellifera*), which are also known pollinators of Pima cotton (Umbeck et al. 1991, Keasar 2010). Both bee species are known to fly many kilometers to forage (Seeley, 1985; Proctor *et al.*, 1996; Pasquet *et al.*, 2008). The small population of Pima cotton at N$\bar{a}$n$\bar{a}$kuli is approximately one km from the nearest Hawaiian cotton population (See Fig. 2.1), easily within the foraging

range of both bee species, therefore, maintaining the risk that gene flow may occur between the two species again in the future.

FUTURE MANAGEMENT AND RESEARCH RECOMENDATIONS

Although our study did not find extant hybrids, Pima and Upland cotton grown in home and public gardens in coastal regions of all islands still present a risk to natural populations of Hawaiian cotton due to their potential to naturalize, spread, and eventually hybridize with Hawaiian cotton. To avoid future hybridization events between Hawaiian cotton and either Pima or Upland cotton, we recommend that landowners or land management agencies remove these species. Specifically, the population of Pima cotton at Nānākuli should be controlled to prevent future hybridization events with Hawaiian cotton. In addition, horticultural sale of these species should be prohibited to limit possible further naturalization events.

We reiterate the suggestions of Pleasants and Wendel (2010) that strategies to mitigate gene flow between Upland (and Pima) and Hawaiian cotton (and vice versa) should be undertaken. To reduce the potential for interspecific gene flow, transgenic cotton production is prohibited in Hawaii with the exception of EPA permitted test plots or breeding nurseries (EPA 2005). Seed companies regularly grow permitted transgenic cotton (herbicide resist and Bt) in Hawai‘i; the last issued test permit (2008-2009) allowed for production on 2,725 acres (ISB 2012). Permitted test plots cannot be planted within three miles of natural *G. tomentosum* populations and must be surrounded by 24 border rows of a “suitable pollinator trap” (EPA 2005). In addition to these regulations,

we recommend the need for field-based pollinator and gene flow studies in Hawai‘i to establish safe planting distances, similar to those that have been undertaken in other parts of the world (Umbeck et al. 1991, Llewellyn and Fitt 1996, Zhang et al. 2005, Heuberger et al. 2010). These measures would help prevent the escape of transgenic and non-transgenic Upland and Pima cotton genes into Hawaiian cotton populations, as well as maintaining the genetic purity of commercial cotton lines grown in Hawai‘i.

CHAPTER 3. ASSESSING THE FITNESS AND FECUNDITY OF RECIPROCAL F_1 HYBRIDS BETWEEN ENDEMIC HAWAIIAN COTTON, *Gossypium tomentosum* AND THE COMMERCIAL COTTON SPECIES *G. hirsutum* AND *G. barbadense*

INTRODUCTION

The Hawaiian archipelago is the most isolated island group in the world, separated by over 800 km to other islands and over 3,000 km to the next major land mass (McDonald *et al.*, 1986). Due to this isolation and 1–5 million years of evolution (McDonald *et al.*, 1986), the Hawaiian Islands are home to one of the highest levels (~90%) of plant endemism in the world (Wagner *et al.*, 1999). This high endemism rate makes any biodiversity loss in the islands even more poignant (Gagné, 1988). Currently, 319 native Hawaiian plant species, accounting for over 25% of the native flora, are threatened or endangered, accounting for 40% of all federally listed plant species (USFWS 2012).

Island populations have a much higher risk of extinction than continental populations (Frankham, 1988; Rieseberg, 1991; Levin *et al.*, 1996) and greater susceptibility to hybridization because of their small numbers and their frequent proximity to numerically larger congeners (Carlquist, 1974; Crawford *et al.*, 1987). Insular species are also particularly vulnerable to hybridization due to gametic wastage, reduced seed-set, and the production of ill-fit progeny on the one hand, and the swamping effect of gene flow on the other (Levin *et al.*, 1996). These effects are not likely to be counterbalanced by immigration from other conspecific populations due to isolation and remoteness.

The Hawaiian flora is especially susceptible to hybridization due to the nature of island endemics having few or no pre- or post-zygotic reproductive barriers (Carlquist, 1974; Ganders and Nagata, 1984; Crawford *et al.*, 1987; Levin *et al.*, 1996) and be prone to outcross (Carlquist, 1974). In addition, poorly differentiated floral architecture and a year round flowering season increase Hawaiian species' vulnerability to hybridization (Carlquist, 1974). Yet natural interspecific hybridization events appear to be relatively rare, highlighting the significance of geological and ecological separation in preventing potentially deleterious gene flow (Carlquist, 1974; Ganders and Nagata, 1984; Crawford *et al.*, 1987). The breakdown of geographical and/or ecological barriers by human disturbances, such as agriculture, urban sprawl and habitat fragmentation, greatly increases the risk of gene flow between native and alien species (Rieseberg, 1991; Levin *et al.*, 1996; Vila *et al.*, 2000).

Ecological barriers often limit hybridization and gene flow, the incorporation of genes from one population into the gene pool of another. Human disturbances, such as agriculture and urban sprawl often reduce or remove these barriers (Levin *et al.*, 1996; Vila *et al.*, 2000). Landscape ornamentals, agricultural and horticultural crops, and naturalized alien species are all potential sources of gene flow (Levin *et al.*, 1996; Vila *et al.*, 2000). Over the past two decades, largely spurred by the advent of genetic engineering (GE) of crops, crop-to-wild-plant gene flow has received significant attention (Klinger *et al.*, 1992; Raybould and Gray, 1993; Klinger and Ellstrand, 1994; Arriola and Ellstrand, 1996; Ellstrand *et al.*, 1999; Rieseberg *et al.*, 1999; Burke *et al.*, 2002; Arnaud

et al., 2003; Ellstrand, 2003a; Jenczewski *et al.*, 2003; Anderson and de Vicente, 2010; Vacher *et al.*, 2011). Evidence for spontaneous hybridization between 12 of the world's 13 most important food crops and their wild relatives has been well documented (Ellstrand *et al.*, 1999). Gene flow between crops and their wild relatives happens regularly, whether the crops are transgenic or conventional (i.e., non-transgenic) (Ellstrand, 2003a) and has played an important role in crop development (Harlan, 1992).

Crop-to-wild gene flow also presents significant ecological risks. Because crop plant varieties have been bred with specific traits such as increased vigor and yield, impacts of crop-to-wild gene flow may be different than that of other introduced species. For example, maladaptive traits such as male sterility, dwarfing or lack of seed dormancy or dispersibility may lead to a selective disadvantage for hybrids while increased pest resistance may prove advantageous to hybrids, increasing their likelihood for persistence (Ellstrand and Hoffman, 1990; Ellstrand, 2003b). Impacts of crop-to-wild gene flow include increased weediness of wild species (Tiedje *et al.*, 1989; Snow and Palma, 1997; Ellstrand *et al.*, 1999; Ellstrand, 2003a), loss of genetic diversity of wild, sexually compatible species (Gepts and Papa, 2003; Haygood *et al.*, 2003) with possible genetic assimilation and species extinction (Levin *et al.*, 1996; Ellstrand, 2003a; Haygood *et al.*, 2003).

Crop-to-wild gene flow is primarily pollen mediated (Ellstrand and Hoffman, 1990), occurring when pollen from one species successfully fertilizes the egg of another. Gene flow can occur in both directions (i.e., crop-to-wild and wild-to-crop), however given the

tendency for modern crop populations to be large, concentrated monocultures, wild populations are more likely to be exposed to crop pollen than visa versa (Kwit *et al.*, 2011). In order for crop genes (transgenic or conventional) to be transferred to wild populations via pollen mediated gene flow, crop plants and their wild relatives need to: 1) be sympatric, 2) have similar pollination biology, 3) share pollinator vectors, 4) overlap in flowering time, 5) be cross-compatible (i.e., capable of producing viable offspring), and 6) persist in the environment (Jenczewski *et al.*, 2003; Kwit *et al.*, 2011). In short, if a crop and wild relative co-exist, the frequency of gene flow under natural conditions will strongly depend both on their relatedness and on the phenology and pattern of pollen dispersal (Lavigne *et al.*, 2002).

One species which is at risk of crop-to-wild gene flow is Hawaiian cotton or maʻo (*Gossypium tomentosum*) (Fryxell, 1979; Ellstrand *et al.*, 1999; Daehler and Carino, 2001; Münster and Wieczorek, 2007; Anderson and de Vicente, 2010; Pleasants and Wendel, 2010). This long lived perennial species is endemic to arid, lowland and coastal sites between sea level and 120 m on all the main Hawaiian Islands, except the Islands of Hawaiʻi and Kauaʻi (Wagner *et al.*, 1999) though it has recently been reintroduced to Kauaʻi (Krakos *et al.*, 2010). Hawaiian cotton has long been valued for it's economic and cultural importance. Flowers of the plant were traditionally and continue to be used to create a yellow dye while leaves are used to create a delicate green dye, both used to dye tapa cloth (Krauss, 1993). More recently *G. tomentosum* has been recognized for it's agronomical value in breeding programs.

Two additional, agriculturally important cotton species, *G. barbadense,* (Pima cotton) and *G. hirsutum* (Upland cotton) have been introduced to the Hawaiian Islands and are now sporadically naturalized; Pima cotton on all the main islands except Kahoʻolawe, and Upland cotton on Oʻahu (Wagner *et al.*, 1999) and East Maui (Wagner *et al.*, 2012). Both commercial species were introduced to the Hawaiian Islands during the 1800's, experiencing a commercial boom during the American Civil War (Crawford, 1937). Cotton (most likely predominately *G. barbadense*) was planted on the leeward sides of Kauaʻi, Oʻahu, and Hawaiʻi, from sea level to 800 feet elevation in areas that received 20–60 inches of annual rainfall (Crawford, 1937). Insect pressure, especially that of the pink bollworm (*Pectinophora gossypiella*), and a USDA quarantine implemented in 1913 which restricted imports of cotton from areas harboring the pink boll worm put an end to any hope of profitable cotton production in the state (Crawford, 1937). There is currently no large-scale commercial cotton production in the state of Hawaiʻi and production of GE cotton is banned in the state. However, seed companies regularly obtain EPA permits to use the Hawaiian Islands as winter nursery sites, primarily for GE (herbicide tolerant or insect resistant (Bt)) Upland cotton and potentially Pima cotton (ISB 2012). Both species, particularly Pima cotton, are also grown as landscape ornamentals in the state of Hawaiʻi (Staples and Herbst, 2005).

The potential risk of gene flow between *Gossypium tomentosum* and *G. hirsutum* and *G. barbadense* in the Hawaiian Islands is high due to the following factors:

Known Interfertility Between the Species

Gossypium tomentosum belongs to the primary gene pool of both commercial species (Percival *et al.*, 1999; Anderson and de Vicente, 2010). Because of this, interspecific crosses have been made between Hawaiian cotton and both commercial cotton species to help decipher their phylogenies (Gerstel and Sarvella, 1956; DeJoode and Wendel, 1992), to introduce the nectariless trait from Hawaiian cotton into agricultural varieties of *G. hirsutum* (Meyer and Meyer, 1961; Meyer and Meredith, 1978), and to develop trait specific molecular markers for cotton breeding programs (Saha *et al.*, 2006).

Shared Insect Pollinators

Carpenter bees (*Xylocopa* sp.) and honey bees (*Apis mellifera*) are known pollinators of all three cotton species in Hawaii (Umbeck *et al.*, 1991; Keasar, 2010; Pleasants and Wendel, 2010). These bee species are known to fly several kilometers to forage; up to 12 km for honey bees (Seeley, 1985) and six km for carpenter bees (Pasquet *et al.*, 2008) potentially moving pollen upwards of 15 km.

Shared Habitat

All three species share similar lowland, coastal habitats (Wagner et al. 1999), increasing chances that Pima and Upland cotton may naturalize in areas where

Hawaiian cotton currently exist. In addition, agricultural zones in the state of Hawai‘i are often within pollinator foraging distances of native cotton populations.

Overlap in Flowering Phenology

All three species are capable of flowering during the same periods of the year (Stephens 1964; Lehman, pers. obs.). In addition, all three species have flowers that are receptive during the day, for one day, opening in the morning and closing by late afternoon (Free, 1970; Oosterhuis and Jernstedt, 1999; Krakos *et al.*, 2010; Pleasants and Wendel, 2010). *Gossypium tomentosum,* though fully self-compatible, favors outcrossing (Pleasants and Wendel, 2005; Krakos *et al.*, 2010) and this increases the odds for outcrossing and potential gene flow.

History of Sporadic Gossypium Hybridization in the Hawaiian Islands

Early reports of hybridization between *G. barbadense* and *G. tomentosum*, first observed at Nānākuli by Dr. Otto Degener in 1950 (Hutchinson 1959) and later described by Stephens (1964) provided detailed morphological and geographical descriptions of two hybrid cotton populations at Nānākuli on the Wai‘anae Coast of O‘ahu. More recently, Kenneth Nagata (then affiliated with Lyon Arboretum) collected herbarium vouchers of *G. barbadense* ×*G. tomentosum* hybrids in 1980, close to one of the sites described by Stephens (1964).

The risk for gene flow between commercial cotton species and native Hawaiian cotton species is apparent yet un-quantified. One way to quantify the risk of gene flow between two species is through controlled artificial hybridization (i.e., hand pollination) (Ellstrand and Hoffman, 1990; Armstrong *et al.*, 2005; Anderson and de Vicente, 2010). If artificial hybridization is relatively easy, then it can be assumed that gene flow is possible given favorable circumstances, thus warranting further research on the likelihood of natural gene flow and the impacts if may have on native Hawaiian cotton (Armstrong *et al.*, 2005). On the other hand, if artificial hybridization is difficult or requires technology such as embryo rescue or chromosome doubling, it can be assumed that spontaneous interspecific gene flow is unlikely in the wild (Armstrong *et al.*, 2005; Anderson and de Vicente, 2010). If artificial hybrids are formed, evaluation under controlled field conditions can help assess their potential ecological impacts (Tiedje *et al.*, 1989; Ellstrand and Hoffman, 1990; Johnston *et al.*, 2006).

To determine how readily *G. tomentosum* hybridizes with *G. hirsutum* and *G. barbadense,* reciprocal crosses were carried out in a controlled setting. Microsatellite DNA markers were optimized for distinguishing between the three study species and their hybrid progeny. To assess the impacts of gene flow on fitness and fecundity, F_1 hybrids were germinated in a greenhouse and then grown in a common garden plot along with parental species.

MATERIALS AND METHODS

Parent Plant Material

Propagules were gathered and cultivated in the spring of 2010 to be used for this project by Richard Pender, of the University of Hawaii's (UH) Botany Department. Seeds or mature plants of *G. hirsutum* and *G. barbadense* were obtained from naturalized plants or landscape specimens to act a parent stock for hybridization studies (See Table 3.1). Because *G. tomentosum* takes up to two years to reach flowering maturity when planted from seed (Culliney and Koebele, 1999; Lehman pers. obs.), mature, flowering landscape plants located on the UH Manoa campus were used to achieve the F_1 generation. Naturalized or landscape plants were chosen as commercial cotton parent stock for this study as they may pose the most significant risk to endemic Hawaiian cotton due to lack of regulation (D. Frohlich, Bishop Museum, pers. comm.) and proven ability to persist in Hawaii's environment (Wagner *et al.*, 1999; Wagner *et al.*, 2012).

TABLE 3.1. PARENT MATERIAL SOURCES USED DURING RECIPROCAL CROSSES TO CREATE F_1 GENERATION.

Species	Source	Total experimental plants
G. tomentosum	UH Manoa campus, existing landscaping plants, aproximatly 10 years of age, near Pope Laboratory, UH Manoa.	6
G. hirsutum	Naturalized population near Waimanalo. One mature fruit was collected from 20 separate plants.	45
G. barbadense	UH Manoa campus, Botany Courtyard. Seeds collected from two mature plants.	21

Commercial species (*G. hirsutum* and *G. barbadense*) were raised to flowering maturity and maintained for hybridization trials at the Magoon Research Facility (MRF), UH Manoa. Plants were initially grown in the greenhouse and later planted outside in irrigated planter beds (with exception of *G. tomentosum*, in which mature landscape plants growing near Pope Laboratory, on the UH campus, were used due to the delayed flowering of young plants). A minimum of thirty successful reciprocal crosses between *G. tomentosum* and each commercial species (*G. barbadense*, *G. hirsutum*) and successful selves among species were achieved (See Table 3.3). Voucher specimens (A. Lehman collection numbers 11A, 10A and 12A) of parent plants are deposited at the Joseph F. Rock Herbarium (HAW).

Hybridization, Harvesting and Fecundity

Flowers were bagged with bridal veil cloth the day prior to anthesis to exclude potential insect visitation. Bags were gently but securely tied at the base of the peduncle with thin gauge wire. Healthy, exposed flowers were selected at approximately waist height on plants, unhealthy or heavily shaded flowers were avoided. Upon reaching anthesis, the bag was removed; the stigma was carefully exposed to minimize the likelihood of self-pollination. Pollen from the experimental male parent was liberally applied until the stigma was visually coated with donor pollen. Stigmas were inspected to ensure that they were free of maternal pollen prior to crossing. All crossed female parent flowers were labeled with parentage and date of cross with plastic nursery labels attached to the peduncle.

Following pollination, flowers were carefully re-bagged to prevent insect visitation. Bags were removed upon petal senescence, typically within one to two days of the pollination event. Flowers that were to be selfed were bagged with the bridal veil prior to anthesis, and bags removed only after the petals senesced.

Time from pollination to mature fruit set was measured as time between hand pollination and boll dehiscence. Fruit was harvested upon boll dehiscence. Fruit abortion was recorded as flowers that were pollinated and either never developed fruit or developed fruit that later senesced prior to maturation. Most fruit abortion occurred within 3-7 days of the pollination event. Upon harvesting, boll mass was measured by weighing the entire boll (excluding the carpel walls). Lint was then removed from seeds with detachable lint (*G. hirsutum* and *G. barbadense*) and seeds per boll were counted. Fiber of *G. tomentosum* adheres tightly to the seed coat and thus was not removed. Immature/undeveloped seeds were removed from bolls and were not included in the seed count. Processed seeds were sealed in labeled paper seed packets and stored in breathable plastic bins at 4°C.

Germination

Seeds of 15 fruits per cross, including selfs representing the F_1 generation, were sown in greenhouses at the MRF on April 2, 2011. An additional control group of 15 *G. tomentosum* fruits collected from open pollinated flowers (no insect exclusion fabric was applied, nor were the flowers hand manipulated) were also sown to test for effects of possible inbreeding depression of selfed *G. tomentosum*. Bolls from open pollinated

flowers were collected from the same parental *G. tomentosum* plants used throughout the trial for reciprocal crosses and selfs.

Seeds were sown into labeled 1"x 8" dibble tubes and placed into 98 cell racks. Seeds were covered with a fine layer or #2 perlite, approximately ½ cm thick, to discourage damping off. Soil mix consisted of a 3:1 ratio of Therm-O-Rock brand perlite to PRO-MIX brand potting mix. Racks were placed at random on the benches and rotated clockwise (both individually and about the bench) weekly to take into account possible environmental and irrigation irregularities of bench space. Germination benches were covered with 40% shade factor saran cloth (applied to the roof) and received five minutes of overhead irrigation, 4 times a week (Mon, Wed, Fri, Sat) at 8am. Upon noticing damping off of seedlings, Subdue Maxx® fungicide (Novartis Crop Protection Inc., Greensboro, North Carolina) was applied as a drench on April 21, 2011 at the rate of 0.25 fl. Oz/ 100 gallons solution (roughly 7.4 mL fungicide per 379 L water). Application was delayed by two weeks due to a spray ban at the greenhouse facility (see discussion for relevance). Once cotyledons fully emerged, to prevent etiolation and dehydration, dibble tubes were transferred to adjacent benches that lacked the 40% saran cloth and received eight minutes of morning overhead irrigation, six days a week (every day excluding Tuesdays). A randomized pattern was maintained, with weekly clockwise rotations.

After two months (on June 3, 2011), seeds that failed to germinate were removed and tested for viability using a 1% solution of 2-3-5 tetrazolium chloride (TTC) following methods outlined by Peters (2005). A two month cut off date was set as viable *G.*

tomentosum seeds can take up to two moths to germinate under favorable conditions (Lilleeng-Rosenberger, 2005). Each seed was first visually inspected for signs of rot and decay by bisecting longitudinally. Seeds that showed signs of rot and decay were recorded as such and discarded. Seeds that appeared viable (white, firm embryonic tissue, no rot or decay) were placed in labeled 1.5 mL vials, saturated with TTC solution (3-4 drops) and placed in a light excluding incubator at 22 C° for 24 hours. Seeds were then scored based on stain evaluation diagrams for Malvaceae outlined in Peters (2005).

Seedlings were maintained in dibble tubes for observations and analysis until a plot was available for outplanting in a common garden on July 17, 2011. Seedlings were fertilized once a month at the product recommended low rate of 1 teaspoon of Miracle-Gro® water-soluble fertilizer per gallon of water (roughly 5 mL fertilizer per 3.8 L water). Applications were made using a 1:16 calibrated Hozon siphon and a Dramm water breaker. Two overhead drenches were made per application.

Days to germination were measured as time from sowing to the emergence of fully expanded cotyledons. Plant height after one month was measured from media surface to the apical meristem. Survivorship was noted as the percentage of germinated seeds that survive for the duration of the two months greenhouse germination experiment.

Molecular Hybrid Confirmation

Because maternal flowers used in hybridization trials were not emasculated, all seedlings, resulting from interspecific crosses were potentially maternal selfs. To determine

parentage, leaf tissue was gathered from randomly selected individuals for analysis with microsatellite (SSR) DNA markers. Due to the high seed set and resulting seedling germination, a representative sample of 100 randomly selected individuals was selected for molecular analysis, using the "RANDBETWEEN" function in Microsoft Excel, for crosses *G. hirsutum* x *G. tomentosum* (HT) and *G. barbadense* x *G. tomentosum* (BT). Due to lower seed set of native cotton and subsequent fewer seedlings, all germinated *G. tomentosum* x *G. hirsutum* (TH) and *G. tomentosum* x *G. barbadense* (TB) were tested, 55 and 53 individuals, respectively. Fifteen randomly selected selfed parental-type progeny, *G. hirsutum* (HX)*, G. barbadense* (BX)*, G. tomentosum* (TX) and *G. tomentosum* open pollinated (T-OP), were also tested to ensure that there was no accidental cross contamination between species. When possible, one seedling per boll was selected using the "RANDBETWEEN" function for all crosses and selfs. If only one seed germinated per fruit it was automatically selected. If no seeds germinated for a fruit, another fruit in the same cross or self was selected at random and a seedling was again selected at random.

Because more than 100 progeny germinated for HT and BT crosses, remaining seedlings not accounted for in the initial 100 randomly selected individuals were assessed visually for morphological hybrid characteristics common for SSR confirmed hybrids. Hybrid characteristics included intermediate leaf pubescence, intermediate number of leaf lobes, and intermediate plant height. To insure accuracy of morphological selections, plants selected as morphological hybrids were randomly screened with SSR markers (see below) along with plants that were deemed to be non-hybrid, maternal selfs. All

morphologically selected hybrids tested positive with SSR markers for hybridization while all those deemed as non-hybrids, based on morphology, tested negative. Because naturalized or landscape plants were used in place of current agriculture varieties of Upland and Pima cotton, the standard lines TM-1 and 3-79 were used to verify marker effectiveness for standard agriculture lines of *G. hirsutum* and *G. barbadense*, respectively.

Leaf material (approximately 100 mg wet weight) was disrupted using TissueLyser (QIAGEN® Industries) by adding lysis buffer and operating the TissueLyser for 2 min at 30 Hz (2x). DNA was subsequently extracted using either Macherey-Nagel's NucleoSpin 96 Plant II Kit (MACHERY-NAGEL®) or Qiagen DNeasy Plant Mini Kit (QIAGEN® Industries). Eluted DNA was diluted to acquire concentrations between 5 and 20 ng/µl for optimal PCR amplification.

Five SSR markers were selected from the Cotton Marker Database (Blenda *et al.*, 2006) and five from Gardunia (2006) for use based on prescreened marker data. Individual PCR reactions contained 0.1 µg bovine serum albumin (BSA), 1x PCR buffer (NEW ENGLAND BIOLABS®), 0.2 mM deoxynucleotides (dNTPs), 2.0 mM $MgCl_2$, and 0.4 ul of Taq polymerase. BSA was added to the individual PCR reactions following difficulty in amplifying samples as suggested by Horne et al. (2004). Individual PCRs were performed in a thermocycler (BIORAD) using a touchdown PCR cycle: initial denaturation at 94 °C for 15 min, denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s, elongation at 72 °C for 2 min, decreasing 1 °C/cycle for 9 cycles followed by 30 cycles

of denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s, elongation at 72 °C for 2 min, and a final elongation at 72 °C for 7 min. Three of the most informative microsatellite markers (BNL1034, DPL0019, DPL0094; See Table 3.2) were selected for the hybrid confirmation study based on successful individual PCR amplification and the variation in size of PCR products. Markers were selected for their capacity of distinguishing between *G. hirsutum, G. barbadense* and *G. tomentosum* as well as their hybrid progeny.

TABLE 3.2 PRIMERS AND MICROSATELLITE LOCI FOR GOSSYPIUM SPECIES (Blenda *et al.*, 2006; Gardunia, 2006).

			Size (bp)		
Name	Primer sequence (5'-3')	Repeat Motif	*G. tom*	*G. hir*	*G. barb*
BNL1034	F- TTGCTTTCAATGGAAAACCC R- CGTCGCAAAGTTGAGAATCA	$(CT)_{16}$	96, 206, 253	118, 206, 224, 254	118, 208, 244, 259, 279
DPL0019	F- ATTCACGAATCTCGTGGTGTATC R- GTTATGATCAAGTCGGGTGGTTAT	$(CT)_{17}$			
DPL0094	F- CCCAAACCACATTCATTTCG R- AGATGTCTGTGATGAGTTTGGAGA	$(AG)_{29}$			

**G. tom* = *G. tomentosum*, *G. hir* = *G. hirsutum*, *G. barb* = *G. barbadense*

A multiplex PCR reaction was designed using the three microsatellite markers. The composition for each multiplex PCR was performed in a total reaction volume of 10 µl consisting of 5µl of 2x Multiplex PCR mix (Qiagen ®), 0.2 µl of each primer (10 pmole), 1 µl Q-solution, 1.4 µl of molecular biology grade water, 1.4 µl of eluted DNA. Multiplex PCRs were performed in a thermocycler (BIORAD) under the following conditions: initial denaturation at 95 °C for 15 min, 32 cycles of denaturation at 94 °C for 30 s, annealing at 58.7 °C for 90 s, elongation at 60 °C for 60 s, and final elongation at 60 °C for 30 min. Following multiplex PCR, samples were run on a 2% gel stained with

GelRed™ (BIOTUM) nucleic acid stain, for 100 minutes at 110 volts. Gels were visualized using the BioDoc-It (UVP) imaging system (See Fig. 3.1). In total, 309 potential hybrid individuals and 60 maternal selfed individuals were screened using molecular markers (See Table 3.5).

F_1 Fitness and Fecundity

Common Garden Plot

Of the SSR confirmed hybrid and confirmed non-hybrid selfed progeny, 15 per cross and parental type (N=120) were selected at random using the "RANDBETWEEN" function in Microsoft Excel to be planted in a common garden at the MRF. The field site was tilled to 20 cm prior to planting to remove weeds and loosen the soil. Simplot Micro Green Brand 10-4-16 fertilizer was applied at a rate of 45 kg/ha and incorporated into the soil to a depth of 10 cm by tilling. Plants were labeled with species type and spaced 0.5 x 0.9 m, on center, in five rows irrigated with t-tape connected to an automatic timer. Once plants were in the ground, pre-emergent herbicide XL 2g was applied at a rate of 337.5 kg/ha to help with weed control.

Half of the field was devoted to progeny resulting from *G. hirsutum* / *G. tomentosum* reciprocal crosses, H Field, while the other half accommodated progeny resulting from *G. barbadense* / *G. tomentosum* reciprocal crosses, B Field. Both sections contained selfed progeny of each respective parental species along with the additional control group of open pollinated G. *tomentosum.* The planting was randomized by using a latin square planting pattern so that no single species or hybrid was adjacent to a like species or

hybrid within or between rows. This was done to ensure competition between hybrids and their parental-type species and vice versa. In the B Field, selfed parents of *G. barbadense* and *G. tomentosum* (7 each) were used to fill spaces left vacant by the lack of *G. barbadense* x *G. tomentosum* hybrids, (n=1). No measurements were taken from these additional parent-type plants. All plants were grown under the same environmental conditions to the extent possible. To assess the fitness of hybrids and paternal selfs, the following measurements were taken while plants were in the common garden:

F_1 Height

Plant height was measured monthly, for six months, from the base of the plant at ground level to the most terminal growing tip.

F_1 Days to First Flower

Days to first flower were measured for each plant as the time from outplanting in the common garden to formation of the first open flower.

F_1 Seed Set

Fecundity measurements were taken to assess the reproductive ability of F_1 hybrids compared to parent F_1 selfs. Pollen fertility was measured two ways, seed set and pollen stainability. F_1 plants were selfed, (see above for selfing protocol), to create the F_2 generation and to evaluate seed set, the most accurate evaluation of pollen viability (Dafni and Firmage, 2000). A goal of three successful selfs per plant was set. If a self aborted, another flower was bagged until three successful selfs were achieved. In some

cases plants ceased flowering before three selfs could be achieved or it became to late in the experiment to continue selfing if a six-month biomass harvest date were to be achieved. Abortion rates were recorded for each plant. Time from self-pollination (anthesis) to formation of mature fruit was measured for each successful self. Despite repeated attempts, some plants, especially in the B Field, failed to produce 3 mature fruits. Boll weight and seeds per boll were determined for each successful self.

Pollen Stainability

In addition to seed set, pollen stainability, a measurement of pollen viability (Aslam *et al.*, 1964; Stone *et al.*, 1995), was measured by evaluating pollen stainability, following procedures outlined by Aslam *et al.* (1964). Pollen was collected on dry, sunny days as water is known to negatively affect cotton pollen viability (Burke, 2002). Pollen from a total of four individual flowers, per trial plant, was assessed for pollen stainability, to the extent possible. In some circumstances, plants ceased flowering during pollen trials (especially *G. hirsutum* F_1 selfs) or no dry flowers were observed on sampling days. In addition to sampling F_1 generation pollen stainability, pollen from parent (F_0) generation plants (those used to attain the F_1 generation) was sampled during the same period and compared against F_1 hybrids and F_1 parent types.

Flowers were collected upon anthesis, typically between 9am and 12pm, to ensure optimal pollen vigor (Thomas *et al.*, 2004). Anthers were tapped to disperse mature pollen from anthers into a drop of TTC solution placed on a microscope slide. Pollen was mixed thoroughly for 30 seconds using a toothpick to ensure complete

pollen/solution immersion. TTC solution consisted of 2% TTC and 60% sucrose in distilled water (Aslam *et al.*, 1964; Barrow, 1983). A cover slide was placed atop the mixed solution and slides were placed in the dark at room temperature for 24 hours.

Pollen stain counts were taken under (2x) 10x magnification no sooner than 24 hours from initial staining. Grains were scored by either scanning the slide, starting in the top left corner, making transects right and down until 200 grains were scored. Edges of the slides were avoided, as accumulated pollen may not have been thoroughly immersed in solution. Dark red to dark pink grains were scored as viable, light pink to white/yellow (no staining) and ruptured grains were scored as non-viable.

F_1 Biomass

After six months, from planting (June 17, 2011- January 27, 2012), trial plants were destructively harvested to measure above ground biomass (both wet and dry). Plants were cut at ground level and sectioned into pieces that were placed into labeled paper bags. Prior to being placed into dryers at MRF at 65 °C, wet weight was measured, using an Ohaus Corp. Model CD-33 digital scale, for each bag. After two weeks, randomly selected bags, located throughout the dryer were weighed every other day. Plants were deemed to be fully dry once all randomly sampled bag weights remained constant at three consecutive weigh-ins. Final dry biomass measurements were then taken. Because plants remained within bags during weighing, to account for bag weight, 500 new, empty bags were weighed in mass and mean bag weight was calculated. This mean bag weight value was then subtracted from each biomass measurement.

F_1 Stem Diameter

Stem diameter, at ground level was measured for each plant as it was harvested for biomass analysis. A tailor's style measuring tape was used to record the stem circumference at ground level. Stem diameter was determined by entering stem circumference (c) into the following equation: $d = c / \pi$.

Statistical Analysis

Comparisons between F_1 hybrid, F_1 parental-type and parental (F_0) generation plants were made using one-way analysis of variance and Kruskal Wallace ANOVA on Ranks (SigmaStat® 3.1 statistical software). Pairwise comparisons of means were made using either a Tukey test for normally distributed data or Dunn's Method for non-normally distributed data. Differences were considered statistically significant if $P < 0.05$. Because only one BT hybrid was germinated, F_1 data from this cross (with exception to "days to boll maturity," "boll weight," and "seed count") was excluded from statistical analysis as to not skew the results. Because TX and T-OP were planted in both fields (H and B) comparisons between the two were made both within and between each respective field.

Phenotypic Observations

Digital photographs were taken of plants grown at MRF for phenotypic documentation. Seedlings, mature plants, flowers (including cross sections of flowers at anthesis), fruits (at various stages of development), leaves (both mature and juvenile growth), leaf and bract nectaries were recorded using a Canon Rebel XTI digital camera. Herbarium

vouchers (A. Lehman collection numbers 1-4), were taken, representing all hybrid and parent-type plants grown in the common garden, at all stages of development (vegetative, flowering and fruiting) and deposited at the Joseph F. Rock Herbarium (HAW) and the National Tropical Botanical Gardens herbarium (PTBG). A brief description was recorded for each hybrid type.

RESULTS

Reciprocal Crossing and Selfing (F_1 and F_2)

All crosses made to achieve F_1 hybrids had boll abortion rates above 40% with BT having the highest abortion rate of 58% and TB having the lowest rate at 41% (See Table 3.3). To achieve F_1 selfs, HX and TX showed low selfing abortion rates at 12% while BX had a high abortion rate at 75%. The low abortion rates for *G. tomentosum* is surprising as most literature states this species to be a facultative outcrosser, with limited to no seed set in the absence of a pollinator (Fryxell, 1979; Krakos *et al.*, 2010; Pleasants and Wendel, 2010).

Among the F_1 hybrids, progeny resulting from *G. hirsutum* and *G. tomentosum* reciprocal crosses showed the lowest abortion rates to achieve the F_2 generation with 6% of HT selfs aborting (vs 42% for F_1) and 8% of TH selfs aborting (vs 42% for F_1). These rates are remarkably lower than initial F_1 crossing abortion rates. Progeny resulting from crosses between *G. tomentosum* and *G. barbadense* showed the highest abortion rates with 52% of TB selfs aborting (vs 41% for F_1) and 40% of BT selfs aborting (vs 58% for F_1). Again, it should be noted that only one BT plant was used for observations so results may

be skewed. See Table 3.3 for data pertaining to the number of flowers used to achieve each generation.

Among the parent-type F_1 plants, HX showed higher abortion rates to achieve the F_2 generation 27% (vs 12% for F_1). Again, BX showed high, though slightly reduced abortion rates in achieving the F_2 generation at 71% (vs 75% for F_1). No data were collected on TX or T-OP F_2 abortion rates as no plants flowered in the common garden within the six-month period allotted.

TABLE 3.3. TOTAL FLOWERS MANIPULATED AND BOLL ABORTION RATES TO OBTAIN F1 AND F2 GENERATION.

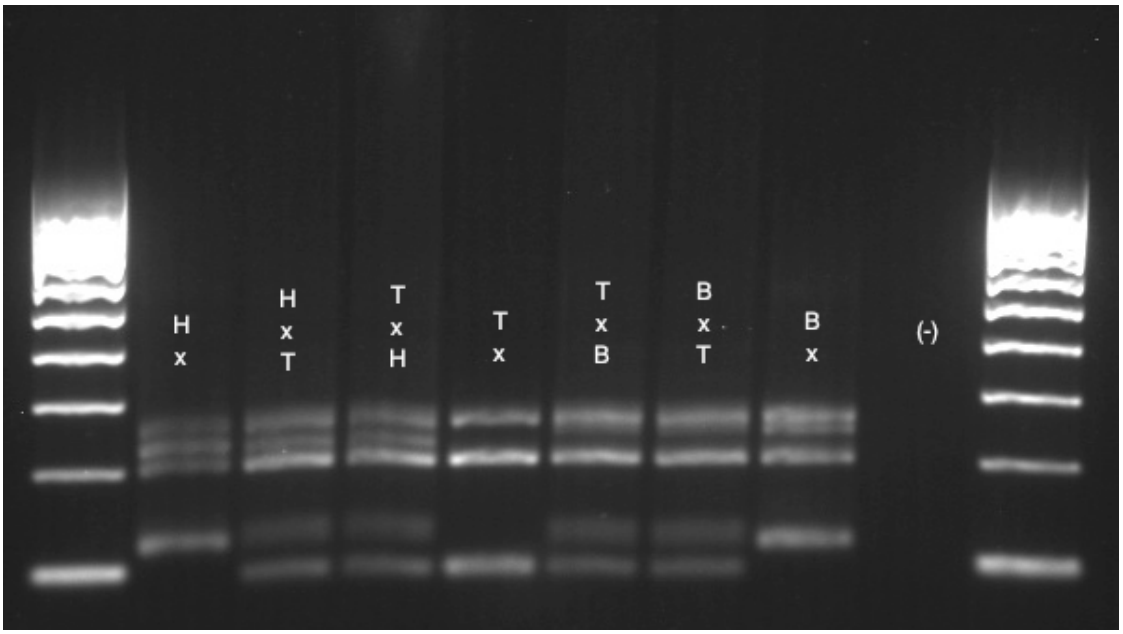
Species	Total flowers manipulated to get F1	Total F1 bolls harvested	Total F1 bolls aborted	% F1 abortion rates	Total flowers manipulated to get F2	Total F2 bolls harvested	Total F2 bolls aborted	% F2 abortion rates
TX	42	37	5	12	--**	--	--	--
T-OP	--*	15	--	--	--**	--	--	--
TH	109	61	48	44	49	45	4	8
HT	55	32	23	42	47	44	3	6
HX	51	45	6	12	48	35	13	27
TB	119	70	49	41	82	39	43	52
BT	95	40	55	58	5	3	2	40
BX	170	42	128	75	92	27	65	71

TX= Gossypium tomentosum (self, ♀ and ♂), T-OP= Open Pollinated G. tomentosum (self, ♀ and ♂), TH = G. tomentosum (♀) x G. hirsutum (♂), HT= G. hirsutum (♀) x G. tomentosum (♂), HX= G. hirsutum (self, ♀ and ♂), TB= G. tomentosum (♀) x G. barbadense (♂), BT= G. barbadense (♀) x G. tomentosum (♂), BX= G. barbadense (self, ♀ and ♂).
* No flowers were manipulated in this treatment. ** TX and T-OP F1 plants did not reach flowering maturity.

Molecular Marker Hybrid Confirmation and Hybridization Rates

Optimized SSR markers were capable of distinguishing between the three parental species and their resulting F_1 hybrid progeny (See Fig 3.1). Unique banding patterns for parental types are as follows: *G. tomentosum* at 96 bp, *G. hirsutum* at 118 and 224 bp, and *G. barbadense* at 118 and 244 bp. F_1 hybrid progeny showed discrete intermediate banding between the two parental types. Progeny arising from manipulated flowers that failed to result in successful hybridization resulted in banding patters identical to the maternal parent and were identified as maternal selfs.

FIGURE 3.1. GEL ELECTROPHORESIS SHOWING BANDING PATTERNS OF PARENT SPECIES AND F_1 HYBRID PROGENY.



HX= G. hirsutum (self, ♀ and ♂), HxT= G. hirsutum (♀) x G. tomentosum (♂), TxH = G. tomentosum (♀) x G. hirsutum (♂), TX= G. tomentosum (self, ♀ and ♂), TxB= G. tomentosum (♀) x G. barbadense (♂), BxT= G. barbadense (♀) x G. tomentosum (♂), BX= G. barbadense (self, ♀ and ♂), (-)= negative control. Using a 100 base pair ladder (far left and far right).

Percent hybrid progeny of germinated seeds ranged from 100% for TH to 0.57% for BT (See Table 3.4). These values are based solely on germinated seedlings; hybrid parentage of non-germinated seeds is unknown. HT showed hybridization rates of 37.46% while TB produced 32.08% hybrid progeny. No maternally selfed individuals tested positive for hybridization. Along with SSR markers, hybridization rates include hybrids determined through morphological observations; these include 75 HT and one BT.

TABLE 3.4. DNA MICROSATELLITE AND MORPHOLOGICALLY CONFIRMED F_1 COTTON HYBRIDS.

Species	Seedlings	Tested with SSR markers	SSR confirmed hybrid	Morphologically confirmed hybrid	Total hybrids	% Hybrid progeny
TX	39	15	0	0	0	0.00
T-OP	59	15	0	0	0	0.00
TH	55	55	55	0	55	100.00
HT	315	108	43	75	118	37.46
HX	161	15	0	0	0	0.00
TB	53	53	17	0	17	32.08
BT	176	101	1*	1*	1	0.57
BX	213	15	0	0	0	0.00

TX= Gossypium tomentosum (self, ♀ and ♂), T-OP= Open Pollinated G. tomentosum (self, ♀ and ♂), TH = G. tomentosum (♀) x G. hirsutum (♂), HT= G. hirsutum (♀) x G. tomentosum (♂), HX= G. hirsutum (self, ♀ and ♂), TB= G. tomentosum (♀) x G. barbadense (♂), BT= G. barbadense (♀) x G. tomentosum (♂), BX= G. barbadense (self, ♀ and ♂). * Of the initial 100 randomly selected BxT seedlings, none had positive hybrid SSR marker results. Of the remaining 76 seedlings, one was determined to be a morphological hybrid and verified using SSR markers.

Germination Rates

The percentage of seeds from each cross and selfing successfully germinated under greenhouse conditions, ranged from 92% for HT seeds to 43% for TB seeds (See Table 3.5). HT had considerably higher germination rates than the maternal HX self in which only 45% of seeds germinated (vs 92% for HT). Both TH (45%) and TB (43%) had lower germination rates than TX (56%). BT had a noticeably lower germination rate of 55% than the maternal self, BX at 72%. Germination rates were similar between TX and T-OP at 56% and 53% respectively.

Two months after sowing, seeds that failed to germinate, were exhumed and examined for decay or dormancy. The majority of these seeds had rotted while a small percentage

had positive TTC staining indicating seed dormancy (See Table 3.5). Exceptions to this were BX and BT seeds where all of those that failed to germinate were decayed upon examination. T-OP had the most dormant seeds, with 17% non-germinated seeds staining positive while TX had 4% stain positive. Only 2% of HX seeds stained positive for dormancy. Crosses with *G. tomentosum* acting as maternal parent had higher dormancy rates than crosses with commercial cotton species as maternal parents; with TH and TB crosses having 2% and 8% seed dormancy, respectively, while HT and BT crosses had dormancy rates of 1% and 0%, respectively.

Days to Germination

In the case of potential hybrid progeny, days to germination data was only included from SSR and morphologically confirmed hybrids. Both commercial cotton species had significantly faster mean germination times than the wild Hawaiian cotton (See Table 3.5). Crosses with *G. tomentosum* as maternal parent (TH and TB) had significantly delayed germination when compared to their commercial paternal parent but did not significantly differ from their maternal parent. The same trend was observed when commercial cotton was the maternal parent (HT, BT), where germination time was significantly quicker than the paternal *G. tomentosum* but not significantly different from the maternal commercial parent. It should be noted that no statistical differences could be detected for measurements involving hybrid BT as data were gathered from only one individual plant. There was no significant difference in mean germination times between TX and T-OP.

Seedling Survival

Of the seeds that germinated, survival rates were high, all above 95%, for the three months that the plants remained in the greenhouse prior to outplanting in the common garden (See Table 3.5). All seedling mortality was a result of damping off and typically happened near the time of germination. Seedling mortality that occurred prior to cotyledon emergence was not recorded.

TABLE 3.5. SEEDS SOWN AND MEAN (±SE) GERMINATION RATES FOR EXPERIMENTAL CROSSES AND PARENTAL TYPE CONTROLS.

Means followed by the same letter are not significantly different ($p > 0.05$) determined by one-way ANOVA.

Species	Fruits sown	Seeds sown	Seeds germinated	Mean days to germination	% Germinated	% Survival**	Non-germinated viable seeds	% Non-germinated viable seeds	Non-germinated non-viable seeds	% Non-germinated non-viable seeds
TX	15	73	41	28.2 ± 1.9 a	56	95	3	4	29	40
T-OP	15	115	61	21.0 ± 1.2 ab	53	97	20	17	34	30
TH	15	123	55	14.3 ± 0.5 b	45	100	3	2	65	53
HT	15	344	315	8.9 ± 0.1 c	92	100	2	1	27	8
HX	15	370	165	9.3 ± 0.2 c	45	98	8	2	197	53
TB	15	131	56	15.7 ± 1.0 a	43	95	10	8	65	50
BT*	15	327	180	8.0	55	98	0	0	147	45
BX	15	307	221	8.7 ± 0.2 d	72	96	0	0	86	28

TX= Gossypium tomentosum (self, ♀ and ♂), T-OP= Open Pollinated G. tomentosum (self, ♀ and ♂), TH = G. tomentosum (♀) x G. hirsutum (♂), HT= G. hirsutum (♀) x G. tomentosum (♂), HX= G. hirsutum (self, ♀ and ♂), TB= G. tomentosum (♀) x G. barbadense (♂), BT= G. barbadense (♀) x G. tomentosum (♂), BX= G. barbadense (self, ♀ and ♂). ** Because only one BT plant was available for observations, data was excluded from statistical analysis. ** Three months in greenhouse conditions.

Greenhouse and Field Plant Height

All hybrids in the greenhouse initially resembled their maternal parents in mean height, with exception of TH. These plants were significantly different from either parent at one month from sowing by being taller than maternal (TX) and shorter than paternal (HX) (See Table 3.6). By the end of month one in the common garden (four months post-sowing), no significant difference existed between HT and TH height means (See Table 3.6). Though not statistically analyzed, mean heights of TB and BT were similar throughout the six-month growing period (See Table 3.7). By the end of month three in the common garden plot, all hybrids showed significantly greater mean height than either of their respective parent species. These trends continued until harvest.

When comparing between TX and T-OP, initially, there was no difference between mean heights, either within or between fields (See Table 3.8). However, by month three in the common garden plot, T-OP (B Field) was significantly taller than TX (H Field), a trend that continued until harvest at six months.

TABLE 3.6. MEAN (±SE) MONTHLY HEIGHT MEASUREMENTS FOR CROSSES BETWEEN *G. tomentosum* AND *G. hirsutum*.

Height one month from sowing includes measurements from all confirmed hybrids and parental selfed seedlings in the greenhouse. Field height measurements are taken from individuals selected for common garden trials. All heights are in cm. Means followed by the same letter are not significantly different ($p > 0.05$) determined by one-way ANOVA.

Species	No.	Height 1 month from sowing	No.	Field Height at 1 month	Field Height at 2 month	Field Height at 3 month	Field Height at 4 month	Field Height at 5 month	Field Height at 6 month
TX F1 (H)	29	3.6 ± 0.2 a	8	9.1 ± 1.0 a	33.2 ± 7 a	47.6 ± 9.9 a	64 ± 13.1 a	75.5 ± 15.8 a	80.0 ± 17.0 a
T-OP F1 (H)	53	4.6 ± 0.1 a	7	12.7 ± 2.1 a	45.9 ± 6.6 a	66.7 ± 10.2 a	86.6 ± 13.0 a	102.6 ± 17.0 a	112.7 ± 20.6 a
TH F1	55	6.9 ± 0.2 b	15	31.6 ± 2.5 b	130 ± 3.0 b	201.2 ± 3.6 b	233.9 ± 5.2 b	243.0 ± 6.4 b	258.4 ± 7.6 b
HT F1	118	10.0 ± 0.2 c	15	32.9 ± 1.5 b	125.7 ± 2.2 bc	193.6 ± 2.8 b	222.5 ± 3.6 b	233.5 ± 4.7 b	247.4 ± 6.0 b
HX F1	161	10.1 ± 0.2 c	15	32.8 ± 1.7 b	110.7 ± 2.1 ac	139.7 ± 2.5 a	141.5 ± 2.4 a	144.5 ± 3.5 a	159.2 ± 5.5 a

TX= Gossypium tomentosum (self, ♀ and ♂), T-OP= Open Pollinated G. tomentosum (self, ♀ and ♂), TH = G. tomentosum (♀) x G. hirsutum (♂), HT= G. hirsutum (♀) x G. tomentosum (♂), HX= G. hirsutum (self, ♀ and ♂). (H) refers to plants grown in the plot containing G. tomentosum / G. hirsutum crosses.

TABLE 3.7. MEAN (±SE) MONTHLY HEIGHT MEASUREMENTS FOR CROSSES BETWEEN *G. tomentosum* AND *G. barbadense*.

Height one month from sowing includes measurements from all confirmed hybrids and parental selfed seedlings in the greenhouse. Field height measurements are taken from individuals selected for common garden trials. All heights are in cm. Means followed by the same letter are not significantly different ($p > 0.05$) determined by one-way ANOVA.

Species	No.	Height 1 month from sowing	No.	Field Height at 1 month	Field Height at 2 month	Field Height at 3 month	Field Height at 4 month	Field Height at 5 month	Field Height at 6 month
TX F1 (B)	29	3.6 ± 0.2 a	7	10.2 ± 0.8 a	46.4 ± 6.0 a	79.0 ± 8.5 a	103.1 ± 9.0 a	124.1 ± 10.0 a	132.9 ± 11.8 a
T-OP F1 (B)	53	4.6 ± 0.1 a	8	12.0 ± 1.6 a	45.5 ± 5.0 a	86.4 ± 6.1 a	116.3 ± 6.8 a	140.6 ± 7.1 a	159.9 ± 8.5 a
TB F1	17	6.9 ± 0.3 a	15	26.3 ± 1.0 b	113.2 ± 2.0 b	187.9 ± 2.6 b	243.7 ± 5.1 b	301.7 ± 6.1 b	352.3 ± 6.7 b
BT F1*	1	13.5	1	27.0	112.0	202.0	270.0	313.0	377.0
BX F1	212	11.1 ± 0.1 b	15	26.6 ± 0.9 b	106.8 ± 2.9 b	152.7 ± 3.4 c	183.7 ± 4.0 c	223.8 ± 4.7 c	258.1 ± 4.8 c

TX= Gossypium tomentosum (self, ♀ and ♂), T-OP= Open Pollinated G. tomentosum (self, ♀ and ♂), TB= G. tomentosum (♀) x G. barbadense (♂), BT= G. barbadense (♀) x G. tomentosum (♂), BX= G. barbadense (self, ♀ and ♂). (B) refers to plants grown in the plot containing G. tomentosum / G. barbadense crosses. * Because only one BT F1 plant was available for observations, data was excluded from statistical analysis.

TABLE 3.8. MEAN (±SE) MONTHLY HEIGHT MEASUREMENTS FOR ALL TX AND T-OP PLANTS.

Height measurements are taken from individuals selected for common garden trials. All heights are in cm. Means followed by the same letter are not significantly different ($p > 0.05$) determined by one-way ANOVA.

Species	No.	Field Height at 1 month	Field Height at 2 month	Field Height at 3 month	Field Height at 4 month	Field Height at 5 month	Field Height at 6 month
TX F1 (H)	8	9.1 ± 1.0 a	33.2 ± 7.0 a	47.6 ± 9.9 a	64.0 ± 13.1 a	75.5 ± 15.8 a	80.0 ± 17.0 a
T-OP F1 (H)	7	12.7 ± 2.1 a	45.9 ± 6.6 a	66.7 ± 10.2 ab	86.6 ± 13.0 ab	102.6 ± 17.0 ab	112.7 ± 20.6 ab
TX F1 (B)	7	10.2 ± 0.8 a	46.4 ± 6.0 a	79.0 ± 8.5 ab	86.6 ± 14.0 ab	124.1 ± 9.6 ab	132.9 ± 11.8 ab
T-OP F1 (B)	8	12.0 ± 1.6 a	45.5 ± 5.0 a	86.4 ± 6.1 b	86.6 ± 15.0 b	140.6 ± 7.1 b	159.9 ± 8.5 b

TX= Gossypium tomentosum (self, ♀ and ♂), T-OP= Open Pollinated G. tomentosum (self, ♀ and ♂). (H) refers to plants grown in the common garden containing G. tomentosum / G. hirsutum crosses while (B) refers to plants grown in the common garden containing G. tomentosum / G. barbadense crosses.

Days to First Flower

Neither TX nor T-OP plants, in either field, reached flowering maturity in the six months allotted for the common garden trial. Accordingly, subsequent analysis involving F_1 flowering data lack F_1 TX and T-OP data. Both TH (57.4 days) and HT (55.3 days) took significantly longer to flower than HX (46.9 days) (See Table 3.9). Similarly, TB (81 days) took significantly longer to flower than BX (72 days) (See Table 3.10); the one BT plant produced took 85 days. It should be noted that all hybrid types, especially TH and HT, flowered continuously and prolifically after reaching flowering maturity up until harvesting for biomass. Beginning in month five, BT and TB flowering, though continuous, became less vigorous and more sporadic. By the end of month three, *G. hirsutum* ceased flowering, only to again flower sporadically beginning in month five. *G. barbadense*, once reaching flowering maturity, flowered throughout the six-month trial.

TABLE 3.9. MEAN (±SE) DAYS TO FIRST FLOWER AND PERCENT POSITIVE POLLEN STAIN FOR CROSSES BETWEEN *G. tomentosum* AND *G. hirsutum* AND THEIR PARENTAL PLANTS.

Means followed by the same letter are not significantly different ($p > 0.05$) determined by one-way ANOVA.

Species	No.	Days to flower	No.	% positive pollen stain
TX F1	8	*	--	--
T-OP F1	7	*	--	--
TH F1	15	57.4 ± 0.06 a	58	72.7 ± 2.31 a
HT F1	15	55.3 ± 1.11 a	58	71.0 ± 2.72 a
HX F1	15	46.9 ± 0.96 b	4	85.3 ± 6.86 a
TX P	--	--	31	61.8 ± 4.48 a
HX P	--	--	24	83.2 ± 1.56 a

TX= Gossypium tomentosum (self, ♀ and ♂), T-OP= Open Pollinated G. tomentosum (self, ♀ and ♂), TH = G. tomentosum (♀) x G. hirsutum (♂), HT= G. hirsutum (♀) x G. tomentosum (♂), HX= G. hirsutum (self, ♀ and ♂). P indicates that data was collected from parental generation plants. * TX F1 and T-OP F1 did not flower in the six months allotted.

TABLE 3.10. MEAN (±SE) DAYS TO FIRST FLOWER AND PERCENT POSITIVE POLLEN STAIN FOR CROSSES BETWEEN *G. tomentosum* AND *G. barbadense* AND THEIR PARENTAL PLANTS.

Means followed by the same letter are not significantly different ($p > 0.05$) determined by one-way ANOVA.

Species	No.	Days to flower	No.	% positive pollen stain
TX F1	7	*	--	--
T-OP F1	8	*	--	--
TB F1	15	81.0 ± 0.86 a	49	63.7 ± 1.58 a
BT F1**	1	85.0	6	67.7 ± 3.26
BX F1	15	72.0 ± 1.16 b	59	78.2 ± 1.89 b
TX P	--	--	31	61.8 ± 4.48 a
BX P	--	--	58	78.7 ± 1.41 b

TX= Gossypium tomentosum (self, ♀ and ♂), T-OP= Open Pollinated G. tomentosum (self, ♀ and ♂), TB= G. tomentosum (♀) x G. barbadense (♂), BT= G. barbadense (♀) x G. tomentosum (♂), BX= G. barbadense (self, ♀ and ♂). P indicates that data was collected from parental generation plants. * TX F1 and T-OP F1 did not flower in the six months allotted. ** Because only one BT F1 plant was available for observations, those data were excluded from statistical analysis.

Days to Boll Maturity

Bolls of HT F_1 reached maturity significantly earlier than maternal HX F_1 selfs while TH F_1 bolls matured significantly later than maternal TX F_1 selfs (See Table 3.11). Mean boll maturation rates of TH F_2 and HT F_2 were not statistically different from each other or from TX F_1 or HX F_2 rates. Both, however, matured earlier than HX F_1, which matured significantly later than any other cross or self in the H field.

Bolls of TB F_1 matured significantly later than those of TX F_1 while mean boll maturation time of BT F_1 did not differ significantly from either parent (See Table 3.12). Bolls of TB F_2 matured significantly later than those of TX F_1. Bolls of BX F_2 matured significantly slower than those of BX F_1. Bolls of BT F_2 matured later than that of either parental type.

Boll Weight

Mean boll weights of TH F_1 and HT F_1 did not significantly differ from that of their maternal F_1 parents (See Table 3.11). Mean boll weight of TH F_2 and HT F_2 were statistically similar to each other and significantly greater than TX F_1 while significantly less than HX F_1 and HX F_2.

Mean boll weight of TB F_1 and BT F_1 did not significantly differ from that of their maternal F_1 parents (See Table 3.12). Boll weight of TB F_2 were significantly less than BX F_1 and BX F_2 and similar to TX F_1. Mean boll weight of BT F_2 is greater that of its paternal parent (TX F_1) while less than that of the maternal parent, either BX F_1 or F_2.

Seeds per Boll

Mean seed counts of TH F_1 and HT F_1 did not significantly differ from that of their maternal F_1 parents (See Table 3.11). Mean seed counts of TH F_2 and HT F_2 were statistically similar to one another and significantly greater than TX F_1 but less than that of HX F_1 and HX F_2. HX F_2 had significantly higher mean seed counts than HX F_1.

Mean seed counts of TB F_1 and BT F_1 did not significantly differ from their maternal F_1 parents (See Table 3.12). Mean seed counts for TB F_2 were statistically similar to those of TX F_1 or TB F_1. Though statistical analysis was not carried out on BT F_2, it's mean seed count, 5.3 seeds per boll, was much less than that of BX F_1 (20) or BX F_2 (20.9) and similar to TX F_1 (5.5). There was no significant difference between mean seed counts of BX F_1 and BX F_2.

TABLE 3.11. MEAN (± SE) DAYS TO BOLL MATURITY, BOLL WEIGHTS AND SEED COUNTS FOR F_1 AND F_2 GENERATIONS FOR CROSSES BETWEEN *G. tomentosum* AND *G. hirsutum* AND PARENTAL TYPE PLANTS.

Means followed by the same letter are not significantly different ($p > 0.05$) determined by one-way ANOVA.

Species	No.	Days to Boll Maturity	No.	Boll Weight (g)	Seed Count
TX F1	37	60.4 ± 1.1 ab	37	0.39 ± 0.04 a	5.5 ± 0.6 a
T-OP F1*	--	--	15	0.62 ± 0.05 a	8.3 ± 0.7 ac
TH F1	54	64.9 ± 1.4 c	61	0.67 ± 0.05 a	7.3 ± 0.4 a
HT F1	31	55.3 ± 1.1 a	35	4.22 ± 0.27 bc	21.9 ± 1.2 b
HX F1	16	74.3 ± 1.5 d	47	5.01 ± 0.28 bd	22.3 ± 1.1 b
TX F2**	--	--	--	--	--
T-OP F2**	--	--	--	--	--
TH F2	45	63.9 ± 0.7 abc	45	2.36 ± 0.15 c	13.9 ± 1.0 c
HT F2	44	64.8 ± 0.8 bc	44	2.30 ± 0.14 c	13.5 ± 0.8 c
HX F2	38	63.2 ± 1.0 abc	38	7.73 ± 0.23 d	33.4 ± 0.8 d

TX= Gossypium tomentosum (self, ♀ and ♂), T-OP= Open Pollinated G. tomentosum (self, ♀ and ♂), TH = G. tomentosum (♀) x G. hirsutum (♂), HT= G. hirsutum (♀) x G. tomentosum (♂), HX= G. hirsutum (self, ♀ and ♂). *Days to boll maturity were not measured for T-OP F1 flowers. ** TX of T-OP did not reach flowering maturity in the six months allotted; no F2 bolls were formed.

TABLE 3.12. MEAN (± SE) DAYS TO BOLL MATURITY, BOLL WEIGHTS AND SEED COUNTS FOR F_1 AND F_2 GENERATIONS FOR CROSSES BETWEEN *G. tomentosum* AND *G. barbadense* AND PARENTAL TYPE PLANTS.

Means followed by the same letter are not significantly different ($p > 0.05$) determined by one-way ANOVA.

Species	No.	Days to Boll Maturity	No.	Boll Weight (g)	Seed Count
TX F1	37	60.4 ± 1.1 a	37	0.39 ± 0.04 a	5.5 ± 0.6 a
T-OP F1*	--	--	15	0.63 ± 0.05 a	8.3 ± 0.7 a
TB F1	69	64.4 ± 1.0 b	69	0.58 ± 0.04 a	6.3 ± 0.4 a
BT F1	39	62.4 ± 1.1 ab	39	6.05 ± 0.27 b	22.5 ± 0.6 b
BX F1	36	63.6 ± 1.0 ab	42	5.34 ± 0.26 b	20.0 ± 0.7 b
TX F2**	--	--	--	--	--
T-OP F2**	--	--	--	--	--
TB F2	39	72.0 ± 0.6 c	39	1.48 ± 0.09 a	8.3 ± 0.6 a
BT F2***	3	75.7 ± 0.9	3	0.97 ± 0.19	5.3 ± 1.5
BX F2	28	71.3 ± 1.3 c	28	4.35 ± 0.21 b	20.9 ± 1.0 b

TX= Gossypium tomentosum (self, ♀ and ♂), T-OP= Open Pollinated G. tomentosum (self, ♀ and ♂), TB= G. tomentosum (♀) x G. barbadense (♂), BT= G. barbadense (♀) x G. tomentosum (♂), BX= G. barbadense (self, ♀ and ♂). P indicates that data was collected from parental generation plants. *Days to boll maturity were not measured for T-OP F1 flowers. ** Because they did not reach flowering maturity in the six months allotted, no F2 flowers were manipulated for TX of T-OP. *** Because only one BT F1 plant was available for observations, those data were excluded from statistical analysis.

Pollen Stainability

There were no significant differences in mean pollen stainability between F_1 TH, HT HX and parent generation TX and HX plants (See Table 3.9). TB showed significantly lower mean pollen stainability when compared to both BX F_1 and parental generation B plants (See Table 3.10). Mean pollen stainability for TB was statistically similar to parental generation TX. BT had greater pollen stainability than TX parent generation and reduced stainability when compared to BX parent and F_1 generations. No statistical differences were observed in pollen stainability between parental and F_1 generations of either commercial cotton species.

Stem Diameter and Biomass

Both F_1 TH and HT plants had significantly greater mean stem diameters than both parent species (F_1 TX, T-OP and HX) and were statistically similar to one another (See Table 3.13). TB stem diameter was not statistically different from BX but was significantly larger than both TX and T-OP (See Table 3.14). BT stem diameter was larger than both parental means. There was no statistical difference observed between TX and T-OP stem diameters either within or between fields (See Table 3.15).

Biomass measurements (both wet and dry) followed the same pattern, with both HT and TH having significantly higher mean biomass than both parental species (See Table 3.13) while TB had biomass significantly greater than TX and T-OP but not statistically different than BX (See Table 3.14). BT biomass, both wet and dry, was greater than both parental means. There was no statistical difference between TX and T-OP mean biomass measurements either within or between fields (See Table 3.15).

TABLE 3.13. MEAN (±SE) STEM DIAMETER AND BIOMASS MEASUREMENTS OF CROSSES BETWEEN *G. tomentosum* AND *G. hirsutum* AND PARENTAL TYPE PLANTS.

All measurements taken after six months in the common garden. Means followed by the same letter are not significantly different ($p > 0.05$) determined by one-way ANOVA.

Species	No.	Stem diameter (cm)	Wet biomass (kg)	Dry biomass (kg)
TX F1 (H)	8	1.10 ± 0.18 a	0.14 ± 0.05 a	0.05 ± 0.02 a
T-OP F1 (H)	7	1.44 ± 0.21 a	0.28 ± 0.14 a	0.09 ± 0.04 a
TH F1	15	3.43 ± 0.18 b	3.40 ± 0.60 b	1.25 ± 0.18 b
HT F1	15	3.31 ± 0.17 b	3.35 ± 0.53 b	1.20 ± 0.18 b
HX F1	15	1.94 ± 0.05 a	0.74 ± 0.07 a	0.25 ± 0.02 a

TX= Gossypium tomentosum (self, ♀ and ♂), T-OP= Open Pollinated G. tomentosum (self, ♀ and ♂), TH = G. tomentosum (♀) x G. hirsutum (♂), HT= G. hirsutum (♀) x G. tomentosum (♂), HX= G. hirsutum (self, ♀ and ♂). (H) refers to plants grown in the plot containing G. tomentosum / G. hirsutum crosses.

TABLE 3.14. MEAN (±SE) STEM DIAMETER AND BIOMASS MEASUREMENTS OF CROSSES BETWEEN *G. tomentosum* AND *G. barbadense* AND PARENTAL TYPE PLANTS.

All measurements taken after six months in the common garden. Means followed by the same letter are not significantly different ($p > 0.05$) determined by one-way ANOVA.

Species	No.	Stem diameter (cm)	Wet biomass (kg)	Dry biomass (kg)
TX F1(B)	7	1.29 ± 0.08 a	0.20 ± 0.04 a	0.06 ± 0.01 a
T-OP F1 (B)	8	1.70 ± 0.15 a	0.40 ± 0.09 a	0.13 ± 0.02 a
TB F1	15	5.04 ± 0.22 b	6.48 ± 0.56 b	2.42 ± 0.20 b
BT F1*	1	4.92	7.46	2.78
BX F1	15	3.96 ± 0.21 b	3.48 ± 0.37 b	1.28 ± 0.12 b

TX= Gossypium tomentosum (self, ♀ and ♂), T-OP= Open Pollinated G. tomentosum (self, ♀ and ♂), TB= G. tomentosum (♀) x G. barbadense (♂), BT= G. barbadense (♀) x G. tomentosum (♂), BX= G. barbadense (self, ♀ and ♂). (B) refers to plants grown in the plot containing G. tomentosum / G. barbadense crosses. * Because only one BT F1 plant was available for observations, those data were excluded from statistical analysis.

TABLE 3.15. MEAN (±SE) STEM DIAMETER AND BIOMASS MEASUREMENTS OF ALL TX F_1 AND T-OP F_1 PLANTS.

All measurements taken after six months in the common garden. Means followed by the same letter are not significantly different ($p > 0.05$) determined by one-way ANOVA.

Species	No.	Stem diameter (cm)	Wet biomass (kg)	Dry biomass (kg)
TX F1 (H)	8	1.10 ± 0.18 a	0.14 ± 0.05 a	0.05 ± 0.02 a
T-OP F1 (H)	7	1.44 ± 0.21 a	0.28 ± 0.14 a	0.09 ± 0.04 a
TX F1(B)	7	1.29 ± 0.08 a	0.20 ± 0.04 a	0.06 ± 0.01 a
T-OP F1 (B)	8	1.70 ± 0.15 a	0.40 ± 0.09 a	0.13 ± 0.02 a

TX= Gossypium tomentosum (self, ♀ and ♂), T-OP= Open Pollinated G. tomentosum (self, ♀ and ♂). (H) refers to plants grown in the common garden containing G. tomentosum / G. hirsutum crosses while (B) refers to plants grown in the common garden containing G. tomentosum / G. barbadense crosses.

TX versus T-OP

In all fitness measurements, there was no statistical difference between TX and T-OP, with exception to field heights between TX H field and T-OP B field starting in month three of the common garden. This was likely due to shading from the more aggressive, sprawling nature of the TH and HT hybrids. No apparent inbreeding depression was observed in this trial for *G. tomentosum.*

Both TX and T-OP bolls were gathered from the same small, isolated population of six individuals occurring outside of the species' native range on the UH campus in Manoa. If T-OP outcrossed, chances of pollen flow to an outside population is relatively low, with flowers likely selfing or mating with individuals from the same population, which may itself already be a product of inbreeding.

DISCUSSION

The goals of this study were: 1) to determine the ease at which interspecific gene flow could occur between endemic Hawaiian and commercial *Gossypium* species, through manipulated hand crosses and 2) what impact gene flow would have on F_1 hybrid fitness and fecundity and 3) how might gene flow affect the ability for *G. tomentosum* to persist. The greater the ease of artificial, manipulated hybridizations, the greater the risk of spontaneous natural hybridization (Armstrong *et al.*, 2005; Anderson and de Vicente, 2010). If interspecific F_1 hybrids are formed but are ill-fit and sterile their impact will largely be limited to a reduction of conspecific seed production with negligible competition for limited resources between hybrid and conspecific offspring (Ellstrand and Hoffman, 1990). If, however, interspecific F_1 progeny are fit and fertile, increased competition, weediness, and risk of introgression and genetic assimilation greatly increase the chance for adverse impacts on native species (Levin *et al.*, 1996; Haygood *et al.*, 2003; Jenczewski *et al.*, 2003; Lu and Yang, 2009).

Hybridization Rates

To varying degrees, both Pima and Upland cotton were readily capable of forming hybrids through reciprocal crosses with Hawaiian cotton. During initial reciprocal crosses, F_1 abortion rates ranged between 41% and 58%, which are not unordinary for cotton (Hawkins, 1934). The low abortion rates of selfed TX (12%) are surprising as literature cites this species as rarely producing bolls in the absence of pollinators. In contrast, the high abortion rates for BX F_1 selfs (75%) is higher than expected for this primarily selfing species and may be a result of interference during the flower bagging

process as this species produces the largest flowers of the three species studied. Abortion rates of F_1 and particularly F_2 crosses largely paralleled phylogenetic relatedness, with crosses between the sister species *G. tomentosum* and *G. hirsutum* resulting in lower abortion rates than crosses between the more distantly related *G. barbadense* (with the exception of F_1 TB which had the lowest abortion rate at 41%). Low abortion rates for F_2 segregates of TH and HT bolls (8% and 6%, respectively) could be a result of greater chromosome compatibility during meiosis of when compared to F_2 TB and BT, which had high abortion rates (52% and 40%, respectively).

Hybridization rates followed the same phylogenetic relationship trend. Crosses with *G. hirsutum*, TH (100%) and HT (37.46%), had substantially higher hybridization rates than those with *G. barbadense*, TB (32.08%) and BT (0.57%). Hybridization rates were significantly higher when *G. tomentosum* acted as maternal parent in crosses involving both *G. hirsutum* and *G. barbadense*. This is likely a result of the extended style of Hawaiian cotton, which limits selfing, both autogamously and accidentally during hand pollinations. Both commercial cotton species have relatively short styles, especially *G. barbadense,* with the stigma in close proximity to the anthers (or in some cases adjacent). Though flowers were bagged before crosses, and stigmas were inspected prior to crossing for absence of pollen, self-pollination likely occurred either accidentally during crossing or post-crossing, once the bridal veil was re-applied. Regardless of how self-pollination occurred for plants that were intentionally hand crossed, it was apparent that *G. tomentosum,* under ideal circumstances, is more prone to accept foreign pollen than either

commercial species tested. It was also apparent that *G. tomentosum* hybridizes with greater ease with *G. hirsutum* than it does with *G. barbadense.*

Fitness and Fecundity

With tone exception, hybrid crosses had somewhat lower germination rates than their parental types. This especially true BT in which only 55% of seeds germinated within two months, versus 72% for BX and 56% for TX. Seeds that failed to germinate could potentially be of hybrid origins and suffering from outbreeding depression, be ill-fit for the germination conditions set or have increased endogenous and or exogenous dormancy. Crosses that had *G. tomentosum* as maternal parent had higher seed dormancy rates than crosses that had commercial species as maternal parents. For example, TB had 8% dormancy, higher than TX (4%), BX (0%) and BT (0%). Higher dormancy rates could lead to increased hybrid seed bank accumulation, increasing chances of hybrid persistence (Jenczewski *et al.*, 2003). The lone exception was for HT crosses, which had substantially higher germination rates than either parent (92% vs 45% for HX and 56% for TX).

Although there were differences in germination rates, there was no statistical difference in mean days to germination between hybrids and their maternal parents suggesting another cause for low germination rates. Of the seeds that failed to germinate, the majority were decayed upon examination two months after sowing. The delayed application of Subdue Maxx fungicide appeared to have adverse impacts on overall seedling germination. In an initial germination pre-trial of crosses between *G.*

tomentosum and *G. hirsutum*, all crosses had substantially higher germination rates, with exception to TX, which had lower rates (Lehman, unpublished data). All conditions during the preliminary trial were identical to the current trial results reported in this paper, aside from an application of fungicidal drench at first signs of seedling damping off. Repeated germination trials should be done to establish more accurate germination rates.

Should a hybrid seedling become established, it is likely to outcompete both of its parents for available resources (soil, sunlight, water nutrients etc.), especially *G. tomentosum* which had substantially lower fitness than all hybrid types. Assuming that cotton seeds germinate in close proximity to their maternal parent, hybrids will be in direct competition for resources with conspecific seedlings and established plants. For example, field heights of hybrids were statistically similar to their maternal parent through month two. However, all hybrids had statistically greater height than either parent type. By month three. In addition, all hybrids had significantly greater biomass (wet and dry) and basal stem diameter than *G. tomentosum*, implying greater vigor and vitality. TB was the only cross to not statistically differ from a parent type (BX) for either biomass or stem diameter. An increase in vegetative vigor leads to an increase in competitive advantage (Jenczewski *et al.*, 2003).

When a hybrid seedling becomes established it would likely outcompete *G. tomentosum* for population establishment, maintenance and growth as well.. Initial reciprocal crosses produced bolls with statistically similar mean boll weights and seed counts as their

maternal parents. Though all hybrids took longer to flower than their commercial parent counterpart, all flowered significantly quicker than Hawaiian cotton, which failed to reach flowering maturity in the six-month field trial. Though not quantified, F_1 hybrids produced substantially more flowers than either commercial species, especially the crosses with *G. hirsutum* as a parent. Both TH F_2 and HT F_2 had statistically greater mean boll weight and mean seed count than TX F_1 (the only data available for comparison). For crosses with *G. barbadense* as a parent mean boll weights and seed counts did not statistically differ from *G. tomentosum* and were less than that of *G. barbadense* (BT F_2 boll weight was greater than TX F_1). Hybrid pollen stainability was either statistically similar to or in the case of BT greater than that of parental generation *G. tomentosum.* Given the ability for hybrids to outcompete their parent-type offspring for resources, as shown with height and biomass, their ability to match or outcompete their parent-type offspring reproductively implies a competitive advantage in population persistence and growth and an increased risk of genetic assimilation for *G. tomentosum* (Haygood et al., 2003).

Conservation Implications

Results show that there are no strong hybridization barriers between *Gossypium tomentosum* and either commercial cotton species examined in this study. However, *G. tomentosum* is most capable of hybridizing with *G. hirsutum* and reciprocal crosses between the two species create the most fecund F_1 hybrids. Today, over 90% of the world's cotton production depends on varieties of *G. hirsutum.* These data are especially poignant as cotton is the third largest GE crop by acreage worldwide, behind only corn

and soybean. As the seed industry continues to grow in the state of Hawaii there may be increased pressure to grow GE cotton in the state. To mitigate the potential for interspecific gene flow, transgenic cotton production is prohibited in Hawaii with the exception of EPA permitted test plots or breeding nurseries (EPA, 2005). Seed companies regularly grow permitted transgenic cotton (herbicide resistant and Bt) in Hawai‘i; the last issued test permit (2008-2009) allowed for production on 2,725 acres (ISB, 2012). Permitted test plots cannot be planted within 3 miles of natural *G. tomentosum* populations and must be surrounded by 24 border rows of a "suitable pollinator trap" (EPA, 2005). These mitigation strategies are intended to limit potential gene flow between GE cotton and *G. tomentosum*; no regulations exist regulating conventional *G. hirsutum*, be it an agricultural crop or a landscape oddity.

Though less prone to produce hybrid offspring, reciprocal crosses between *G. tomentosum* and *G. barbadense* produce highly fit offspring capable of outcompeting Hawaiian cotton. *Gossypium barbadense* is a relatively common landscape ornamental in the state with no regulatory oversight making gene flow management difficult. Foraging distances by honeybees has been documented as being up to 12 km (Seeley, 1985). Thus, it is recommended that plants (naturalized or otherwise) of both *G. barbadense* and *G. hirsutum* be eradicated when they occur within this range to *G. tomentosum* populations. In addition, horticultural sale of these species should be prohibited to mitigate hybridization risk.

FUTURE RESEARCH

To further quantify risk under field conditions, additional field-based pollinator and gene flow studies should be implemented in Hawai‘i, similar to those that have been undertaken in other parts of the world (Umbeck *et al.*, 1991; Llewellyn and Fitt, 1996; Zhang *et al.*, 2005; Heuberger *et al.*, 2010). Such research would establish safe planting distances directly relevant to the unique conditions of Hawai‘i. These measures would help prevent the escape of GE and non-GE Upland and Pima cotton genes into Hawaiian cotton populations, as well as maintaining the genetic purity of commercial cotton seed lines grown in Hawai‘i. In addition, field studies should address the ability for conspecific and hybrid seeds to establish, compete and persist in a “natural” field setting without human aid or greenhouse protection. These studies would better address how sporadic hybrids may compete with Hawaiian cotton for resources. As hybrid decline often begins with the F_2 generation (Edmands, 1999), analysis of future generation fitness and fecundity would be beneficial in assessing the long term risk hybrids may pose to Hawai’i’s ecosystems and *G. tomentosum*.

CHAPTER 4. IN CONCLUSION

INTRODUCTION

Ecological barriers often limit hybridization and gene flow, the incorporation of genes from one population into the gene pool of another. Human disturbances, such as agriculture and urban sprawl often reduce or remove these barriers (Levin *et al.*, 1996; Vila *et al.*, 2000). Landscape ornamentals, agricultural and horticultural crops, and naturalized alien species are all potential sources of gene flow (Levin *et al.*, 1996; Vila *et al.*, 2000). Over the past two decades, largely spurred by the advent of genetic engineering (GE or transgenic) of crops, crop-to-wild plant gene flow has received significant attention (Klinger *et al.*, 1992; Raybould and Gray, 1993; Klinger and Ellstrand, 1994; Arriola and Ellstrand, 1996; Ellstrand *et al.*, 1999; Rieseberg *et al.*, 1999; Burke *et al.*, 2002; Arnaud *et al.*, 2003; Ellstrand, 2003a; Jenczewski *et al.*, 2003; Anderson and de Vicente, 2010; Vacher *et al.*, 2011).

One species which is at risk of crop-to-wild gene flow is Hawaiian cotton or maʻo (*Gossypium tomentosum*) (Fryxell, 1979; Ellstrand *et al.*, 1999; Daehler and Carino, 2001; Münster and Wieczorek, 2007; Anderson and de Vicente, 2010; Pleasants and Wendel, 2010). This long lived perennial species is endemic to arid, lowland and coastal sites between sea level and 120 m on all the main Hawaiian Islands, except the Islands of Hawaiʻi and Kauaʻi (Wagner *et al.*, 1999) though it has recently been reintroduced to Kauaʻi (Krakos *et al.*, 2010). Two additional, agriculturally important cotton species closely related to Hawaiian cotton, *G. barbadense,* (Pima cotton) and *G. hirsutum*

(Upland cotton) have been introduced to the Hawaiian Islands and are now sporadically naturalized; Pima cotton on all the main islands except Kahoʻolawe, and Upland cotton on Oʻahu (Wagner *et al.*, 1999) and East Maui (Wagner *et al.*, 2012).

MATERIALS AND METHODS

History of Spontaneous Hybridization in Hawaii

Historically, spontaneous hybrids have been documented between *G. barbadense* and *G. tomentosum* on the Waiʻanae coast of Oahu at Nānākuli (Hutchinson, 1959; Stephens, 1964). Field surveys were conducted to assess whether these hybrid populations still remain and if so to evaluate their spread and to measure the gene flow that had potentially taken place between the two species.

F_1 Hybrid Fitness and Fecundity

To determine how readily *G. tomentosum* hybridizes with *G. hirsutum* and *G. barbadense,* reciprocal crosses were carried out in a controlled setting. Microsatellite DNA markers were developed for distinguishing between the three study species and their hybrid progeny. To assess the impacts of gene flow on plant fitness and fecundity, F_1 hybrids were germinated in a greenhouse and then grown in a common garden plot along with parental species.

Species Distribution Maps

In order to properly assess where gene flow is likely to occur it must be known where native populations exist. Because access to sites was often difficult or impossible to

obtain, species distribution models for *Gossypium tomentosum*, developed by Price *et al.* (2012) where used to show the species potential native range and highlight areas with a high risk for gene flow.

Distribution maps were constructed using ArcGis® ArcMap™ 10 software. Maps consist of the *G. tomentosum* distribution layer, as defined by Price *et al.* (2012), overlain atop a Hawai'i land use layer that includes agricultural, urban and conservation zoned land downloaded from the Hawai'i State GIS Program. A three-mile buffer, based on EPA regulations for growing transgenic cotton in the sate of Hawaii, encompasses and extends from the boundaries of the species distribution layer. GPS survey points of current Oahu and Maui *G. tomentosum* populations were added to the map to assess the validity of the distribution model (Maui survey points courtesy of Forest and Kim Starr of Starr Environmental). Extant Oahu populations were located using herbarium records at the B. P. Bishop Museum (BISH) and the Joseph F. Rock Herbarium at the University of Hawaii at Manoa, (HAW), as well as by field surveys. On Kauai, survey points represent *G. tomentosum* re-introduction efforts by the National Tropical Botanical Garden (NTBG) and Lida and David Burney (NTBG) and are not necessarily representative of where natural populations would have historically occurred. GPS data at each population located (Oahu and Kauai) were taken using a Garmin 60CSx.

RESULTS

Spontaneous Hybridization

Field surveys at and near Nānākuli failed to locate any extant hybrid individuals as described by Stephens (1964). DNA analysis of one herbarium voucher collected near Stephens' 1964 population did test positive for hybridization indicating that at least one hybrid occurred at a nearby site until as late as 1980. No extant hybrids between Pima and Hawaiian cotton in populations of either species indicates that no recent gene flow has taken place and that hybrid plants that did occur in the past likely have not persisted and spread. The landscape has changed significantly since Stephens' described hybrid cotton at Nānākuli; the lack of evidence of current hybridization may be a product of human development rather than the ability of these cotton hybrids to persist and spread.

Impacts of Gene Flow: Ease of Hybridization and Fitness of F_1 Hybrids

Results show that there are no strong hybridization barriers between *Gossypium tomentosum* and either commercial cotton species. In hand pollination trials, *G. tomentosum* is more prone to accept foreign pollen than either commercial species, with higher rates of hybridization. The same hand pollination trial showed that *G. tomentosum* hybridizes more freely with *G. hirsutum* or Upland cotton than with *G. barbadense* (Pima cotton). All F_1 hybrids show significantly greater fitness (plant height biomass etc.) and fecundity (flower production, seed production and weight etc.) than conspecific F_1 *G. tomentosum* suggesting that natural F_1 hybrids could outcompete native cotton populations for habitat and resources should they become established. Because all three

species can readily hybridize amongst each other, isolation between species is critical to conserve the genetic integrity of Hawaiian cotton and to ensure its long-term survival.

Species Distribution and Risk Maps

Much of the habit suitable for *G. tomentosum* is now urban (especially Oahu) or has been cleared for various agricultural endeavors (See Figs. 4.1-4.4). Though likely greatly reduced in abundance and distribution, *G. tomentosum* populations remain and all remnant populations surveyed on Oahu and Maui fall within the prescribed species distribution justifying the model's validity.

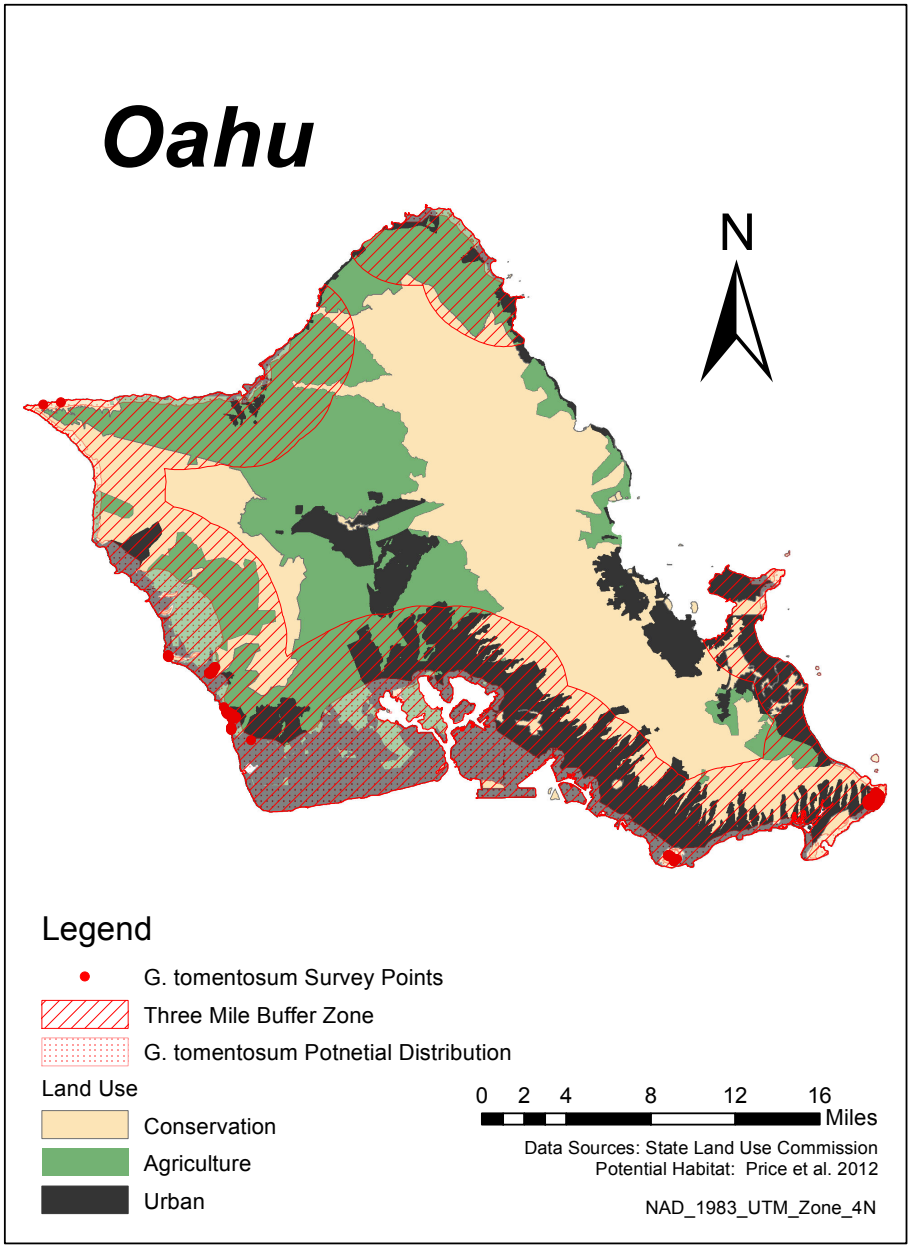
Areas that fall within the three-mile buffer zone are at the highest risk of gene flow. These include both agriculturally zoned lands, which are most likely to be sites for permitted transgenic cotton production as well as urban land where commercial cotton species, especially *G. barbadense*, are commonly planted as landscape ornamentals (Staples and Herbst, 2005). Areas outside of the three-mile (4.8 km)buffer are still at considerable risk of gene flow; carpenter bees are known to forage up to 6 km (3.7 miles) (Pasquet *et al.*, 2008) and honey bees up to 12 kilometers (7.5 miles) (Seeley, 1985), both of which are known pollinators of both Hawaiian cotton and either commercial cotton species. Aside from the risk of gene flow, parties who intend to plant transgenic cotton in Hawaii cannot legally do so within three miles of *G. tomentosum* (EPA, 2005); thorough botanical surveys must be conducted prior to planting transgenic cotton in the state (Hank Oppenheimer pers. com., 2011).

RECOMMENDATIONS

The only way to fully ensure that no gene flow occurs, in either direction, whether it be from transgenic cotton or landscape ornamentals to Hawaiian cotton or vice versa, is to exclude non-native cotton from the state or limit its production to the Big Island where native cotton does not naturally occur. Naturalized populations of commercial cotton species should be eradicated and sale of commercial varieties for ornamental purposes should be regulated or halted all together.

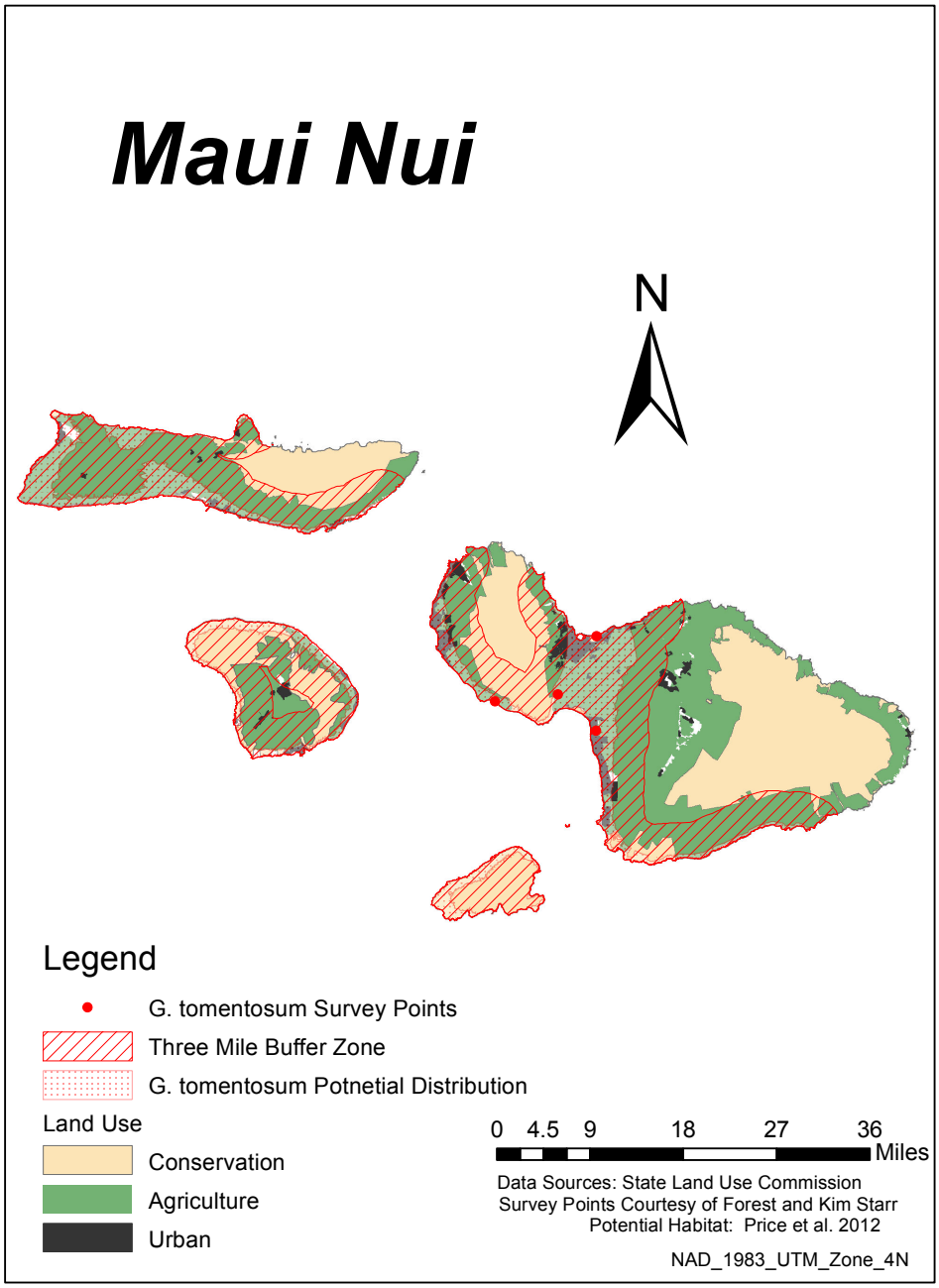
Field-based pollinator and gene flow studies should be conducted in Hawai'i; similar to those which have been undertaken in other parts of the world (Umbeck et al. 1991, Llewellyn and Fitt 1996, Zhang et al. 2005, Heuberger et al. 2010). Such research would establish safe planting distances directly relevant to the unique conditions of Hawai‘i. In addition, thorough field surveys should be conducted throughout the native range of *G. tomentosum* to definitively establish where populations of the species remain as well as to evaluate the populations overall vitality and assess for cases of sporadic hybridization and introgression. These measures would help prevent the escape of transgenic and non-transgenic Upland and Pima cotton genes into Hawaiian cotton populations, as well as maintaining the genetic purity of commercial cotton lines grown in Hawai‘i. In addition, further field tests assessing the ability for hybrid seeds to germinate and persist in a field setting should be conducted to better understand a hybrid's capability of establishing and persisting in a more "natural" setting.

FIGURE 4.1. PROJECTED NATIVE RANGE OF *Gossypium tomentosum* WITH A THREE MILE ISOLATION BUFFER FOR THE ISLAND OF OAHU.



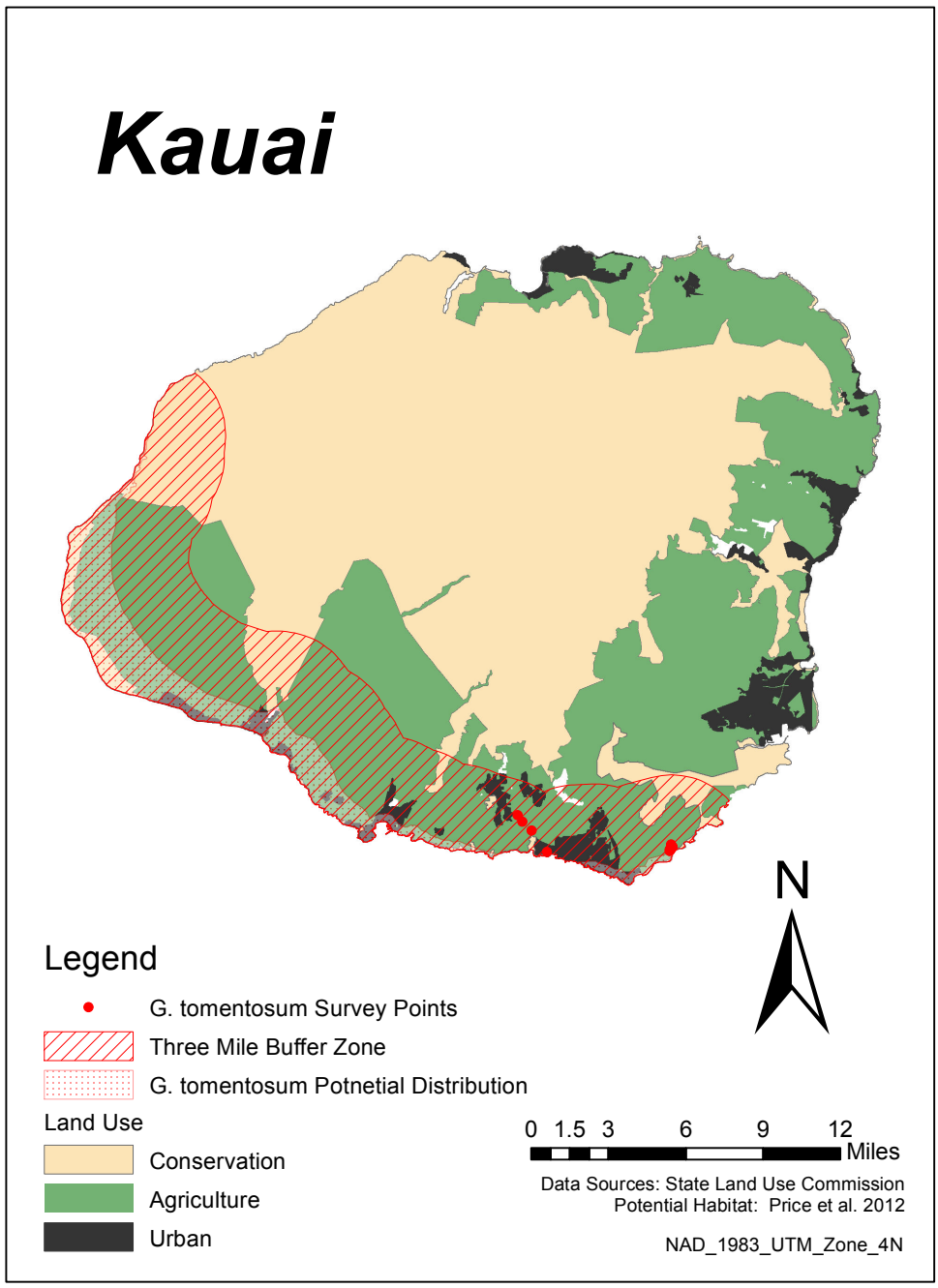
Survey points represent all known extant *G. tomentosum* populations on the island. Areas within the three-mile buffer are at the highest risk of interspecific cotton gene flow and legally cannot be planted with transgenic cotton if native cotton does indeed occur.

FIGURE 4.2. PROJECTED NATIVE RANGE OF *Gossypium tomentosum* WITH A THREE MILE ISOLATION BUFFER FOR THE ISLANDS OF MAUI NUI.



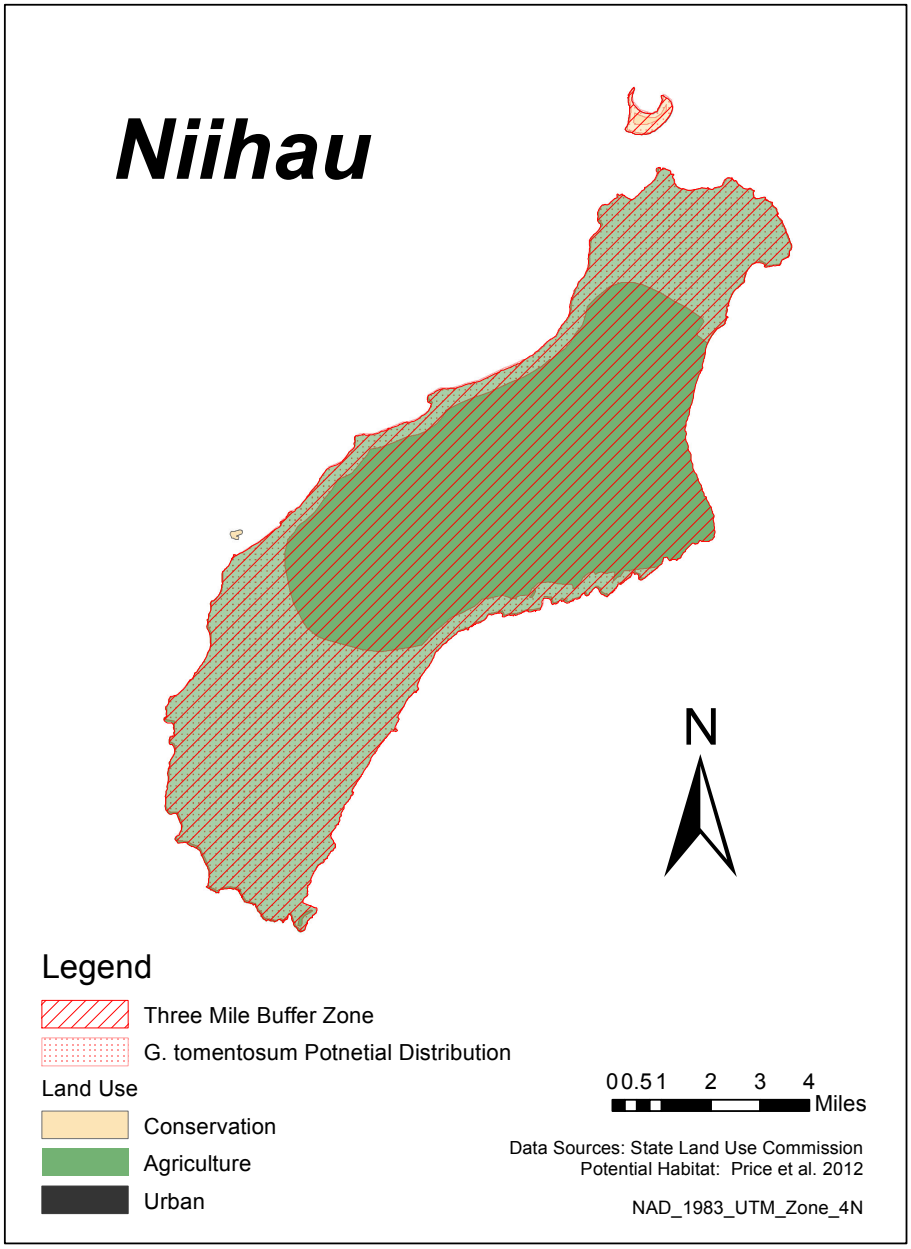
Survey points represent GPS points collected by Forest and Kim Starr of extant *G. tomentosum* populations (all populations are not surveyed). Areas within the three-mile buffer are at the highest risk of interspecific cotton gene flow and legally cannot be planted with transgenic cotton if native cotton does indeed occur.

FIGURE 4.3. PROJECTED NATIVE RANGE OF *Gossypium tomentosum* WITH A THREE MILE ISOLATION BUFFER FOR THE ISLAND OF KAUAI.



Survey points were collected from the National Tropical Botanical Gardens and Lida and David Burney's restoration sites. Areas within the three-mile buffer are at the highest risk of interspecific cotton gene flow and legally cannot be planted with transgenic cotton if native cotton does indeed occur.

FIGURE 4.4. PROJECTED NATIVE RANGE OF *Gossypium tomentosum* WITH A THREE MILE ISOLATION BUFFER FOR THE ISLAND OF NIIHAU.



Areas within the three-mile buffer are at the highest risk of interspecific cotton gene flow and legally cannot be planted with transgenic cotton if native cotton does indeed occur.

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