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# Genetic impoverishment and cross-incompatibility in remnant genotypes of *Ziziphus celata* (Rhamnaceae), a rare shrub endemic to the Lake Wales Ridge, Florida

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Abstract. The loss of genetic diversity in fragmented populations of self-incompatible plant species may result in sexual reproductive failure and local extinctions. Florida ziziphus (*Ziziphus celata*) is a self-incompatible clonal shrub known only from five genetically depauperate populations on the Lake Wales Ridge, Florida, USA. Recovery of thix species requires identification of cross-compatible genotypes that can be used to create viable (i.e., sexually reproducing) populations. To further development of a recovery program for this highly imperiled species, we investigated its genetic structure and sexual reproductive viability. We used random amplified polymorphic DNAs (RAPDs) to investigate genetic variability within remnant populations and we conducted experimental compatibility trials to determine the truss-compatibility of remnant genotypes. One hundred and ninety-nine unique stem samples collected from one *ex situ* and five *in situ* populations were assayed for the presence or absence of a band for 32 RAPD markers. Based on unweighted pair-group mean cluster analysis ( UPGMA), only I I multi-locus genotypes (MLGs) were identified. Eight of these MLGs correspond lo MLGs identified in an earlier allozyme study. In addition. we identified three new RAPD-based MLGs. Three of the five natural populations consisted of only one MLG, while the largest and most genetically diverse population comprised only four MLGs. Coefficients of similarity ranged from 96.6% for the most closely related MLGs to 20.7% for the most distantly related. The compatibility trials demonstrated that most MLGs are cross-incompatible. With 69% of all possible one-way crosses tested (38/55 ), we have identified only eight compatible crosses via germination trials. Based on the results of the compatibility trials, we assigned MLGs to self-incompatibility (SI) mating types. On present evidence. the current breeding population of Florida ziziphus may comprise as few as two SI mating types. These SI mating types will be used to guide future breeding efforts and an experimental introduction.

## Introduction

Habitat loss and fragmentation are the major threats for many species considered at risk for extinction (Wilcove et al. 1986: Noss and Cooperrider 1994; Lande 1998; Harrison and Bruna 1999; Luijten 200 1). Plant species occupying fragmented landscapes are imperiled both by the ecological (Huenneke 1991; Saunders et al. 199 l) and the genetic (Barrett and Kohn 199 1) consequences of reduced population size and isolation of populations. Habitat fragmentation causes the loss of genetic

variation by creating bottlenecks such that remnant populations represent only a subset of the pre-fragmentation gene pool (Young et al. 1996). Genetic diversity in small. isolated populations may be further eroded by random genetic drift (Young et al. 1999) and by the interruption of gene flow via pollen exchange and seed dispersal (Brown 1992; Kwak et al. 1998).

Habitat fragmentation reduces the pool of available mates, leading to increased selfing in self-compatible species and increased mating among closely related individuals in both self-compatible and self-incompatible species (Luijten 2001). Self-incompatibility (SI) systems are genetically regulated via mating types based on SI alleles (Richards 1986). Remnant populations of species with a self-incompatiblc mating system are consigned to sexual reproductive failure (sterility) once all members of the population share the same SI genotype (Les et al. 1991; DeMauro 1993: Weller 1994). A sterile population may be a clone or may comprise genetically distinct individuals that share the same SI alleles.

The loss and erosion of genetic diversity in remnant populations, compounded by the effects of inbreeding, particularly in self-incompatible species, may result in extinction without aggressive intervention to re-create genetically diverse and sexually reproductive populations (DeMauro 1993, 1994; Godt et al. 1995; Young et al. 1999: Warburton et al. 2000; Luijten 2001). Thus, conservation biologists seeking to restore viable populations of a self-incompatible species must assess both the genetic variability within remnant populations and the cross-compatibility of remnant genotypes.

Florida ziziphus, *Ziziphus celata* Judd and D. Hall (Rhamnaceae), is a xeromorphic clonal shrub restricted to five sites on the Lake Wales Ridge of central Florida, USA. Thought to be extinct at the time of its description (Judd and Hall 1984), Florida ziziphus was federally listed as an endangered species following its rediscovery (DeLaney et al. 1989; US Fish and Wildlife Service 1999). Allozyme electrophoresis determined that four of the five extant populations of Florida ziziphus each consist of a single MLG, while the fifth population includes at least seven MLGs (Godt et al. 1997). Breeding system experiments involving these 11 allozyme-based MLGs demonstrated that Florida ziziphus is an obligate outcrosser and that some MLGs are cross-incompatible (Weekley and Race 2001).

Agricultural, commercial and residential development on the Lake Wales Ridge has resulted in the loss of 85% of the xeric uplands (Peroni and Abrahamson 1986) that once supported an endemic-rich mosaic of scrub and sandhill habitats (Christman and Judd 1990). Twenty-two Lake Wales Ridge plant species are currently federally-listed as endangered or threatened (US Fish and Wildlife Service 1999) and eight others are listed by the State of Florida (Coile 2000). The major causes of endangerment for most of these species are habitat loss and fragmentation (US Fish and Wildlife Service 1999).

Genetic diversity within and among populations may go undetected by allozyme electrophoresis (Hartl 1988; Hamrick and Godt 1990; Godt et al. 1997). Molecular DNA-based techniques may be better suited to detecting potentially compatible, genotypes within small populations (Steinger et al. 1996). The random amplified polymorphic DNA (RAPD) technique has been increasingly employed for genetic

studies in recent years (Frankel et al, 1995). RAPDs reveal similar patterns of genetic diversity when compared with other marker types (Isabel et al. 1995; Aagaard ct al. 1998), but RAPDs tend to provide more diagnostic, population-, race-, and species-specific markers. RAPDs have been successfully used to examine population genetic structure or genetic relationship for a number of rare endemic plant species (e.g. Eriksson and Bremer 1993; Van Buren et al. 1994; Palacios and Gonzalez-Candelas 1997; Gordon and Kubisiak 1998; Ayers and Ryan 1999; Jones and Glicldon 1999).

Most self-incompatible angiosperms have a single-locus multi-allelic gametophytic self-incompatibility (GSI) system (Richards 1986). In a GSI system, both parents and progeny are by definition heterozygous at the SI locus and the SI reaction is determined by the haploid pollen grain (Richards 1986). Closely related individuals can mate as long as the pollen donor contributes an SI allele not possessed by the pollen recipient. Cross-incompatible individuals belong to the same SI mating type.

We employed a four-tiered approach to investigate the genetic variability and the cross-compatibility of remnant genotypes of Florida ziziphus: (1) we used RAPD markers to assess the genetic variability of extant populations of Florida ziziphus and to assign individuals to RAPD-based multi-locus genotypes (MLGs); (2) we compared RAPD-based MLGs to previously defined allozyme-based MLGs (Godt et al. 1997); (3) we conducted experimental hand pollinations to test cross-compatibility between remnant genotypes; and (4) based on the results of the RAPD analysis and the cross-compatibility trials, we assigned RAPD-based MLGs to SI mating types. Our findings provide essential information for implementation of the Recovery Plan (US Fish and Wildlife Service 1999) for this highly imperiled species.

## Materials and methods

### *Species description and study sites*

Florida ziziphus is a single- or more often multi-stemmed shrub to 2.5 m in height. It spreads vegetatively through production of root shoots, which may arise several meters from the 'parent' plant and which may become physiologically independent through fragmentation or other means. Mature 'individuals' (often comprising several stems) drop their leaves in the dry season (early winter) prior to flowering. Flowering occurs in late December/early January when plants are leafless except for the previous year's extension growth (Weekley and Race 1999). Generally, there is a second flowering flush in late January/early February after plants have refoliated; this second flush occurs on the previous year's new growth. Flowers are perfect, ~5 mm in diameter, and typical of the Rhamnaceae in their possession of a nectar disk that surrounds the pistil and of anthers initially enclosed by clasping petals (Judd and Hall 1984). The flowers are fragrant and attract a diversity of insect visitors (Weekley and Race 2001). Mature plants may produce tens of thousands of flowers

and hundreds of fruits (Weekley and Race 2001). The fruit is a single-seeded drupe that matures in late April/early May. Drupes are fleshy fruits containing one or more woody stones or pits (pyrcnes) enclosing the seeds (Zomlefer 1989).

The five in situ populations of Florida ziziphus fall along a 45-km stretch of the Lake Wales Ridge in south-central Florida (Figure 1). The mean distance between adjoining populations is 10.8 km (t6.1 km), with the two closest populations being only 2.7 km apart. All populations occur on yellow sand soils in xeric uplands (Table 1), which historically supported longleaf pine/wiregrass (*Pinus palustris* Mill. /*Aristida beyrichiana* Trin & Rupr.) snndhill, turkey oak (*Quercus laevis* Walter) sandhilt, or oak/hickory (*Quercus myrtifolia* Willd./*Carya floridana* Sarg.) scrub (Christman and Judd 1990; Myers 1990; Menges 1999). (Nomenclature follows Wunderlin 1998.) However, three of the five known sites have been converted to pastures. On these sites, Florida ziziphus occurs as remnant populations within a landscape otherwise denuded of native vegetation (Weekley and Race 1999). Only one extant site (PO 1) is currently afforded legal protection at Lake Wales Ridge State Forest. In addition to the five *in situ* populations, there is an *ex situ* Florida ziziphus poputation in the Center for Plant Conservation's National Collection at Bok Tower Gardens in Lake Wales, Florida (Table 1). The *ex situ* population was propagated from root cuttings collected from the five *in situ* populations between 1989 and 1995.

## *RAPD analysis*

Leaf samples were collected from 223 stems in the five in *situ* populations and from the *ex situ* population at Bok Tower Gardens (Table 1). At the four uniclonal sites (*sensu* Godt et al. 1997; POI-PO4 in Table 1), an effort was made to collect at least one sample from each clump of stems separated by at least 0.25 m from other clumps; we considered each clump a physiologiwlty independent 'plant'. Where differentiation of individual plants was difficult, multiple samples were collected. At HOI. where hundreds of stems occur in three separate subpopulations. a different strategy was employed. In the smallest subpoputetion all plants were sampled (as in P01 –P04). But in the two larger subpopulations, the density of stems was too great to confidently separate individual plants; instead we attempted to sample stems uniformly across the subpopulation.

At all sites, each sampled stem was tagged, branches were collected and placed in labeled zip-lock bags in a cooler, and shipped overnight to the USDA Forest Service, Southern Institute of Forest Genetics in Saucier, Mississippi for RAPD analysis.

Total nucleic acids were isolated from approximately 1 .O g of leaf tissue using a modification of the CTAB-based procedure outlined by Wagner et al. ( 1987). The RNA component of these individual extracts was removed by incubation in the presence of RNase A as described by Ausubel et al. (1987). Oligonucleotide IO-mer primers were obtained from Operon Technologies (Alameda, California). The quality and quantity of DNA in the individual extracts was assessed using a Beckman DU 7500 spectrophotometer (Beckman Instruments, Inc., Fullerton.

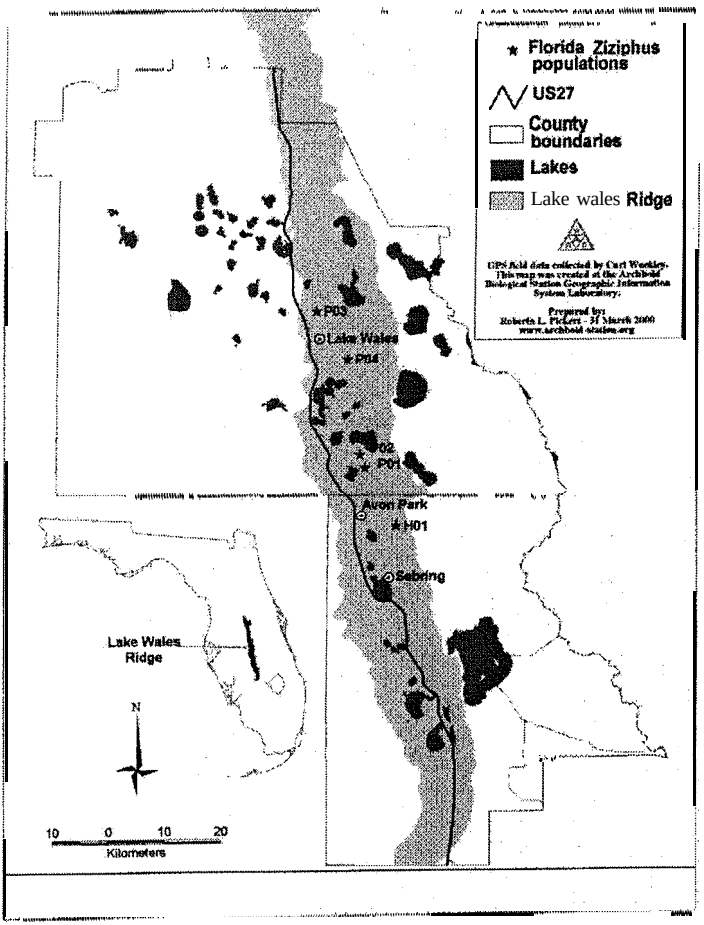


*Figure 1.* Current distribution of Florida ziziphus populations in Highlands and Polk Counties (H01, P01, P02, P03, and P04) on the Lake Wales Ridge in south-central Florida.

California). An aliquot of these individual extracts was diluted to 25 ng $\mu l^{-1}$ and used as stocks for the PCR amplification of RAPD fragments.

DNA amplification via the polymerase chain reaction (PCR) was based on the protocol reported by Williams et al. (1990). The reaction consisted of the following in 24 $\mu l$ total volume: 6.25 ng template DNA, 1 $\mu l$ primer DNA (5 $\mu M$ stock), 3.6 $\mu l$ dNTPs (1 mM stock), 2.4 $\mu l$ 10X *Taq* DNA polymerase reaction buffer (500 mM KCl, 100 mM Tris–HCl, 1.0% Triton X-100, 15 mM $MgCl_2$), and 0.X U *Taq* DNA polymerase. Reactions were loaded in flexible microtitre plates and overlaid with 25 $\mu l$ of mineral oil. Microtitre plates were placed in preheated (85 "C) MJ Research

*Table 1.* Site characteristics of five *in situ* and one *ex situ* populations of *Ziziphus celata.*

| Site | Soil | Habitat | Size ($m^2$) | Number of MLGs | Number of plants | Number of leaf samples |
|---|---|---|---|---|---|---|
| P01 | Tavares[a] | Oak-hickory scrub; turkey oak sandhill | 1600 | 1 | 6 | 8 |
| P02 | Tavares[a] | Pasture | 3 | 1 | 1 | 4 |
| P03 | Candler[a] | Remnant sandhill | 200 | 1 | 17 | 16 |
| P04 | Tavares[a] | Pasture | 500 | 1 | 49 | 60 |
| H01-1 | Astatula[b] | Pasture | 335 | At least 6' | NA | 57 |
| H01-2 | Astatula[b] | Pasture | 60 | At least 3' | NA | 8 |
| H01-3 | Astatula[b] | Pasture | 20 | At least 2' | NA | 14 |
| BTG/P01 | Candler | BTG planting beds | 50 | 1 | 9 | 9 |
| BTG/H01 | Candler | BTG planting beds | 100 | 7 | 47 | 47 |

For the *in situ* H01 site three subpopulations are shown: similarly. the *ex situ* population at Bok Tower Gardens is shown as two subpopulations based on the in *situ* source population. The number of plants is based on the 1999 census (plant cluster of stems within a 0.25-m radius). MLGs are based on Godt et al. (1997). Also shown is the number of leaf samples collected in 1999 for RAPDs analysis. [a]Soil Conservation Service ( 1989). [b]Soil Conservation Service (I 990). [c]Based on MLGs in *ex* situ population at Bok Tower Gardens.

PTC- 100 programmable temperature cyclers (Watertown. Massachusetts) and covered with mylar film (Dynex Technologies, Chantilly, Virginia). The DNA samples were amplified using the following thermal profile: 5 s at 95 °C; 1 min 55 s at 92 "C; followed by 45 cycles of 5 s at 95 °C, 55 s at 92 °C, 1 min at 35 "C, and 2 min at 72 °C; followed by 7 min at 72 °C. The reactions ended with an indefinite hold at 4 "C.

Amplification products were electrophoresed in 2% agarose gels and TAE buffer (40 mM Tris base, 20 mM sodium acetate, 2.0 mM EDTA, glacial acetic acid to pH 7.2) for approximately 3.5 h at 3 V $cm^{-1}$ (150 V). A total of 3.0 µl loading buffer ( 10X TAE, SO% glycerol, and 0.25% bromophenol blue) was added to each reaction prior to electrophoresis. After electrophotesis, the gels were stained with ethidium bromide (0.4 µg $ml^{-1}$) for 45 min, washed in distilled water for 1.0 h, and photographed under ultraviolet light using a Polaroid MP-4 camera and Polaroid 667 instant film (Cambridge, Massachusetts).

To identify informative RAPD fragments, a total of 132 oligonucleotide primers were screened against a panel of DNAs consisting of eight randomly selected Florida ziziphus samples. Seven of these samples had been previously assigned to five unique MLGs (Godt et al. 1997). Those fragments found to be polymorphic among these eight samples were scored as potentially informative. Markers were subjectively chosen based on the intensity of amplification (only intensely amplified bands were scored) and absence of co-migrating DNA fragments (see for example Figure 2). For those cases in which a reaction completely failed, or the presence or absence of bands was unclear, samples were either re-amplified or DNA was re-extracted and re-amplified. RAPD fragments were identified by the manufacturer primer code corresponding to the primer responsible for their amplification. followed by a subscript four-digit number indicating the approximate fragment size in base pairs.

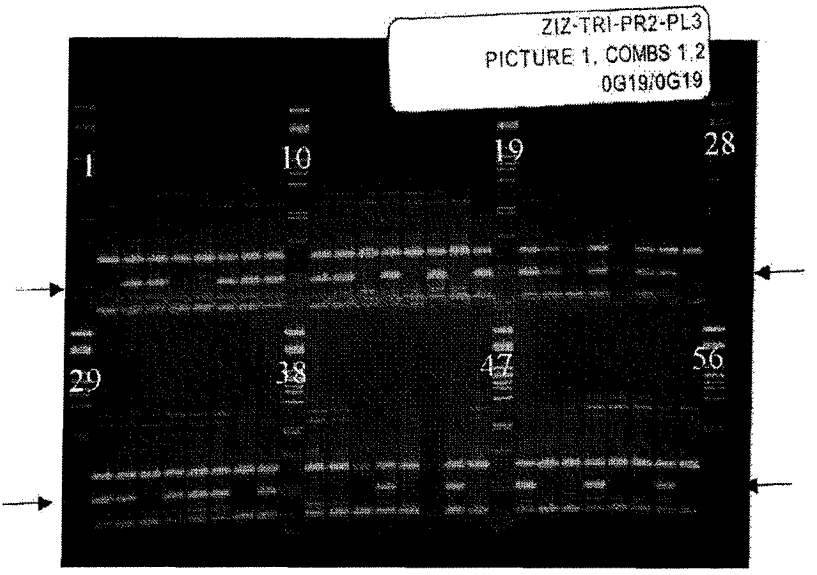


*Figure 2.* Products obtained by amplifying DNA from 48 Florida ziziphus samples with Operon Technologies Inc. 10-mer primer G19 (5'-GTCAGGGCAA-3'). Arrows indicate RAPD marker G190475. Lanes 1. 10, 19, 28, 29. 38. 47. and 56 contain λ DNA cut with restriction enzyme PstI.

Florida ziziphus samples were scored for the presence or absence of a band at each of the RAPD fragments. Coefficients of similarity – simple matching [(*n*1 1 + *n*00)/*n*] and Jaccard's [*n*11 /(*n* − *n*00)] – were calculated between pairwise comparisons of samples, where *n*1 I is the number of markers at which both samples were band-present, *n*00 is the number of markers at which both samples were band-absent, and *n* is the total number of markers. Pair-wise distance coefficients between Florida ziziphus samples were clustered using the unweighted pair-group mean cluster analysis (UPCMA) available in the software package NTSYS-pc version 1.80 (Rohlf 1994), and verified using the CLUSTER procedure in the statistical analysis software SAS (SAS Institute Inc.. Cary, North Carolina). Although the presence of a RAPD band cannot be used to directly infer a sample's genotype (i.e.. cannot distinguish between a homozygous and heterozygous band-present individual) but only a band-present phenotype, for purposes of this study we will refer to a unique cluster or group as an MLG.

*Cross-compatibility and germination trials*

We tested for cross-compatibility between MLGs (*sensu* Cmdt et al. 1997) of Florida ziziphus by experimental hand-pollinations. On plants representing selected MLGs we bagged fowering branches prior to bud-break and also marked unbagged branches to serve as open-pollinated controls. We used 25 X 30 $cm^2$ Delnet$^R$

(PQ2 18) heat-sealed, non-woven pollination bags (Applied Extrusion Technologies, Inc. Delnet Nonwoven Fabrics, Middleton, Delaware) to prevent contamination of pollen-donor flowers and to prevent unwanted pollen transfer by insects on pollen-recipient flowers. Pollen recipients were emasculated shortly after bud-break to prevent contamination with self pollen. Pollen-donor flowers in transit between sites were transported in an ice chest in labeled petri dishes to keep them fresh. We swiped pollen-donor anthers across the stigmatic surface of the pollen recipient, using magnifying optivisors to observe the deposition of pollen grains.

Interpretation of cross-compatibility experiments was complicated by the production of parthenocarpic (seedless) fruits and by high rates of fruit and seed abortion. Con firmation of cross-compatibility between genotypes thus necessitated germination trials. Germination trials on fruits obtained from open-pollinated crosses prior to the current study suggested that germination was enhanced by after-ripening and by pre-soaking seeds prior to planting (Race and Weekley, unpublished data).

Florida ziziphus fruits obtained from the 1999 and 2000 cross-compatibility trials were harvested as they ripened and cleaned to remove the mucilaginous pulp that surrounds the pit. The pit was stored in the dark in a brown paper bag at ambient room temperature (-25 °C) for 2-6 months. Prior to planting, seeds were soaked in water for at least 6 h. Seeds were planted in yellow sand in small pots and irrigated as needed (usually every 2-3 days). The 1999 trial ran for -90 days and the 2000 trial for -150 days. At the end of the trials, non-germinants were exhumed and dissected.

### *Assignment of self-incompatibility (SI) genotypes*

On the assumption that Florida ziziphus has a single-locus multi-allelic GSI system, typical of most SI dicots (Richards 1986), and based on the results of between-genotype crosses (*sensu* Godt et al. 1997) conducted over 4 years, we assigned cross-incompatible MLGs to a common SI mating type.

## Results

### *RAPD analysis of genetic diversity*

Two hundred twenty-three Rorida ziziphus samples were subjected to the PCR and scored for the presence or absence of a band for 32 RAPDs (Table 1). Of the 223 samples, five were dropped from the data set, as they could not be unambiguously scored at all 32 RAPDs even after re-extraction and re-amplification of DNA. Another 19 samples representing replicate collections were dropped from the final analyses. For the 199 unique samples. 19 900 pairwise comparisons were made and a similarity matrix was constructed. Based on UPGMA cluster analysis, WC identified 11 RAPD-based MLGs (Figure 3). Three of the five natural populations of Florida ziziphus (PO1 , PO2 and P03) were found to consist of single unique MLGs.

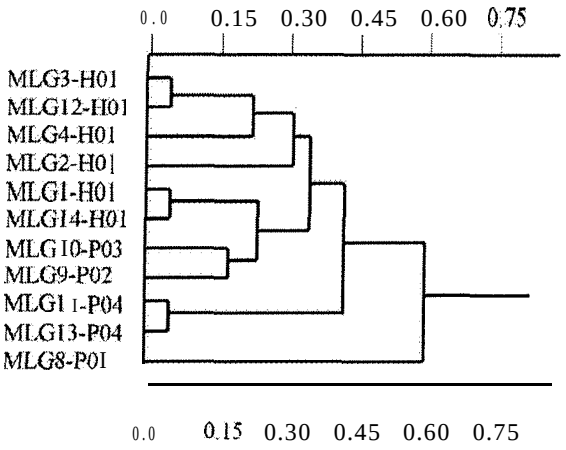


*Figure* 3. UPGMA dendrogram for Florida ziziphus collected from five natural sites (1101, P01, POZ. P03, and P04) on the Lake Wales Ridge, Florida.

*Table* 3. Comparison of the allozyme-based and RAPD-based multi-locus genotype (MLG) groupings for 199 Z. *celata* samples collected from five in *situ* sites and from Bok Tower Garden *ex situ* collection on the Lnkc Wales Ridge in south-central Florida. Also shown are SI mating typos for MLGs as determined by *cross-compatibility* and germination *trials.*

| site | MLC | Allozyme[a] | RAPDs" | SI mating type |
|---|---|---|---|---|
| BTG/H01 | 1 | 6 | 6 | *SIS2* |
| HOI-I | 1 | N A | 57 | *SIS2* |
| BTG/H01 | 2 | 3 | 3 | *SIS2* |
| HOI-2 | 2 | NA | 8 | *SIS2* |
| BTG/H01 | 3 | 19 | 17 | *SIS2* |
| H01-3 | 3 | N A | 14 | *SIS2* |
| BTG/H01 only | 4 | 5 | 4 | *SIS3* |
| BTG/H01 only | 5 | 1 | N A | N A |
| BTG/H01 only | 6 | 1 | 0' | *SIS2* |
| BTG/H01 only | 7 | 1 | 0" | *SIS2* |
| P01 | 8 | 17 | 8 | *SIS3* |
| H01/P01 | 8 | N A | 9 | *SIS3* |
| PO2 | 9 | 4 | 4 | *SIS2* |
| PO3 | 10 | 16 | 16 | *SIS2* |
| P04 | 11 | 3X | 19 | *SIS2* |
| PO4 | 11 | N A | 21 | *SIS2* |
| BTG / HO 1 | 12 | N A | 4" | N A |
| PO4 | 13 | N A | 4[d,f] | N A |
| H01-1 | 14 | N A | 3[1] | N A |
| Total | | 111 | 199[g] | |

"Number of samples grouped based on five polymorphic allozyme loci (Godt et al. 1997 ). [b]Number of samples grouped based on 32 RAPDs markers. 'Grouped by RAPDs into MLG I. "Grouped by RAPDs into MLG 2. [e]Three of these samples grouped in MLG 3 or 4 by Godt et al. (1997). 'Sot sampled by Godt et al. (1997). 'Includes two samples assigned to MLG 1 or 2 by RAPDs.

*Table 3.* Distance (*D*) between pairwise comparisons of *Z. celata* MLGs based on the simple matching (SM) coefficient.

| | MLG1 | MLG2 | MLG3 | MLG4 | MLG8 | MLG9 | MLG10 | MLG11 | MLG12 | MLG13 | MLG14 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MLG1 | - | | | | | | | | | | |
| MLG2 | 0.241 | - | | | | | | | | | |
| MLG3 | 0.379 | 0.176 | - | | | | | | | | |
| MLG4 | 0.241 | 0.345 | 0.207 | - | | | | | | | |
| MLG8 | 0.4X8 | 0.552 | 0.759 | 0.621 | - | | | | | | |
| MLGY | 0.207 | 0.379 | 0.380 | 0.310 | 0.517 | - | | | | | |
| MLG10 | 0.241 | 0.414 | 0.414 | 0.345 | 0.621 | 0.172 | - | | | | |
| MLG11 | 0.345 | 0.310 | 0.517 | 0.448 | 0.586 | 0.414 | 0.370 | - | | | |
| MLG12 | 0.414 | 0.310 | 0.034 | 0.241 | 0.793 | 0.414 | 0.448 | 0.552 | - | | |
| MLG13 | 0.379 | 0.345 | 0.552 | 0.483 | 0.621 | 0.448 | 0.414 | 0.034 | 0.517 | - | |
| MLG14 | 0.034 | 0.276 | 0.414 | 0.376 | 0.483 | 0.241 | 0.276 | 0.379 | 0.370 | 0.345 | - |

SM = $n11+n00/n$, where $n11$ is the number of markers at which both samples were band-present, $n00$ is the number of markers at which both samples were band-&sent, and $n$ is the total number of markers scored; $D=(1-\mathrm{SM})$.

Populations HOI (including the Bok Tower Gardens *ex situ* population in part) and PO4 were found to consist of six and two unique MLGs, respectively.

Ninety-one of the 199 samples included in this study had been previously genotyped at five polymorphic allozyme loci by Godt et al. (1997); 20 additional plants sampled for the allozyme analysis had died or lacked leaf material for the RAPD analysis. Godt et al. (1997) grouped their 111 samples into 11 MLGs (Table 2). RAPDs also detected I | MLGs, but only eight MLGs were common between the two studies. Two samples designated as unique single-individual MLGs based on allozymes, MLGs 6 and 7 (Godt et al. 1997), were subsumed into MLGs I and 2, respectively (Table 2). Two samples grouped into MLG 3, and one sample grouped into MLG 4 (Godt et al. 1997), were grouped by the RAPDs into a new MLG (MLG 12). Four samples from P04, grouped by the allozyme analysis into MLG 11 (Godt et al. 1997), were grouped by the RAPDs into a new MLG (MLG 13).

Of the 108 samples for which allozyme data were unavailable, 82 were collected from the recently accessible HOI site, 25 from new root shoots at P04, and one from the *ex situ* HO1 population at Bok Tower Gardens. Based on the RAPD data, most of the additional HOI plants represent stems from previously sampled MLGs (Table 2). Fifty-seven of the samples collected at HOI were grouped into MLG I, eight were grouped into MLG 2, and 14 into MLG 3. However, one sample from the *ex situ* H01 population grouped to the new MLG 12, and three samples from the in *situ* HO1 population formed a new MLG (MLG 14). Of the 25 additional stems sampled at P04, 21 grouped to MLG 11 and four grouped to MLG 13.

The phenogram produced by UPGMA analysis using the simple matching coefficient was identical to that obtained using the Jaccard coefficient, except that the similarity coefficients obtained using Jaccard's approach were slightly smaller. Coefficients of similarity ranged from 96.6% for the most closely related MLGs to 20.7% for the most distantly related (Table 3). Relationships among the MLGs were mostly as expected based on known collection information. One exception, how-

*Table 4.* Fruit yield (1997–2000) and germination (1999–2000) percentages for hand-pollinated crosses, involving 11 MLGs (*sensu* Godt et al. 1997). Fruit yield is below the diagonal and germination above the diagonal.

| | H01-G1 | H01-G2 | H01-G3 | H01-G4 | H01-G5 | H01-G6 | H01-G7 | P01 (G8) | P02 (G9) | P03 (G10) | P04 (G11) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| H01-G1 | | | 0% | 14.8% | | | | 28.6% | | | |
| | | | 0/1 | 4/27 | | | | 2/7 | | | |
| H01-G2 | | | | 30.0% | | | | | | | |
| | | | | 3/10 | | | | | | | |
| H01-G3 | 0.8% | 0% | | 22.2% | | | | | | | |
| | 1/132 | 0/178 | | 4/18 | | | | | | | |
| H01-G4 | 8.7% | 10.1% | 7.5% | | | 10.0% | | | | 25.0% | 16.7% |
| | 27/309 | 10/99 | 18/240 | | | 1/10 | | | | 1/4 | 1/6 |
| H01-G5 | 0% | | 0% | | | | | | | | |
| | 0/48 | | 0/130 | | | | | | | | |
| H01-G6 | 0% | | 0% | 3.6% | | | | 40.0% | | | |
| | 0/263 | | 0/140 | 10/280 | | | | 2/5 | | | |
| H01-G7 | | 0% | OR | 0% | | | | | | | |
| | | 0/228 | 0/116.5 | 0/29 | | | | | | | |
| P01 (G8) | 5.2% | | 0% | 0% | | 4.8% | | | | 0% | 0% |
| | 7/135 | | 0/32 | 0/239 | | 5/105 | | | | 0/1 | 0/7 |
| P02 (G9) | | | | 0% | | | | 0% | | 0% | |
| | | | | 0/25 | | | | 0/82 | | 0/2 | |
| P03 (G10) | 0% | 0% | 0% | 1.0% | 0% | 0% | 0% | 0.5% | 1.3% | | |
| | 0/203 | 0/218 | 0/172 | 4/404 | 0/8 | 0/66 | 0/13 | 1/202 | 2/158 | | |
| P04 (G11) | 0% | 0% | 0% | 1.7% | 0% | 0% | 0% | 3.2% | 0% | 0% | |
| | 0/155 | 0/35 | 0/28 | 6/358 | 0/34 | 0/27 | 0/53 | 7/221 | 0/145 | 0/182 | 7 |

The upper number in each cell is the percentage. For fruit yield, the lower number is the ratio of number of fruit obtained to number of flowers hand-pollinated; for germination, the lower number is the ratio of number of germinants to number of fruit in the germination trial.

*Table 5.* Summary of cross-compatibility and germination trials of Florida ziziphus conducted between 1997 and 2000.

| Year | MLG recipients | MLG donors | Number of hand pollinations | Number of fruit (%) | Number of germinants |
|---|---|---|---|---|---|
| 1997 | 7 | 6 | x29 | 12 (1.45) | NA |
| 1998 | 2 | 8 | 482 | 0 | 0 |
| 1999 | 7 | Y | 1330 | 56 (4.21) | 12 |
| 2000 | 10 | 10 | 3335 | 30 (0.9) | 6 |
| Total | 10 | 11 | 5976 | 98( 1.64) | 18 |

ever, is for MLGs 1 and 14, both of which represent collections from the HOI site. These MLGs were found to be more genetically similar to MLGs 9 and 10 from sites PO2 and P03, respectively, than to the otber MLGs found at the H01 site. However, placement within this cluster was only weakly supported based on 100 bootstrap data sets (57%).

## *Cross-compatibility and germination trials*

Hand-pollinated crosses between allozyme-based MLGs and germination trials on the resulting fruits confirmed the cross-compatibility of only eight of 38 experimental crosses (21%) conducted between 1997 and 2000 (Table 4). Experimental crosses included all 11 allozyme-based MLGs aud we conducted 38 of the 55 possible one-way crosses (69%). Wc hand-pollinated ~6000 flowers and an approximately equal number served as open-pollinated controls. While we obtained 98 fruits from hand-pollinated crosses, only 18 (18.4%) of those fruits resulted in germinants (Table 5). There was no significant difference in the percent germination of the 1999 and 2000 hand-pollinated crosses (22.8%) as compared to open-pollinated controls (27.3%; $G = 3.177$, df = 1, $P = 0.075$). Most successful crosses (i.e., resulting in germinants) involved a single MLG (MLG 4) as either pollen donor or pollen recipient (Table 4). The new RAPD-based MLGs were not included in the cross-compatibility trials because (1) they were not identified until the trials were completed, and (2) most plants representing those MLGs did not flower.

In most non-germinants, the pyrene (pit or stone) was either an empty chamber or a solid mass of lignificd tissue; in either case no embryo was contained within the pyrene. However, about one-third (12/37) of the non-germinants from the 90-day 1999 trial appeared to contain intact but ungerminated seeds, indicating the possibility of seed dormancy and suggesting that the germination trial had been ended prematurely. Consequently, the 2000 trial was exteuded to 150 days, but only five additional gcrminants were obtained between day 90 and day 150.

## *Assignment of SI mating types*

Based on the results of the cross-compatibility and germination trials, we assigned SI mating types to the 11 known allozyme MLGs (Table 2). Six MLGs (MLGs 1.2.

3, 9, 10 and 11) most likely have the same SI genotype (*S1S2*), as crosses between these MLGs are incompatible (Table 4). MLGs 4 and 8 each harbor at least one unique SI allele (*S3*), as these MLGs are cross-compatible with one or more of the *S1S2* mating types. However, crosses between MLGs 4 and 8 are incompatible (Table 4), suggesting that these two MLGs share the same SI alleles (e.g., these two MLGs could both be *S1S3* or *S2S3*). Thus, the current breeding population of Florida ziziphus may comprise only three SI alleles and two cross-compatible SI mating types.

## Discussion

Plant species imperiled by the genetic consequences of habitat loss and fragmentation constitute a special challenge for conservation biologists (Knapp and Dyer 1998; Luijten 2001 ). The loss of genetic diversity and/or sexual viability in fragmented populations may doom some species to extinction without aggressive, interveution to increase genetic variability and to ensure sexual reproductive success (DeMauro 1993, 1994; Godt et al. 1995; Young et al. 1999; Warburton et al. 2000; Luijteu 2001). The goal of our work on the genetic diversity and cross-compatibility of Florida ziziphus is the establishment of viable (i.e., sexually reproducing) populations, as mandated by the US Fish and Wildlife Service (1 999), through the translocation of cross-compatible genotypes.

Our results confirm that Florida ziziphus is a genetically depauperate and reproductively challenged species. Most *in situ* populations are self-sterile and probably uniclonal and most. of the 11 genotypes that make up the current breeding population are cross-incompatible. The most likely explanation for the cross-incompatibility of genotypes is that they belong to the same SI mating type. The paucity of SI mating types complicates recovery efforts, since translocation of MLGs does not guarantee that genetically enhanced or experimentally created populations will be sexually viable. Moreover. sexually viable populations based on the same few SI mating types wilt necessarily involve crosses between closely related genets and will entrain the potentially deleterious consequences of inbreeding depression. Inbreeding depression within experimental populations is probably unavoidable because the limited number of cross-compatible genotypes available for the introductions makes it likely that some introduced individuals will be siblings.

### *Genetic diversity*

We used RAPDs to re-assess the genetic diversity of Florida ziziphus because some studies suggest that RAPDs can detect genetic variation undetected by allozyme electrophoresis in genetically depauperate populations (Brauner et al. 1992; Ayers and Ryan 1997; Wong and Sun 1999; Warburton et al. 2000). However, the results obtained from the RAPD analysis arc nearly identical to those reported by Godt et al. (1997). RAPDs aud allozymes agree that three of the Polk County sites (P01–P03) comprise single unique MLGs and that the HOI population *sensu lato* (i.e.,

including the *ex situ* plants at Bok Tower Gardens) is the most genetically diverse. Although two allozyme-based MLGs were subsumed by the RAPD analysis, three new MLGs were uncovered. At first, we were suspicious of the three new MLGs because representatives of these groups were found to differ from the most closely related MLG by only a single marker ($G14_{0500}$). However, based on re-extraction and re-amplification of DNAs, this marker was found to be 100% repeatable (T.L. Kubisiak, unpublished dataj.

The most important difference between the allozyme analysis and the RAPD analysis involves the HOI MLGs. The seven MLGs identified by Godt et al. (1997) within H01, the most genetically diverse population, were based on the Bok Tower Gardens *ex situ* subset of the *in situ* population (because the *in situ* site was inaccessible at the time of their collections). Given that the *in situ* HOI population comprises three subpopulations (with subpopulation 1 over 500 m distant from subpopulations 2 and 3) and over 400 plants, we anticipated that we would identify additional MLGs from the HO | site. However, our data indicate that there may be as few as four MLGs *in situ*, with each of the two smaller subpopulations constituting separate clones. The largest subpopulation, which comprises about two-thirds of the overall HOI population, appears to be virtually uniclonal as well, with only three individuals belonging to the new MLG 14. The apparent absence of MLG 4, which occurs in the *ex situ* population, from the HO1 site is noteworthy because it is the only MLG confirmed to be compatible with the three (mutually cross-incompatible) MLGs that constitute most of the HO1 *in situ* population.

Thus, the HO1 *in situ* population, despite its size, distribution and relatively greater gcnetic variation compared to the other in situ populations, may also be self-sterile. This population was recovering from moving just prior to our gaining access to it (in April 1999) and there was virtually no flowering in 2000. Determination of the sexual reproductive viability of the *in situ* HO | population must await its return to full flowering status and will require additional experimental crosses (e.g., involving the new MLG 14, known only from three in *situ* plants).

Due to the dominant nature of RAPD markers, genetic diversity statistics could not be calculated from the RAPD data. However, given the congruence of the RAPD and the allozyme studies, genetic diversity statistics calculated on the allozyme results seemed warranted. Based on Godt et al. (1997), Weekley and Race (1999) calculated the mean number of alleles per locus ($A = 1.25$) and the percent polymorphic loci ($P = 25.0$) for Florida ziziphus, two standard measures of allelic diversity. These values are lower than those reported by McDonald and Hamrick (1996) for *Ceratiola ericoides*, a relatively widespread shrub in Florida scrub ecosystems, and for several species of rare Lake Wales Ridge endemic subshrubs in the genus *Dicerandra*. The Florida ziziphus values are also lower than those reported by Hamrick and Godt (1990) for the 8 1 endemic plants they surveyed across a broad geographical and habitat range. The values of $A$ (mean number of alleles per locus) and $P_p$ [percent polymorphic loci) for Florida ziziphus are about the same as or slightly higher than those reported by Mcnges ct al. (2001) for seven narrowly endemic Lake Wales Ridge herbs ($A = 1.24$ and $P_p = 14.6$). However,

long-lived woody species typically have higher values than short-lived herbs or subshrubs (Hamrick and Godt 1990).

Overall, Godt et al. (1997). using Nei's genetic diversity statistics (Nei 1973, 1975), estimated relatively high total genetic diversity ($H_T$ = 0.377) for Florida ziziphus at the five polymorphic loci analyzed in the allozyme study, but low species diversity ($H_{es}$ = 0.079). $H_T$ for Florida ziziphus is slightly higher than mean values for other woody species, but $H_{es}$ is lower (Hamrick and Godt 1990). The relatively high level of total genetic diversity ($H_T$) is not inconsistent with the other results, because loss of alleles exceeds reductions in heterozygosity when bottlenecks occur and populations remain small (Barrett and Kohn 1991). While the historical distribution, range and abundance of Florida ziziphus are not known, low allelic diversity in extant populations suggests a recent history of population loss and fragmentation (Barrett and Kohn 1991: Young et al. 1996; Godt et al. 1997). However, we cannot rule out the possibility that Florida ziziphus has always been rare (Weekley and Race 2001) or that genetic diversity may have always been low.

*Sexual reproductive failure*

Loss of genetic diversity by a species can be offset by sexual reproduction, particularly by outcrossing. But in a fragmented, self-incompatible species such as Florida ziziphus, outcrossing is virtually precluded once all members of the population share the same few SI alleles (Weller 1994). The five remnant *in* situ populations of Florida ziziphus may be self-sterile because they are self-incompatible clones or because individuals genetically distinct at a level undetected by allozymes and RAPDs belong to the same SI mating type.

Thirty of 3X between-MLG crosses (involving over 4000 hand pollinations) conducted over 4 years have failed to yield a single viable fruit. Most crosses have been repeated on more than one 'individual' and in more than 1 year, sample sizes exceed 100 hand pollinations for about two-thirds of these crosses, and reciprocal crosses are typically performed (i.e., each MLG serves as both pollen donor and pollen recipient). Germination trials have demonstrated the viability of only eight crosses. Six of the eight successful crosses involved MLG 4 as either pollen donor or pollen recipient and the other two involved MLG 8. Sexual reproductive failure due to incompatibility between MLGs sharing the same SI alleles provides a cogent explanation of these results, but cannot rule out other factors (e.g., non-viable pollen) in the sterility of some genotypes.

Another aspect of sexual reproductive failure in Florida ziziphus is the low level of seed production. Even among compatible crosses, both fruit yield and the production of viable seeds are minimal. Fruit yields based on the 1999 and 2000 compatibility trials (number of fruits/number of flowers pollinated) are less than 5% annually (Table 5). In addition to fruits obtained from experimental crosses, over 20 000 fruits have been harvested from open-pollinated plants in the multi-genotype *ex situ* population at Bok Tower Gardens. Germination trials of fruits obtained from experimental hand-pollinated and open-pollinated (control) crosses. as well as the

non-experimental open pollinations, demonstrate that ~75% of Florida ziziphus fruits lack viable seeds (Weekley and Race, unpublished data). An experimental study by Perez (200 1) of the germination biology of Florida ziziphus also obtained germination rates below 25%. Parthenocarpy (the production of seedless fruits) and seed abortion apparently account for the tow level of germination.

In many species, low rates of fruit or seed production are due to resource limitation (Stephenson 1981). But resource limitation is an unlikely explanation for the total reproductive failure of many Florida ziziphus crosses. Thus, crosses involving some MLGs consistently fail even when those genotypes mature a substantial crop of fruits when crossed with other MLGs (e.g., MLG 4 has consistently produced viable fruits when crossed with MLGs I and 2, but has never produced fruits when crossed with MLGs 8 or 9; Table 4).

The most parsimonious explanation of sexual reproductive failure in Florida ziziphus is the reduction of atlelic diversity at the SI locus (assuming that the species has a single-locus GSI system, as is most likely; Richards 1986). Selection favors the maintenance of SI alleles within a population, the more the better since the chance of compatible crosses increases with the number of SI mating types. Typically plant species with a GSI system maintain dozens to hundreds of SI alleles (Richards 1986). Our data suggest that Florida ziziphus may comprise as few as three SI alleles.

The main obstacle to the restoration of sexually viable populations of Florida ziziphus is the limited number of SI mating types. If, as appears likely on present evidence, Florida ziziphus comprises only three SI alleles (*S1*, *S2*, *S3*), then there are only three possible Sl mating types (*S1S2*, *S1S3*, *S2S3*), given the mechanics of the GSI system (Richards 1986). While progeny of crosses involving these SI mating types are genetically unique individuals, all are either full siblings (with both SI genotype parents in common) or half siblings (with one SI genotype parent in common). Inbreeding and its possible deleterious consequences (Charlesworth and Charlesworth 1987) appear unavoidable in genetically enhanced or experimentally created populations of Florida ziziphus.

### *Implications for restoration*

The literature on restoration of plant biodiversity is replete with case studies of plant species suffering the consequences of habitat destruction and fragmentation (DeMauro 1993, 1994; Godt et at. 1995; Falk et al. t 996; Young et at. t 999; Warburton et al. 2000; Luijten 2001). Many translocation projects have been implemented to re-introduce species to sites from which they have been extirpated or to introduce species to new sites containing appropriate habitat (Falk et al. 1996 and references therein), However, few translocation projects have been carried out to restore sexual viability to populations of self-incompatible species reduced to sterility by habitat loss and isolation, Notable exceptions include the work of DeMauro (1993, 1994) and Pavlik et al. ( 1993) to restore or create viable populations of *Hymenoxys acaulis* **var.** *glabra* and *Amsinckia grandiflora*, respectively.

Using seedlings genotyped by altozyme and/or RAPD markers and assigned to SI

mating types, we are conducting an experimental introduction of Florida ziziphus to a protected site containing appropriate habitat (US Fish and Wildlife Service 1999). Our introduction follows established guidelines [e.g., Gordon 1994; Guerrant 1996; Pavlik 1996) for the translocation of imperiled species. Potted seedlings representing cross-compatible MLGs will be transplanted to the site aud monitored for survival, growth, flowering and fruit production. The experimental introduction will allow us to further evaluate the microhabitat and management requirements of Florida ziziphus and to further assess its potential for rccovcry.

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