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RAPD ANALYSIS OF THE LAST POPULATION OF A LIKELY FLORIDA KEYS ENDEMIC CACTUS

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ABSTRACT: *The semaphore cactus in the Florida Keys has until recently been considered a disjunct location of the Jamaican* **Opuntia spinosissima.** *Loss of all but one population in the Keys coupled with recent suggestions that the species should be taxonomically separated from the Jamaican cactus and is, therefore, a Florida Keys endemic, makes this population of conservation concern. Random amplified polymorphic DNA (RAPD) analysis was conducted on the remaining 12 individual large stems in the wild to determine whether this small population contains multiple genotypes. Other accessions of the cactus, mainly from private collections and from the Caribbean were also included. Analysis of 42 RAPD markers reveals that all Florida accessions are closely related, with unique genotypes being separated by differences at only one to as many as jive polymorphic markers (2.4–11.9% of markers). Within the wild population. seven genotypes could be uniquely identified. The Jamaican accession was separated from the Florida cacti by an average of 22 marker differences (52.3% of the markers). The difference of the Florida accessions from the Jamaican accession by a large number of markers suggests possible species-level differentiation, providing additional supportive evidence that the Keys population may be one of the rarest and most threatened plants in the continental United States.*

THE semaphore cactus, ***Opuntia spinosissima*** Miller (1768), is an erect tree cactus historically known from only three locations within the United States. These locations were all within Monroe County, Florida: on Big Pine Key, Key Largo, and Little Torch Key. The first two of these populations were noted by Small (1930), who found a record of the Big Pine Key population in 1919. The third population was not discovered until 1965 (The Nature Conservancy, 1990). Some authors have hypothesized that the Little Torch Key population was introduced from propagated stock (Avery, 1981), but no evidence documents this hypothesis.

The only remaining population in the Florida Keys is that on Little Torch Key, the two other populations having been extirpated as land development progressed (The Nature Conservancy, 1990). The Little Torch Key population was significantly threatened by collection in 1977, but has been protected from further such damage since 1988 by The Nature Conservancy. At that time, there were 13 large separately rooted stems, most surrounded by several smaller rooted stems. The smaller stems are likely to be vegetative, forming when pads drop from the larger "adult" plants and take root.

Most of the pads that drop are aborted fruit pads. Viable seeds were not recorded from the plants until 1996. Currently, it is not known whether any of the smaller stems are progeny derived from seed.

While the primary location for this rare cactus has been thought to be in the Blue Hills of south coastal Jamaica, recent work suggests that the populations in Jamaica and Florida are sufficiently morphologically and genetically different to be taxonomically separated. The Florida cacti have now been proposed as more appropriately identified as ***Opuntia corallicola*** Small (Austin et al., 1997). This assessment is supported by earlier work by Howard and Touw (1982), who listed 0. ***spinosissima*** as endemic to Jamaica. However, the species has also been cited from Cayman Brac (Adams, 1972).

Three lines of evidence suggest that the taxonomic differentiation is appropriate, and that the species is a Florida Keys endemic. First, Austin and Binninger (1994) determined that the Florida cacti differ from the Jamaican ones in areole, corolla, ovary, pad, and spine characteristics, height, and habitat. Chromosome numbers could not be used for this analysis because while ***2n*** = 66 for the Florida accessions, the data are not available for the Jamaican population (Austin et al., 1997). Accession material for this work, and for all the work discussed below, was obtained from Fairchild Tropical Garden, which maintains a Center for Plant Conservation collection of this species.

Second, enzyme electrophoresis of 18 accessions from the Florida Keys and 29 from Jamaica (all from a single fruit) was conducted on 13 loci (Hamrick and Godt, 1996). The analysis showed no allozyme diversity among the Florida Keys individuals, which were homozygous at all loci except one, a fixed heterozygote. Four loci were polymorphic in the Jamaican plants, with the mean number of alleles per locus at 2.25. One locus was fixed for different alleles in the Florida and Jamaican plants, while the remainder overlapped. Nei's genetic identity between the two groups was 0.801 (Hamrick and Godt, 1996). The authors concluded that the groups are closely related but could be different species if the variation in the Jamaican accessions was artificially low because of their related source.

Third, Dougherty (1996) examined ribulose bisphosphate carboxylase-oxygenase (*rbc*L) genes of chloroplast DNA in the cacti. The nucleotide sequence variation demonstrated differentiation between the Florida and Jamaican plants sufficient to support the taxonomic differentiation. The author did not evaluate whether there were differences in sequence among the individuals in Florida (Dougherty, 1996).

In order to clarify whether the Little Torch Key semaphore cactus population is composed of one or multiple genetic individuals, we embarked on an additional examination of DNA using random amplified polymorphic DNAs (RAPDs). This information would aid in The Nature Conservancy's management of the remaining wild individuals on the preserve. Two introduction efforts have been undertaken with propagules from 11 of the individuals on the preserve. A collaborative experimental out-planting has been

initiated in two areas disjunct from the wild population on the preserve using propagules from four of the parent plants. An additional series of out-plantings has been conducted by Fairchild Tropical Garden on Big Pine Key and Key Largo. In all cases, accession identity has been recorded. Genetic identities of these accessions may clarify any patterns in growth and survival that are detected.

MATERIALS AND METHODS—*Plant material*—Pads from 21 accessions at Fairchild Tropical Garden were placed in separate paper bags, frozen, placed on dry ice, and shipped overnight to the USDA Forest Service, Southern Institute of Forest Genetics, in Saucier, Mississippi. The accessions included: 12 of the large cacti from the Little Torch Key population (LTK); three cacti from private collections on Little Torch Key (PLTK); one cactus from a private collection from an unspecified location in the lower Keys (LK); one cactus from lower Matecumbe Key (MK); two cacti that had been planted at Castello Hammock Park (PCHP) in Miami-Dade County, Florida; one cactus from a private collection derived from Cayman Brac (CAY); and one cactus from Jamaica (JAM).

DNA extraction—Total nucleic acids were isolated from approximately two grams of cactus pad tissue using a modification of the CTAB-based procedure outlined in Wagner and co-workers (1987). The RNA component of these individual extracts was removed by incubation in the presence of RNase A as described in Ausubel and co-workers (1987). Oligonucleotide 10-mer primers were obtained from Operon Technologies (Alameda, Calif., USA).

RAPD amplification—DNA amplification was based on the protocol reported by Williams and co-workers (1990). The reaction consisted of the following in 24 μl total volume: 6.25 ng of template DNA, 1 μl of primer DNA (5 μM stock), 3.6 μl of dNTPs (1 mM stock), 2.4 μl 10×*Taq* DNA polymerase reaction buffer (500 mM KCl, 100 mM Tris-HCl, 1.0% Triton® X-100, 15 mM $MgCl_2$), and 0.8 U *Taq* DNA polymerase. Reactions were loaded in flexible microtitre plates and overlaid with 25 μl of mineral oil. Microtitre plates were placed in preheated (85°C) programmable thermal cyclers (MJ Research PTC-100) and covered with mylar film. 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.

Electrophoresis-The completed reactions 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 (150 V). A total of 3.0 μl of loading buffer (10× TAE, 50% glycerol, 0.25% bromophenol blue) was added to each reaction prior to electrophoresis. After electrophoresis, the gels were stained with ethidium bromide (0.4 μg/ml) for 45 min, washed in dH_2O for 1.0 h, and photographed under UV light using a Polaroid MP-4 camera and Polaroid 667 instant film.

Primer screening and marker scoring-To identify informative RAPD fragments, a total of 20 oligonucleotide primers (Operon Technologies Inc. primer sets A and B) were screened against a panel of DNAs extracted from the 21 different cacti. Those fragments found to be polymorphic among the 21 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 DNAs (Fig. 1). Those cases in which a reaction completely failed or the presence or absence of bands was unclear, were recorded as missing data. RAPD fragments were identified by the manufacturer primer code corresponding to the primer responsible for their amplification, followed by a four digit number indicating the approximate fragment size in base pairs.

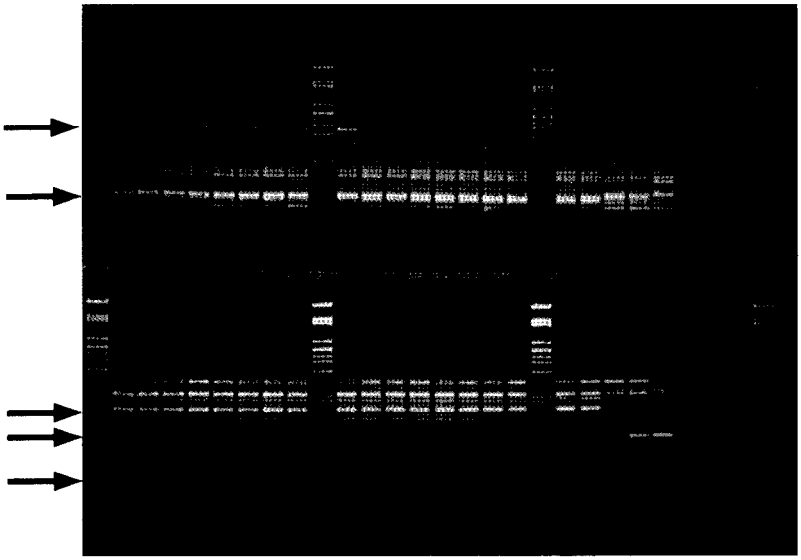

FIG. 1. Ethidium bromide-stained 2.0% agarose gel showing polymorphic markers among the 21 cacti accessions amplified by Operon Technologies primer A01(upper panel) and A07 (lower panel). RAPDs are identified by arrows. Lanes 1, 10, 19, and 28 are lambda-*PstI* molecular weight size ladders. Lanes 2-9 contain samples LTK 90-192 through 90-199; lanes 11–18 contain samples LTK 90-200 through 90-203, PLTK 86-103, 93-416, and LTU1, and LK 92-372; and lanes 20-24 contain MK 92-534, PCHP 89-620, PCHP CHU2, CAY 96-131, and JAM 90-253.

Cluster analysis-Cacti samples were placed into groups or clusters using the unweighted pair-group mean method (UPGMA) available under the CLUSTER procedure in the statistical analysis software SAS (SAS Institute Inc., Cary, NC). A distance or dissimilarity matrix was constructed based on the RAPD fragment data. Cacti samples were scored for the presence or absence of a band at each of the RAPD fragments. The distance matrix was constructed by tallying the total number of marker differences found between pair-wise comparisons. For the 21 samples, a total of 231 pair-wise comparisons were made.

RESULTS-A **total of 42** RAPD **markers were identified in the cactus samples. The distance matrix constructed for cluster analysis is shown in Table 1. UPGMA analysis suggested two primary groups of individuals (Fig. 2). One primary group included all of the Florida cacti except for one of the two cacti planted at** Castello **Hammock Park. The other primary group included the other** Castello **Hammock Park cactus, the** Cayman Brac **accession, and the Jamaican accession.**

Within the larger grouping, additional differentiation among accessions was evident. This separation was based on differences at only a few (one to five) RAPD markers. Of the 12 large cacti from the Little Torch Key population, seven appear to be unique genotypes (Fig. 2). The three cacti from private collections on Little Torch Key (PLTK LTU1, **86-103 and 93-416), are all indistinguishable based on these data. These cacti were also**

TABLE 1. Dissimilarity matrix for the 21 cacti accessions based on the number of marker differences found between pair-wise comparisons of samples at 42 RAPD markers.

Cactus accession	Symmetric dissimilarity matrix																				
PLTK 86-103	0																				
PCHP 89-620	2	0																			
LTK 90-192	0	2	0																		
LTK 90-193	0	2	0	0																	
LTK 90-194	2	4	1	2	0																
LTK 90-195	4	4	3	4	4	0															
LTK 90-196	1	3	1	1	1	5	0														
LTK 90-197	3	1	3	3	5	4	4	0													
LTK 90-198	2	0	2	2	4	4	3	1	0												
LTK 90-199	1	3	1	1	3	3	2	2	3	0											
LTK 90-200	3	1	3	3	5	3	4	0	1	2	0										
LTK 90-201	1	3	1	1	1	5	0	3	3	2	4	0									
LTK 90-202	1	3	1	1	1	5	0	4	3	2	4	0	0								
LTK 90-203	2	4	1	2	0	4	1	5	4	3	5	1	1	0							
JAM 90-253	24	26	23	21	26	25	22	27	26	25	27	25	23	26	0						
LK 92-372	1	3	1	1	3	5	2	4	3	2	4	2	2	3	2	5	0				
MK 92-534	0	2	0	0	1	4	0	3	2	1	3	0	0	1	2	3	1	0			
PLTK 93-416	0	2	0	0	2	4	1	3	2	1	3	1	1	2	2	4	1	0	0		
CAY 96-131	17	17	17	14	19	14	17	18	17	18	18	18	18	19	24	16	17	17	0		
PCHPCHU2	23	25	23	20	24	20	23	26	25	24	26	24	24	24	12	24	22	23	18	0	
PLTK LTU1	0	2	0	0	1	3	1	3	2	1	3	1	1	1	21	1	0	0	16	22	0

indistinguishable from two of the 12 cacti in the wild Little Torch Key population (LTK 90-192 and 90-193). It is not possible to say exactly which wild cacti may have provided material for the private collections, however, it is possible they are clones of one or both of these cacti. One of the cacti planted at Castello Hammock Park in Dade County, Florida (PCHP 89-620) could not be distinguished from one of the 12 cacti in the wild Little Torch Key population (LTK 90-198), suggesting that it may have originally been collected from Little Torch Key.

Within the smaller group, the Jamaican accession (JAM 90-253) and the other cactus planted at Castello Hammock Park (PCHP CHU2) differed at only 12 markers. Both accessions differed from the Florida accessions by an average of 24 markers, suggesting that PCHP CHU2 was probably not collected from Little Torch Key. The Cayman Brac accession (CAY 96-13 1) differed from the Florida accessions by an average of 16 markers, the Castello Hammock Park cactus (PCHP CHU2) by 18 markers, but differed from the Jamaican accession by 24 markers. Currently, we do not know any additional information about the Cayman Brac accession, but it clusters more closely with the Florida accessions than with the Jamaican accession.

The large number of marker differences observed among the Florida accessions and the Jamaican accession provides further evidence that the Florida population may not be 0. *spinosissima* as originally classified. The differences reported here are suggestive of possible species level differences,

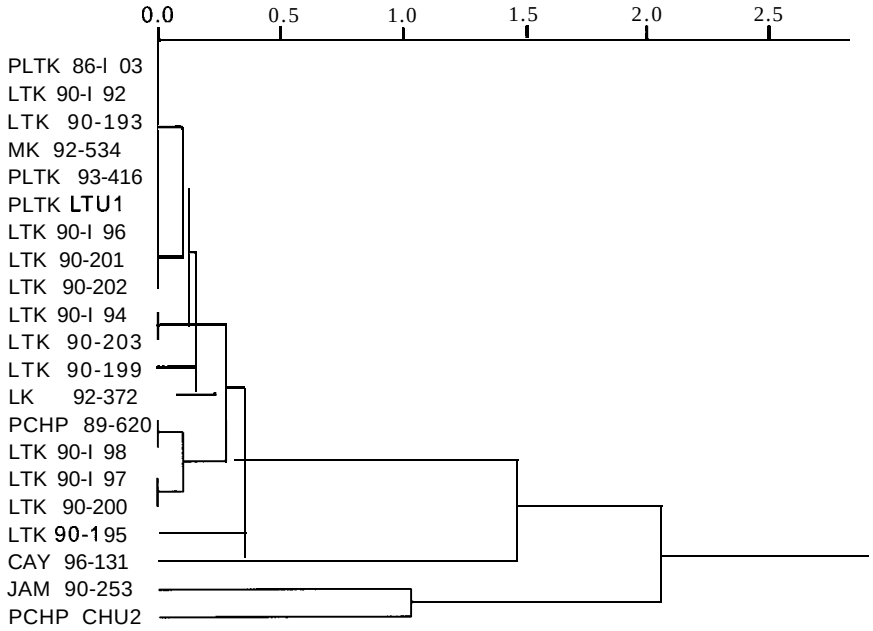

FIG. 2. Unweighted pair group mean cluster dendrogram constructed from a distance matrix based on data from 42 random amplified polymorphic DNA markers collected on 21 cacti accessions. Those accessions beginning with the prefix LTK were collected from Little Torch Key, those beginning with PLTK were from private collections on Little Torch Key, and those beginning with PCHP had been planted at Castello Hammock Park in Dade County, Florida. The accession beginning with PLK was from a private collection from an unspecified location in the Lower Keys, that beginning with MK was from lower Matecumbe Key, that beginning with CAY was from a private collection derived from Cayman Brac, and that beginning with JAM was collected from Jamaica.

however, we cannot overlook the fact that these differences may simply be due to sampling as only a single Jamaican accession was available.

The clustering pattern within the larger Florida-derived group is consistent with differences identified in Dougherty's (1996) *rbc*L DNA sequence work. The DNA sequence data suggested that Florida accessions LTK 90-194 and 90-199 are different, and that LTK 90-200 is different from 90-202 and 90-203. The RAPD marker data confirm the differences among these cacti, as well as providing additional evidence for further differentiation among the Little Torch Key accessions.

Comparison of the RAPD clustering pattern of individuals from the Little Torch Key population with their geographic location on the preserve reveals that geographic proximity does not always correlate with genetic relatedness. Some of the accessions within a meter to a few meters of each other (e.g., LTK 90-195 and 90-196 or LTK 90-200 and 90-201) are less related than are accessions approximately 50 m apart (e.g., LTK 90-192 and

90-201). Thus, assumptions about relatedness that might have been made based on location can now be avoided.

DISCUSSION—These data provide additional support that the Little Torch Key opuntia, if really a Florida Keys endemic species, may be among the most endangered plant species. The data also increase the likelihood that the remaining population is not the result of either an anthropogenic introduction or rafting material carried by storm events from the Caribbean, both of which would probably have been of one individual. Greater proximity and species exchange among the Caribbean islands and with the Florida Keys in earlier geologic times may have allowed spread and then isolation of the species (Dilcher, 1997). Loss of genetic variability through founder effects or inbreeding is possible. Regardless of origin, protection of the imperiled wild population and recovery of the species is critical.

Fortunately, several steps toward recovery are already underway. Viable seeds are now being produced in both the wild and ex *situ* populations. Current research is directed toward identifying the breeding system and should identify techniques for increasing successful pollination (Negron-Ortiz, 1997). Several introduced or re-introduced populations have now been established by Fairchild Tropical Garden and The Nature Conservancy. Differences among accessions in quantitative traits may now be evaluated from these out-plantings. On the preserve, four of the genotypes identified in this study were planted. Phenotypic differentiation in different environments may provide additional clarification of the differences among individuals.

The greatest threats to recovery of this species appear to be collection and predation by the introduced cactus moth, ***Cactoblastis cactorum.*** However, for a species not yet listed, much progress toward recovery has already been made.

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