Comparison of RAPD Marker Patterns To Morphological and Physiological Data In the Classification of *Opuntia* Accessions

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ABSTRACT

An analysis of morphological, physiological, and molecular data from five cactus fruit varieties from Mexico and Chile, two ornamental Texas accessions, and one vegetable accession from Mexico are presented. Molecular analysis was performed using the RAPD (random amplification of polymorphic DNA) method. Phenotypic and molecular analyses distinguished ornamental, vegetative, and fruit market accessions of *Opuntia ellisiana*, *O. lindheimerii*, *O. cochinellifera*, *O. hyptiacantha,* and *O. ficus-indica* from each other, and suggested significant differences among accessions of different market classes. Differences among fruit cultivars were smaller, and, in conjunction with the previously reported cross-compatibility among them, suggests that species designations may need to be reconsidered. The results demonstrate the feasibility of DNA fingerprinting in *Opuntia.*

Keywords: *Opuntia*, genetic diversity, DNA marker, genetics and breeding.

INTRODUCTION

The genus *Opuntia* consists of 170 species (Gibson and Nobel, 1986), with phenotypes varying greatly in ecology, reproduction (Scheinvar, 1995), and ploidy (2x, 3x, 4x, 6x, 8x, etc.) (Pimienta-Barrios and Munoz-Urias, 1995). *Opuntia* is distributed from below sea level in the deserts of California to elevations of over 4700 m in the mountains of Peru; and from tropical regions of Mexico, where temperatures are always above 5°C, to regions in Canada that experience -40°C each winter (Nobel, 1988; Keeley and Keeley, 1989). The great genotypic variability of *Opuntia* has arisen via natural hybridization associated with polyploidy and geographical isolation (Gibson and Nobel, 1986). *Opuntia* has become increasingly important as a crop, with many varieties used for exotic fruit, vegetable, and forage production in Mexico, the U.S.A., Chile, Argentina, Israel, Italy, Spain, and South Africa (Pimienta-Barrios and Munoz-Urias, 1995; Flores-Valdez, 1995; Felker, 1995). Modern commercial plantations have been established using vegetative material from outstanding phenotypes from backyards of rural homes (Pimienta-Barrios and Munoz-Urias, 1995). To facilitate genetic improvement of *Opuntia*, a germplasm collection and hybridization program has been initiated at Texas A&M University-Kingsville.

Classification of cacti in general, and *Opuntia* specifically, needs further work. There are approximately 11,000 binomials published, many of which are considered incorrect (Gibson and Nobel, 1986). Within the genus *Opuntia*, for example, the species *O. compressa* has been described by 35 different binomials. *O. cochenillifera* Miller is sometimes referred to as *Nopalea cochenillifera* (Linneaus) Salm-Dyck, and four additional binomials have been used previously, along with several synonyms (Britton and Rose, 1963). Reasons for improper classification include: Cactaceae, being a large family, is difficult to know thoroughly; poor representation or poor quality of specimens in herbaria due to the difficulty in drying and preserving succulent cladodes; and convergent evolution of morphological features in independent taxa (Gibson and Nobel, 1986).

In an experiment to test experimental conditions and determine whether molecular analysis can contribute to evaluation or improvement of *Opuntia*, we have performed a RAPD (random amplification of polymorphic DNA) analysis of eight selected *Opuntia* accessions, and compared results of molecular data to morphological and physiological data. The RAPD method was chosen because of its simplicity and applicability to a wide range of species without need for prior molecular work (e.g., cDNA or genomic libraries). RAPDs have proven useful for species identification, elucidation of genetic relationships of numerous plant species, and parentage testing (Williams *et al*., 1991; Halward *et al*., 1992; Keil and Griffin, 1994; Novy *et al*., 1994; Kindiger and Dewald, 1996; Cjuric and Smith, 1996; Levi and Rowland, 1997). Our immediate objective was to a determine whether polymorphism was sufficient to distinguish *Opuntia* accessions and to assess the patterns of genetic diversity among a selected group of accessions.

MATERIALS AND METHODS

Plant Material

Opuntia clones were collected from Texas, Mexico, and Chile and were planted in Kingsville, Texas. Eight clones were chosen for this study (Table 1). Accession 1464 is classified as *Opuntia ellisiana*; this species was described by Griffiths (1915) and was the subject of a detailed field water-use-efficiency study (Han and Felker, 1997). Accession 1464 is the most cold hardy of the spineless types in the Texas A&M collection, undamaged at B20EC in December 1989 in San Angelo, Texas, whereas all *Opuntia ficus-indica* and *O. robusta* types froze to the ground at -12°C, 500 km south in Kingsville. Accession 1233 (*Opuntia spp.*) is a thornless accession that survived freezing at B12EC. This clone has undulating cladode margins, is morphologically distinct from the wild spiny *Opuntia lindheimerii*, and is widely planted as an ornamental, but is not found in the wild. *Opuntia cochinellifera* (1308) is distinct from all the other accessions in having a corolla that does not open but stays appressed to the stigma, is the most frost sensitive of the eight varieties in this study, and has no glochids. The spineless accession 1281 is similar to *Opuntia ficus-indica* as described by Britton and Rose (1963) and Scheinvar (1995). However, 1281 differs significantly from *Opuntia ficus-indica* in cladode morphology and gross plant architecture. We are uncomfortable assigning this accession to *Opuntia ficus-indica* and thus list it as *Opuntia spp.* Thorny clone 1287 is an excellent specimen of *O. hyptiacantha* as described by Britton and Rose (1963). The accessions 1279, 1281, 1287, 1294, and 1321 have been used commercially to produce fruits in either Mexico or Chile.

Plant Measurements

Plant and fruit characteristics studied in six-year-old plants were cladode size, spine and glochids, cold hardiness, flower color, fruit color, fruit weight, TSS (total soluble solids) and pH of pulp, pulp/peel ratio, and 100 seed weight. Tests of means were performed by the least-significant-difference method, using a pooled error variance. Cladode frost survival was estimated as 100%, 75%, 50%, 25%, or 0% after a series of freezes in December 1996 and January 1997. As measured with electronic temperature data loggers, the minimum temperature was -7.8°C and there were 62 hr below 0°C, 11 hr below -4°C and 5 hr below -6.6°C from 12 December 1996 through 14 January 1997. Categorical phenotypes were scored as integers (geographic origin, 0=Texas, 1=Mexico, 2=Chile; spine and glochids, 0=absent, 1=present; flower color, 0=yellow, 1=red; fruit color was scored as 4 variables, each representing one color, to avoid assuming additive genetic effects for flower color). Integer data were combined with continuous data (listed in Table 3), and analyzed by cluster analysis using Pearson correlation as distance and the average linkage (UPGMA) method (Swofford and Olsen, 1990; Sokal and Michener, 1958).

DNA Extraction and Molecular Analysis

Cladodes were collected, sliced into portions of 2 g to 3 g each, frozen in liquid nitrogen, and stored at -80°C. DNA was extracted using the method of Bernatzky and Tanksley (1986), then passed

through Sephadex G-50 spin columns. DNA concentrations were determined by comparing with uncut phage l DNA standards (100 to 500 ng) in 0.8% agarose gels run at 22 Vdc (0.75 V/cm) for 16 hr., and visualized under ultraviolet light after staining with ethidium bromide.

DNA amplification and electrophoresis conditions were performed as described previously by Burow *et al*. (1996). Template samples that amplified uniformly using 2.5, 5, 10, 20, and 40 ng/reaction using primer UBC-162 (5=-AACTTACCGC-3=) were deemed of satisfactory quality and were used in further experiments. For analysis, 10 ng DNA samples were amplified in 10 ml final volume, including 3 ml of gelatin (1 mg/ml) added to each well before addition of DNA. Samples were electrophoresed in 1.6% agarose gels at 57 Vdc (2 V/cm) for 3 to 4 hr. DNA from two different clonal plants of each accession was evaluated, and only repeatable results were used for analysis. Polymorphic DNA bands were scored as present (1) or absent (0). Similarity among RAPD marker patterns of accessions was calculated as the simple matching dichotomy coefficient, r, (Apostol *et al*., 1993), in which a match is defined as both samples either possessing or lacking a given DNA fragment. This was converted to dissimilarity, 1 - r. UPGMA (unweighted pair group means analysis) was performed by SYSTAT version 7.0.1 (SPSS, Chicago, Ill.), by cluster analysis of the distance matrix using the average-linkage method.

RESULTS

PCR amplification of cactus DNA produced useful marker patterns, given sufficient care, and analysis gave strong evidence of major genetic differences among accessions representing fruit, vegetable, and ornamental market classes of *Opuntia*.

Distinct marker patterns were observed for different combinations of primers and *Opuntia* accessions (Figure 1) when a standard protocol was modified for cactus. DNA was purified initially by the method of Bernatzky and Tanksley (1986), but often required further purification through spin columns. Phenol-chloroform extraction did not improve quality, and reduced yield (data not shown). DNA recovery typically varied from 0.5 to 3 mg DNA per 2 g cladode. In addition, use of younger cladodes yielded better quality DNA. As a test of DNA quality, only samples that amplified uniformly over concentrations of 2.5 to 40 ng/reaction were used.

Approximately fifty 10-base RAPD primers were tested for utility, and 22 of these produced bands that were legible and reproducible. From these, a total of 95 reproducible DNA fragments were scored, of which 74 (78%) revealed variation among the full set of 8 accessions and were retained for analysis of genetic variation. From 38 to 49% of these markers were polymorphic between fruit and ornamental clones, but only 18% revealed variation among the 5 fruit clones. Pairwise polymorphism between fruit varieties ranged from 1.5% to 6.0%.

Cluster analysis of the accessions demonstrated distinct grouping of the accessions into fruit and ornamental types (Figure 2). The one vegetable clone, 1308, did not cluster tightly with either fruit or ornamental material, but was more similar to the cultivated material. Differences among fruit clones were relatively small and did not correspond with species designations.

Observation of morphological and physiological characteristics of the eight *Opuntia* accessions examined revealed considerable differences that were correlated with the market class of the accession (Table 3, Figure 1). Cladodes of vegetable accession 1308 and ornamental accession 1464 were shorter and thinner than those of fruit accessions 1279, 1294, 1321, 1281, and 1287, which were of similar lengths. All accessions except the vegetable type 1308 had glochids. The most frost-resistant accessions were the ornamental types 1233 and 1464. Both mature and immature cladodes of accession 1308 were frost sensitive. The greatest differences observed were in fruit characteristics. Ornamental accessions did not produce commercially-acceptable fruit, as fruit weights were less than 25% of the weight of the fruit types. Ornamental types also differed from fruit types in pulp/peel ratio, total soluble solids, and fruit pH. The cluster tree of 7 clones (clone 1308 was excluded due to difficulty in obtaining fruit measurements on its small, inedible fruits) is

shown in Figure 1. The ornamental and fruit accessions are separated into distinct branches on the tree.

DISCUSSION

The data demonstrate the feasibility of DNA fingerprinting in cactus. Distinct, reproducible differences were observed, and three-fourths of the bands were polymorphic among the 8 clones tested. In addition, all eight accessions could be identified uniquely by their DNA banding patterns. This illustrates that molecular analyses can be useful in cultivar identification and recognition of duplicate accessions in collections.

Future experiments may benefit from changes to experimental methods. Improvements in DNA extraction would be beneficial, as several DNA extractions were sometimes required to obtain satisfactory DNA. In addition, results had to be repeated to check for reliability, because of the inherent variability in amplification using short primers. Other methods of marker analysis can be considered. Use of RFLP analysis (Beckmann and Soller, 1980), while requiring generation of a cDNA library and use of a radioisotope, is more reliable and allows for comparison of chromosome structural conservation across species and genus boundaries (Paterson *et al.*, 1996). AFLP analysis (Vos *et al*., 1995) is a more accurate nonisotopic PCR-based substitute for RAPD analysis.

Comparisons of molecular to morphological and physiological data produced generally similar conclusions of relatedness among accessions, confirming the utility of molecular analysis. Results indicated that fruit accessions were closely related to each other. The vegetable accession and ornamental accessions were distinct from fruit clones, but less closely related to each other. This was in agreement with the results of artificial hybridization studies, in which hybridization among these fruit clones was more successful than crosses between the fruit and ornamental clones (Wang *et al*., 1996).

In comparing fruit types, there were differences in grouping of accessions based on marker *vs.* morphological and physiological data. Three factors may contribute to this. Some of the phenotypic traits (fruit size and TSS) have been selected under domestication. Therefore, phenotypic similarity may not reflect evolutionary relationships across the entire genome that are more likely to be detected by molecular markers. Additionally, phenotypic data consisted primarily of fruit and cladode measurements, and did not represent a random sampling of gene effects. Finally, phenotypic expression may be influenced by many non-genetic factors that are not expected to affect DNA marker patterns.

Given the preliminary nature of the current work, it was not possible to use markers to differentiate fruit varieties by species or by geographic origin. One serious limitation was use of one or two accessions to represent each species or region, too few to be representative of the range of variation expected to exist. As important, however, was the relatively small degree of difference among fruit varieties, which was also not consistent with the current species classification. The most similar accessions based on RAPD patterns (1281, 1287, and 1321) were designated as two or three different species, from two different countries. Clones 1279, 1294, and 1321, all considered to be *Opuntia ficus-indica,* were not the most closely related among the clones tested.

In conjunction with the similarity in morphological measurements and marker patterns of the five fruit accessions, one may question whether the five fruit accessions represent different species. Based on morphology and physiology, accession 1287 (*O. hyptiacantha*) is closer to 1279 and 1294 (both *O. ficus-indica*) than is 1321 (*O. ficus-indica*). Furthermore, there was no significant difference in either fruit set from hybridization from crosses of different pairs of fruit clones or in F_1 seed germination, except for low seed germination in the cross 1281 X 1321 (Wang *et al*., 1997). Additional evidence suggesting the need to reconsideration the classification of some accessions involves a cross between two *O. amyclea* clones, in which progeny matched the morphological typing of 5 different "species" (Facundo Barrientos Perez, personal communication). The low level

of DNA variation among the 5 fruit cacti, from different countries and considered to represent 3 different species, shows that even at this early stage of domestication there may already have been a considerable "genetic bottleneck" in the gene pool of fruit cacti.

The experiment presented herein demonstrates the potential usefulness of molecular markers in classification of cactus accessions, and indicates the feasibility of a comprehensive effort to determine the relationships among *Opuntia* species using molecular markers. Further collection, evaluation, and utilization of additional germplasm, especially wild germplasm, is clearly a high priority in building the foundation for *Opuntia* improvement. Increased scope of results would be possible by the use of at least two, and preferably more, accessions per species. Use of parsimony analysis would aid in interpretation of the statistical significance of genetic relatedness.

Molecular methods may prove useful also for accelerating transfer of economically important traits from wild germplasm to cultivated *Opuntia* species by marker-assisted selection. Given the long intergenerational time in many cacti, significant time could be saved by selection for markers associated with desired traits. Finally, molecular markers may be useful in verifying hybridity. In some *Opuntia* species, reproduction may occur by either apomixis or amphimixis (Mondragon-Jacobo and Pimienta-Barrios, 1995; Velez-Gutierrrez and Rodriguez-Garay, 1996). DNA markers will be beneficial in determining the mode of reproduction, and providing proof that putative hybrids are not the results of apomixis.

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TABLES

Table 1. *Opuntia* Clones Used in This Study

Accession	Species	Market Use	Collection Location
1279	*O. ficus-indica*	Fruit	Chapingo, Mexico
1281	*O. spp.*	Fruit	Chapingo, Mexico
1287	*O. hyptiacantha*	Fruit	Mexico
1294	*O. ficus-indica*	Fruit	Milpa Alta, Mexico
1321	*O. ficus-indica*	Fruit	Chile
1308	*O. cochenillifera*	Vegetable	Mexico
1233	*O. lindheimeri var. inermis*	Oramental, Forage	Hargil, Texas, USA
1464	*O. ellisiana*	Ornamental	Texas, USA

Table 2. RAPD Primers Used and the Number of Scorable Markers Amplified

Primer	Sequence	Total Markers	Polymorphic Markers	
			Among 8 Clones	Among 5 Fruit Clones
UBC-162	AACTTACCGC	7	4	1
UBC-204	TTCGGGCCGT	2	1	0

UBC-211	GAAGCGCGAT	10	10	2
UBC-212	GCTGCGTGAC	1	1	0
UBC-213	CAGCGAACTA	3	3	0
UBC-225	CGACTCACAG	5	4	0
UBC-226	GGGCCTCTAT	6	5	0
UBC-227	CTAGAGGTCC	7	6	2
UBC-230	CGTCGCCCAT	4	2	0
UBC-231	AGGGAGTTCC	8	5	1
UBC-232	CGGTGACATC	6	4	1
UBC-235	CTGAGGCAAA	3	3	0
UBC-238	CTGTCCAGCA	1	1	0
UBC-239	CTGAAGCGGA	2	2	0
UBC-241	GCCCGACGCG	1	1	0
UBC-243	GGGTGAACCG	3	2	0
UBC-245	CGCGTGCCAG	8	5	0
UBC-246	TATGGTCCGG	2	2	1
UBC-248	GAGTAAGCGG	3	3	2
UBC-259	GGTACGTACT	3	3	1
UBC-261	CTGGCGTGAC	5	5	0
UBC-264	TCCACCGAGC	5	2	1

Table 3. Plant and Fruit Characteristics of *Opuntia* Clones

Accession	**Cladode Measurements[1]**					**Cold Hardiness (survival %)[2]**	
	Length (cm)	**Width (cm)**	**Thickness (cm)**	**Spine[3]**	**Glochids[3]**	**Immature**	**Mature**
1279	35 a	19 b	2.7 ab	-	+	75 ab	100 a

1281	36 a	18 bc	2.6 ab	-	+	85 ab	100a
1287	38 a	20 b	3.1 a	+	+	90 ab	100a
1294	38 a	17 bc	2.7 ab	-	+	85 ab	100a
1321	36 a	16 bc	3.1 a	-	+	70 b	100a
1308	23 b	12 c	1.1 b	-	-	0 c	0c
1233	34 a	26 a	1.9 b	-	+	100 a	100a
1464	17 b	13 c	1.7 b	-	+	100 a	100a

Fruit Characteristics

Accession	Flower Color	Fruit Color	100-Seed Weight (g)	Fruit Weight (g)	Pulp/peel Weight (g)	TSS	pH
1279	Red	Purple	1.76	121 ab	0.6 b	13.2 b	5.6 a
1281	Yellow	Red	1.73	105 b	1.0 a	13.7 b	5.7 a
1287	Yellow	Orange	2.41	138 a	0.8 ab	14.2 ab	5.9 a
1294	Yellow	Orange	1.65	121 ab	0.9 a	13.0 b	5.8 a
1321	Yellow	Lt. yellow	1.65	103 b	0.7 b	16.2 a	5.7 a
1308	Yellow	n.d.[4]	n.d.[4]	n.d.[4]	n.d.[4]	n.d.[4]	n.d.[4]
1233	Yellow	Purple	1.13	26 c	0.2 c	6.1 c	4.6 c
1464	Yellow	Purple	0.3	14 c	0.2 c	8.0 c	[illegible]

							b

[1] Values are the means (n=6 or n=10). Different letters following numbers indicate statistically significant differences among means.

[2] Cladode survival was estimated as 100%, 75%, 50%, 25%, or 0% per experiment after a freeze of -7.6°C in December 1996, as described in the methods section. Fruit data were collected in 1996.

[3] Present (+); absent (-)

[4] n.d., not determined

FIGURES

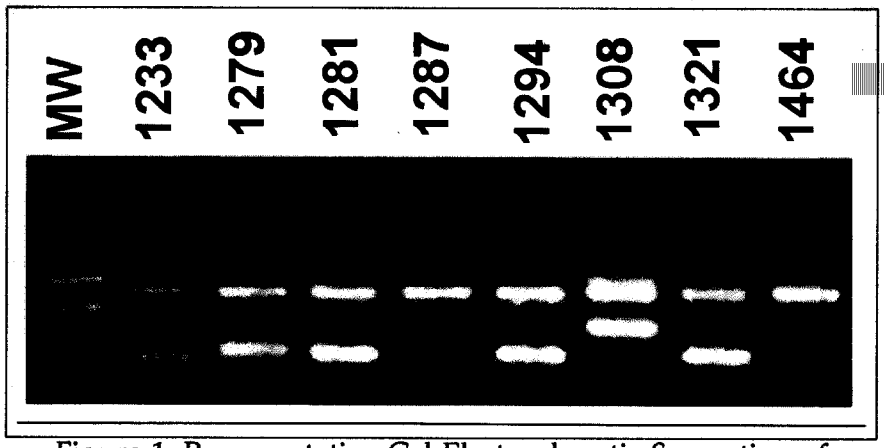

Figure 1. Representative Gel Electrophoretic Separation of Amplification Products of *Opuntia* Accessions. Amplification was performed using primer UBC-259 (5=-GGTACGTACT-3=). MW denotes size markers, HaeIII-digested phage ΦX174. Marker sizes are 1353, 1078, 872, and 603 (faint) bp.

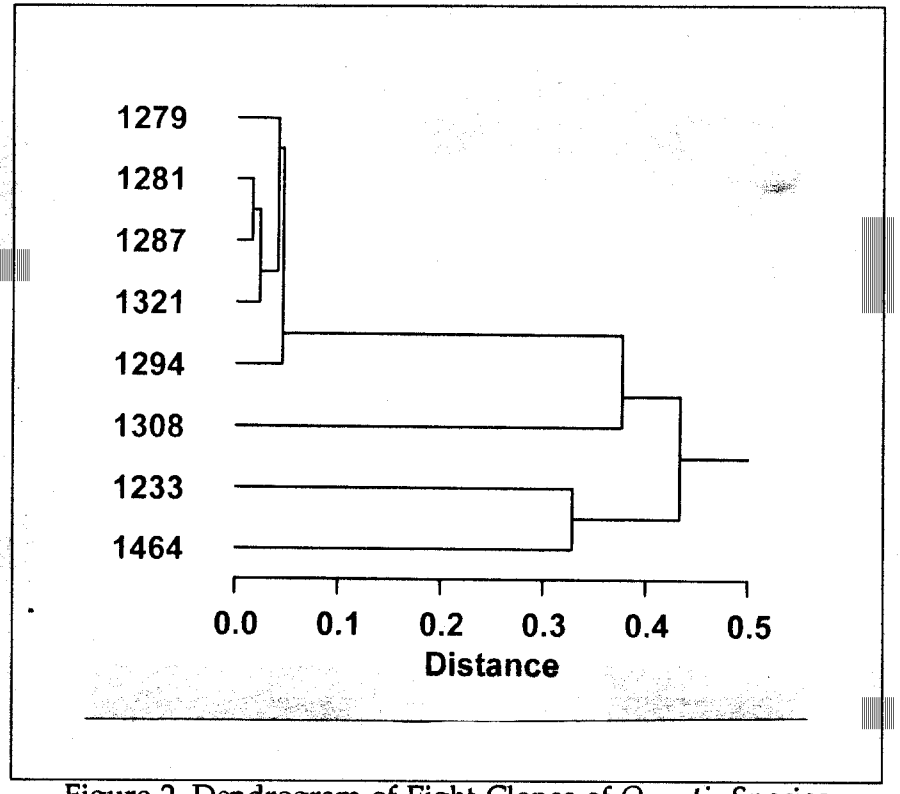

Figure 2. Dendrogram of Eight Clones of *Opuntia* Species Derived from UPGMA Analysis of Distance Value, Based on RAPD markers

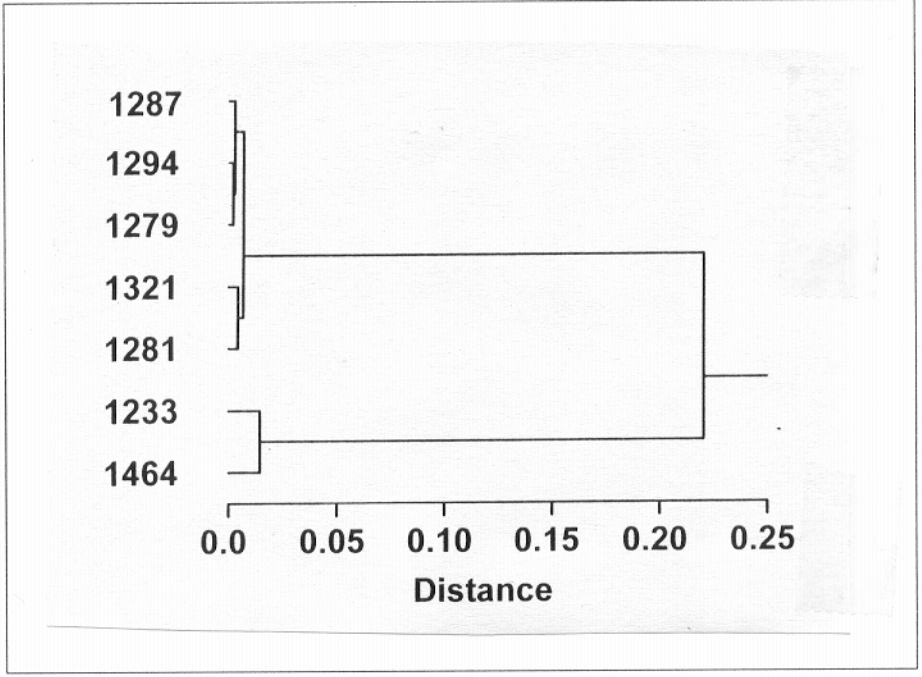

Figure 3. Dendrogram of Seven Clones of *Opuntia* Species From Morphological and Physiological Data. Cluster analysis was performed using Pearson correlation and average linkage (UPGMA).