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USING MOLECULAR EVIDENCE TO ELUCIDATE RETICULATE EVOLUTION IN *OPUNTIA* (CACTACEAE)

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ABSTRACT

The hypothesized natural interspecific hybrid origin of two cacti, *Opuntia* ×*rooneyi* and *O.* ×*spinosibacca*, has been investigated by a variety of non-molecular based techniques. Herein I explore DNA sequence and random amplified polymorphic DNA (RAPD) banding pattern data as it relates to these two cases of putative hybridization. Traditional parsimony-based analyses of nuclear ribosomal and chloroplast DNA sequences do not resolve the phylogenetic position of these two species among closely-related taxa, but a median network analysis is presented that yields an approach to interpreting these relationships. Finally, an analysis of RAPD banding pattern data provides evidence of the additive genetic pattern expected for these two interspecific hybrids. These results support the inferences of non-molecular based studies.

Key Words: Hybridization, parsimony analysis, median network, *Opuntia*, RAPDs.

Hybridization has long been thought to contribute to biodiversity, often leading to the formation of new taxa (Keck 1937; Grant 1954; Lewis and Lewis 1955; Stebbins 1957, among many others). Many types of data have been used to infer hybrid origin of taxa. Morphological data far surpass any other data type in giving researchers inference into hybrid origin, but geographical, ecological, biosystematic, and cytological data have also given insight into reticulate speciation. More recent work downplays the importance of these data types, instead stressing that (macromolecular) genetic evidence confers a greater (and independent) power of inference (Gallez and Gottlieb 1982; Barker et al. 1996; Allan et al. 1997). Putative hybrid taxa have been examined with a variety of genetic techniques, including enzymatic methods (Gallez and Gottlieb 1982; Barrington 1990; Krutovskii and Bergmann 1995), restriction fragment length polymorphisms (Kron et al. 1993; Milne et al. 1999), random amplified polymorphic DNA (RAPD) data (Barker et al. 1996; Díaz Lifante and Aguinagalde 1996; Padgett et al. 1998), and DNA sequence data (Alice et al. 2001).

Cacti illustrate this progression of methodologies particularly well. Hybridization is a well-established means of establishing novel taxa of Cactaceae (Moran 1962; Rowley 1982, 1994; Powell et al. 1991; Powell 1995, 1999). Hybridization as a means of speciation is especially common within the subfamily Opuntioideae (Benson and Walkington 1965; Grant and Grant 1971, 1979; Baker and Pinkava 1987, 1999; Griffith 2001a, b; Pinkava 2002; Hernandez et al. in press). Many of the above types of evidence have previously been used to investigate the hybrid origins of opuntioid taxa, including morphological (Walkington 1966; Grant and Grant 1979; Baker and Pinkava 1987; Hernandez et al. in press), phytochemical (Walkington 1966), geographical (Hernandez et al. in press), artificial hybridization (Griffith 2001b), cytological evidence (Baker and Pinkava 1987; Pinkava and Parfitt 1988), and more recently, molecular evidence in the form of RAPD banding pattern data (Mayer et al. 2000).

At least two Northern Chihuahuan Desert taxa of *Opuntia* sensu stricto are believed to be of hybrid origin. *Opuntia* ×*spinosibacca* is believed to be the result of hybridization between *O. camanchica* and *O. aureispina* (Pinkava and Parfitt 1988; Powell 1998; Powell and Weedin 2001), and *O.* ×*rooneyi* is thought to be the result of hybridization between *O. aureispina* and *O. macrocentra* (Griffith 2001a). The ranges of these putative hybrid taxa intersect with those of the putative parental taxa (Fig. 1). In addition, the hybrid taxa exhibit intermediate morphology between their putative parents (Table 1), and in the case of *O.* ×*spinosibacca*, intermediate karyotype; *O. camanchica* is hexaploid ($2n = 66$), *O. aureispina* is diploid ($2n = 22$), and *O.* ×*spinosibacca* is tetraploid ($2n = 44$) (Pinkava and Parfitt 1988; Powell and Weedin 2001). For the current study, I explored the putative hybrid origin of *O.* ×*spinosibacca* and *O.* ×*rooneyi* through the analysis of DNA sequence data and RAPD banding pattern data.

MATERIALS AND METHODS

I extracted total genomic DNA from specimens of *Opuntia* representing the hybrid taxa *O.* ×*spinosibacca* and *O.* ×*rooneyi*, and putative parental

TABLE 1. MORPHOLOGICAL CHARACTERS OF *OPUNTIA* SPECIES INCLUDED IN THIS STUDY. Data adapted from Powell (1998) and Griffith (2001a).

	Central spines per areole, length	Radial spines per areole, length	Spination	Spine color	Pad color
O. macrocentra	1–2, 7–12 cm	0	most distal areoles	black, white tips	purple
O. ×rooneyi	2–3, 4–5 cm	1–4, to 2 cm	upper two thirds of areoles	brown to red, yellow tips	green-purple
O. aureispina	3–6, 2–6 cm	2–7, to 3 cm	over entire cladode	uniformly yellow	yellow-green
O. ×spinosibacca	2–5, to 7 cm	0	most of cladode	red to brown	yellow-green
O. camanchica	1–3, 3–7 cm	0	upper half of cladode	dark brown	bluish-green

taxa. Additional DNA extractions were performed, for a total of 29 specimens of opuntioid taxa for use in phylogenetic comparisons (Table 2). I employed a protocol (Griffith and Porter 2003) for nucleic acid extraction from mucilaginous tissues, modified from Doyle and Doyle (1987). Amplification of templates for sequencing was performed with the primers *trnE*, *trnF* (Taberlet et al. 1991), ITS5, and ITS4 (White et al. 1990). Thermal cycling parameters for the nrITS and *trnL-F* regions follow Columbus et al. (1998) and Porter et al. (2000), respectively. Templates were purified by precipitation in PEG (Morgan and Soltis 1993), and washing once in 100 μl 80% ethanol. Purified templates were then sequenced directly with 6 primers: ITS5, ITS4, ITS3, and ITS2 (White et al. 1990), *trnE* and *trnF* (Taberlet et al. 1991). For cycle sequencing, I used ''Big Dye'' chemistry (Applied Biosystems), according to the manufacturer's specifications. An Applied Biosystems 3100 Genetic Analyzer gathered all DNA sequence data. Raw sequences were assembled into contigs and edited using Sequencer v4.1 (Gene Codes Corporation). The program Se-Al (Rambaut 1996) assisted with man-

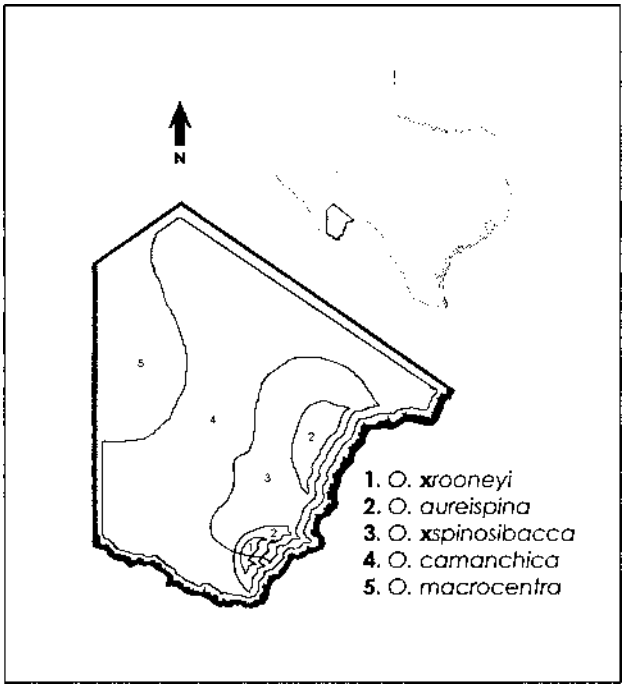

FIG. 1. Ranges of two putative hybrid *Opuntia*, *O. ×rooneyi* and *O. ×spinosibacca*, and putative parental taxa *O. aureispina*, *O. camanchica*, and *O. macrocentra* in Brewster County, Texas, USA. *Opuntia macrocentra* is distributed throughout the county, and *O. camanchica* is found throughout, except the northwestern portion; the other taxa are more restricted in range. Adapted from Griffith (2000).

TABLE 2. SPECIMENS USED FOR MOLECULAR ANALYSES. DBG = Desert Botanical Garden, Phoenix, Arizona; HBG = Huntington Botanical Gardens, San Marino, California; MG = Mesa Garden, Belen, New Mexico.

Taxon	Specimen	Voucher
Brasiliopuntia brasiliensis (Wildenow) Berger	DBG 1990-0559	
Consolea spinosississima (Miller) Lemaire	DBG 1995-0389	
Nopalea cochenillifera (L.) Salm-Dyck	DBG 1997-0395	
Miqueliopuntia miquelii (Monville) Ritter	DBG 1997-0129	
Opuntia aureispina (Heil & Brack) Pinkava & Parfitt	*Griffith 73*	SRSC
Opuntia boldinghii Britton & Rose	DBG 1977-0391	
Opuntia bravoana Baxter	HBG 47063	
Opuntia camanchica Engelmann & Bigelow	*Weedin 374*	SRSC
Opuntia chisosensis (Anthony) D. J. Ferguson	*Powell 5771*	SRSC
Opuntia durangensis Britton & Rose	*Griffith 156*	RSA
Opuntia engelmannii Salm-Dyck ex Engelmann	*Powell 6009*	SRSC
Opuntia erinacea Engelmann & Bigelow	*Honer 658*	RSA
Opuntia ficus-indica (L.) Miller	*Griffith 326*	RSA
Opuntia lindheimeri Engelmann	*Weedin 1670*	SRSC
Opuntia macrocentra Engelmann	*Raun 94-01*	SRSC
Opuntia megasperma Howell	DBG 1994-0075	
Opuntia phaeacantha Engelmann	*Griffith 214*	RSA
Opuntia pubescens Wendland ex Pfeiffer	*Griffith 308*	RSA
Opuntia pumila Rose	DBG 1999-0035	
Opuntia ×*rooneyi* M. P. Griffith	*Griffith 71*	SRSC
Opuntia santa-rita (Griffiths & Hare) Rose	*Griffith 227*	RSA
Opuntia setispina Engelmann ex Salm-Dyck	*Griffith 145*	RSA
Opuntia ×*spinosibacca* Anthony	*Hughes 801*	SRSC
Opuntia strigil Engelmann	*Powell 6008*	SRSC
Opuntia sulfurea G. Don	DBG 1995-0372	
Opuntia stricta (Haworth) Haworth	HBG 71091-1	
Pterocactus decipiens Gürke	MG 1179.2	
Pterocactus valentinii Spegazzini	MG 1179.68	
Tunilla corrugata (Salm-Dyck) Hunt & Iliff	*Hunt 66371*	DES

ual alignment of consensus sequences. Phylogenetic relationships among sequences were determined by a heuristic search of the sequence data using Fitch parsimony, as implemented by PAUP 4.10b (Swofford 1998). Estimations of confidence in the recovered clades were obtained by bootstrapping (Felsenstein 1985) with 1,000 pseudoreplicates, as implemented in PAUP. In addition, the median network (Bandelt et al. 1995) of all possible pathways among these sequences was constructed using Spectronet v1.2 (Langton 2001), pruned to k=3 (Bandelt et al. 1995) to enable interpretation.

The RAPD method (Williams et al. 1990) was also used to scan for genetic additivity. Stringent amplification, visualization, and scoring conditions were maintained to ensure repeatability (Hadrys et al. 1992). Each 25 μl reaction contained PCR buffer at 1.5 mM MgCl, 2.5 mM of each dNTP, 20 mM of a specific primer, 1 unit of *Taq* polymerase, and 10 ng of template DNA. A 96-well Robocycler (Strategene, Inc.) provided thermal cycling: 94°C for two minutes, followed by 44 cycles at 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min, and a final extension of 7 min at 72°C. Fifteen 10 mer primers were used for RAPD amplifications: Op-B-18, Op-D-2, Op-D-5, UBC-101, UBC-103, UBC-108, UBC-111, UBC-149, UBC-165, UBC-188, UBC-190, UBC-417, UBC-446, and UBC-489 (Fritsch et al. 1993). Entire 25 μl reactions were loaded onto 2.0% agarose gels, immersed in 1× TBE, and electrophorectically separated with 30 mV for 12 h. Gels were stained with ethidium bromide for 15 min, destained in water for 30 min, visualized under UV light, and photographed. Rf values of observed bands were compared with Rf values of known molecular weight markers to estimate weight of observed amplicons. For each primer, each band was scored as either present or absent for each molecular weight. Only markers which could be scored unambiguously for presence or absence were used to estimate additivity (Friar et al. 1996; Robichaux et al. 1997). Banding patterns were interpreted manually, and statistically analyzed for correlation and factor analysis using Statview 5.0.1 for Windows (SAS corporation, Chicago, IL).

RESULTS AND DISCUSSION

Phylogenetic analysis of the aligned DNA data matrix yielded a well supported (94% bootstrap) clade of *Opuntia* native to the Chihuahuan and Sonoran Desert regions of North America (with the notable exception of *O. boldinghii*, a native of the Caribbean), which includes both putative hybrid taxa, as well as all three putative parental taxa (Fig. 2). The combined ITS and *trnL-F* data do not resolve the relationships among this monophyletic

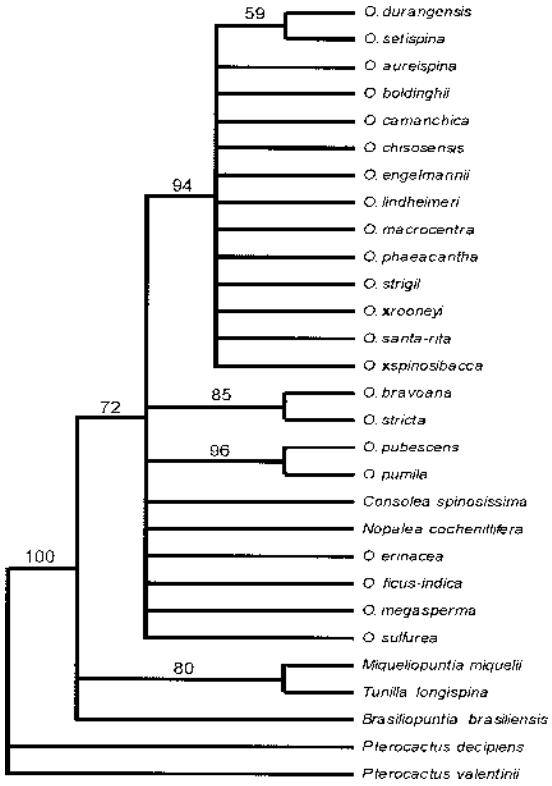

FIG. 2. Phylogenetic analysis of *Opuntia* ×*rooneyi*, *O.* ×*spinosibacca*, putative parental taxa, and related taxa. A strict consensus of 931,500 most parsimonious trees produced by the heuristic search of the combined ITS and *trnL-F* sequence data. Figures above branches represent bootstrap percentages above 50. With uninformative characters excluded, length = 51; CI = 0.8763; RI = 0.9048.

group. Median network analysis is useful for resolving relationships between closely related individuals, however (Bandelt et al. 1999); this analysis of the same data produced an unrooted network showing all most parsimonious pathways between the ITS and *trnL-F* sequences for these taxa (Fig. 3). Note that sequences obtained from the putative hybrid taxa *O.* ×*rooneyi* and *O.* ×*spinosibacca* are very proximal to those obtained from putative parental taxa. *Opuntia* ×*rooneyi* is merely one step removed from both putative parental taxa *O. aureispina* and *O. macrocentra*. *Opuntia* ×*spinosibacca* is one step removed from one putative hexaploid parent, *O. camanchica*, and two steps removed (via one of two shortest pathways) from another putative hexaploid parent, *O. phaeacantha* var. *major*, and the putative diploid parent, *O. aureispina*. Although this analysis cannot resolve reticulate evolution (via genetic additivity, *sensu* Gallez and Gottlieb 1982), the short pathways between the putative parental and hybrid taxa suggest a close relationship among these entities, consistent with the morphological, geographical, and (in the case of *O.* ×*spinosibacca*) cytological evidence of hybridization.

TABLE 3. SUMMARY OF OBSERVED RAPD BANDING FOR *O.* ×*ROONEYI* AND PUTATIVE PARENTAL TAXA.

	O. ×*rooneyi*	*O. aureispina*	*O. macrocentra*
Total bands:	53	26	44
Unique bands:	9	0	13
Shared bands with *O.* ×*rooneyi*	—	21	31
Private bands with *O.* ×*rooneyi*	—	10	19
Private bands between *O. aureispina* and *O. macrocentra*		1	

Banding patterns obtained by RAPD analysis can be used to screen for genetic additivity (Mayer et al. 2000). Of the fifteen primers used for the RAPD analysis, a total of 53 bands were scored for *O.* ×*rooneyi* (Table 3), and 51 were scored for *O.* ×*spinosibacca* (Table 4). An example of the banding pattern observed by amplification with a specific primer is presented in Fig. 4. A correlation matrix of the banding pattern data is presented in Table 5, and a factor plot of these data is presented in Fig. 5. The two putative parents of *O.* ×*rooneyi* (*O. aureispina* and *O. macrocentra*) had a total of 26 and 44 bands present, respectively. Few unique bands (17% of the total) were observed for this complex, and a high proportion (73%) of the bands present in the parental taxa was also observed in *O.* ×*rooneyi*. More relevantly, 41% of the bands observed in the parental taxa are privately shared with the putative hybrid *O.* ×*rooneyi*, while only 1 parental band (1.4%) is privately shared between the parental taxa (Table 3). Thus, while a sizable portion of bands observed in the putative hybrid has identity with the putative parents, the parents have little identity with each other. Additionally, high correlation with *O. aureispina* and *O. macrocentra* was found for *O.* ×*rooneyi* when analyzed

TABLE 4. SUMMARY OF OBSERVED RAPD BANDING FOR *O.* ×*SPINOSIBACCA* AND PUTATIVE PARENTAL TAXA.

	O. × *spinosibacca*	*O. aureispina*	*O. camanchica*
Total bands:	51	26	46
Unique bands:	12	7	9
Shared bands with *O.* ×*spinosibacca*	—	16	33
Private bands with *O.* ×*spinosibacca*	—	6	23
Private bands between *O. aureispina* and *O. camanchica*		3	

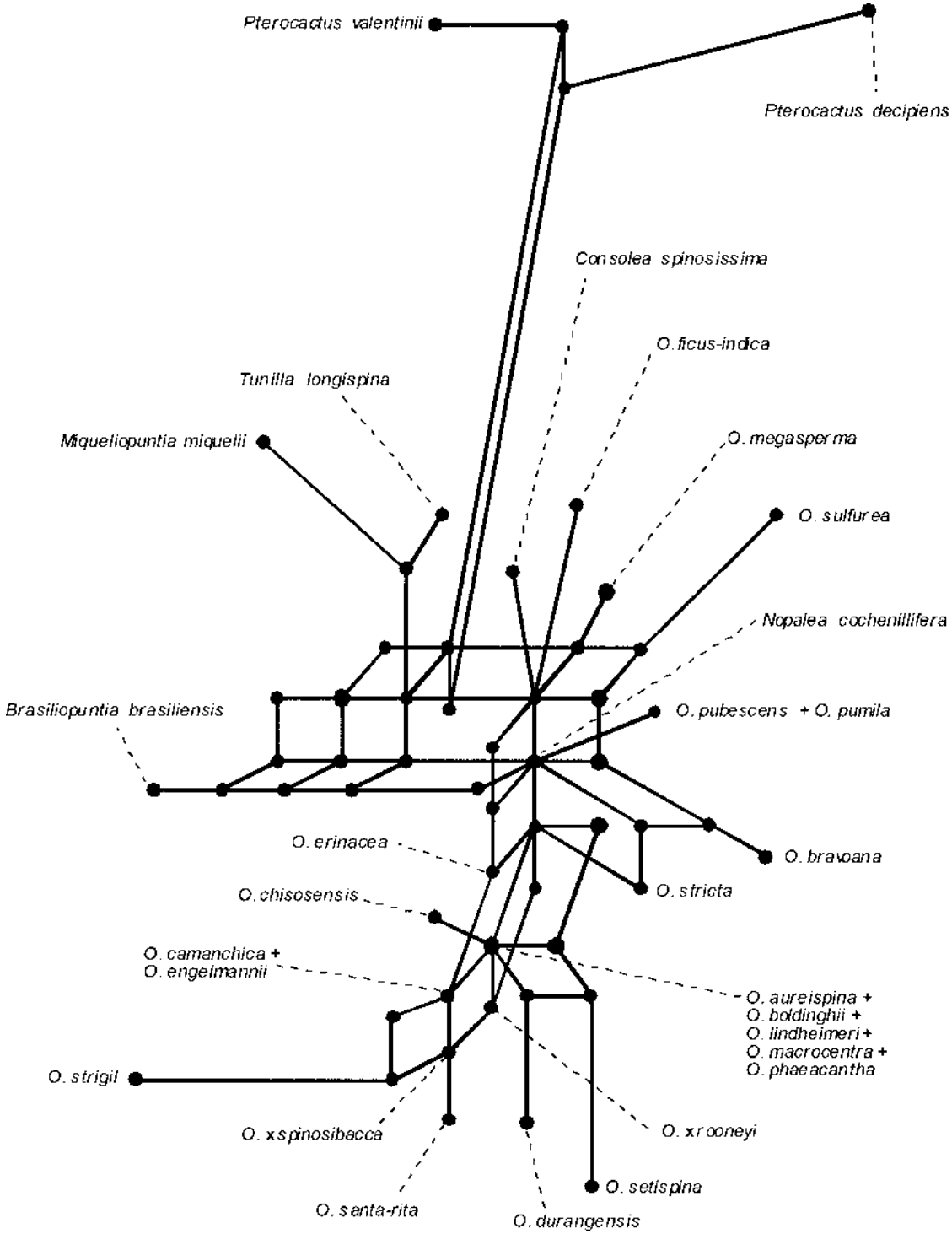

FIG. 3. Median network showing all most-parsimonious pathways between the combined ITS and *trnL-F* sequences for the sampled *Opuntia* taxa (Table 2). Points that correspond to observed sequences are labeled with those taxa; unlabeled points represent hypothetical sequences that must be ''passed through'' in order to reach other observed sequences.

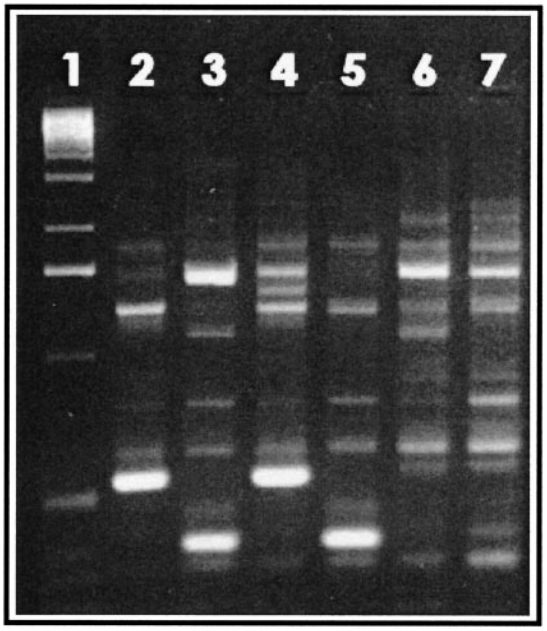

FIG. 4. Example RAPD amplification: six specimens of *Opuntia* amplified with primer Op-B-18. Lanes are as follows: 1) molecular weight markers; 2) *O. aureispina*; 3) *O. macrocentra*; 4) *O. ×rooneyi*; 5) *O. macrocentra*; 6) *O. camanchica*; 7) *O. ×spinosibacca.*

statistically (Table 5), while negative correlation was observed with *O. camanchica* and *O. ×spinosibacca.* A factor plot of these data (Fig. 5) shows a proximal and somewhat intermediate position for *O. ×rooneyi* between its putative parents. Although these data are consistent with the additive genetic pattern expected for hybrid speciation, the restricted sample warrants caution against interpreting these results alone as conclusive evidence of the hybrid origin of *O. ×rooneyi.* However, when these data are viewed in context with the morphological intermediacy (Griffith 2001a), observed interfertility of the parental taxa (Griffith 2001b), and the phylogenetic and network analyses presented above, the hypothesis of hybrid origin for *O. ×rooneyi* is difficult to reject.

Similarly, RAPD banding patterns observed for *O. ×spinosibacca* (Table 4) are consistent with the hybrid origin of that taxon. For this hybrid complex, a total of 28 unique bands were observed overall (22% of the total). The putative parents *O. camanchica* and *O. aureispina* shared a total of 33 (71%) and 16 (62%) bands respectively with the putative hybrid, and 23 (50%) and 6 (23%) of those bands (respectively) were privately shared. Only 3 (4%) of the parental bands are privately shared between the parental taxa. It is interesting to note that a much greater number of bands present in *O. ×spinosibacca* have identity with one parent, *O. camanchica.* This strong identity is also apparent in the factor plot (Fig. 5) and correlation matrix (Table 5). The RAPD banding pattern of *O. ×spinosibacca* correlates positively with both putative parents, and correlates negatively with the other two taxa (Table 5). As in the example above, these data are not conclusive proof of hybrid origin on their own, but in context with the morphological intermediacy (Powell 1998), cytogenetic intermediacy (Pinkava and Parfitt 1988; Powell and Weedin 2001), and the phylogenetic and network relationships presented above, *O. ×spinosibacca* certainly seems to represent a product of reticulate evolution.

Further investigations, involving increased sample sizes, increased sampling of genes, and perhaps other genetic techniques may give increased insight into the exact nature of the hybridization events investigated here; and perhaps growing artificial F1 hybrids to maturity (as in Powell et al. 1991) would also yield valuable information. A broad study using all of the above techniques with very inclusive sampling would be a desirable project that could greatly expand our understanding of how reticulate evolution occurs in cacti. While molecular data is often useful for evaluating hybrid origins of taxa, such investigations may greatly benefit from the integration of as many additional data types as possible (see also Pinkava 2002).

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TABLE 5. CORRELATION MATRIX OF RAPD BANDING PATTERN DATA. [1] *macro. = O. macrocentra*; *roon. = O. ×rooneyi*; *aurei. = O. aureispina*; *spino. = O. ×spinosibacca*; *caman. = O. camanchica.*

	macro.[1]	*roon.*	*aurei.*	*spino.*	*caman.*
O. camanchica	−.131	−.040	−.070	.338	1.000
O. ×spinosibacca	−.154	−.045	.014	1.000	
O. aureispina	−.046	.317	1.000		
O. ×rooneyi	.277	1.000			
O. macrocentra	1.000				

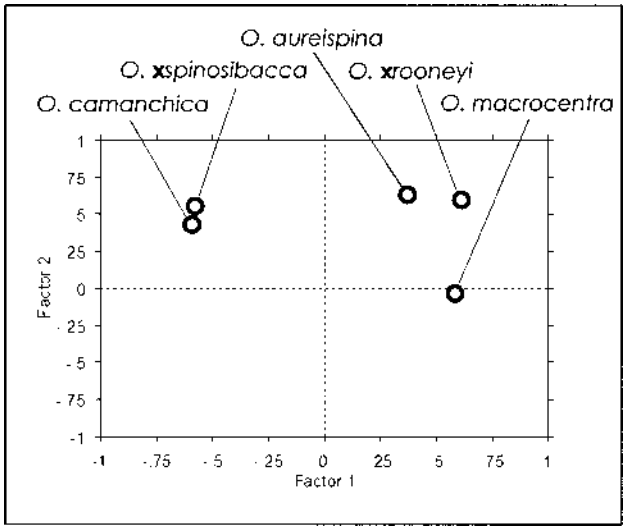

FIG. 5. Unrotated factor plot of RAPD banding pattern data observed for the five taxa studied. *Opuntia* ×*spinosibacca* groups closely with *O. camanchica,* a putative parent. The next most proximal taxon is *O. aureispina,* the other putative parent. *Opuntia* ×*rooneyi* is proximal to and intermediate between the putative parental taxa *O. aureispina* and *O. macrocentra.*

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