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ORIGINAL ARTICLE

Comparative study (AFLP and morphology) of three species of *Prosopis* of the Section *Algarobia*: *P. juliflora*, *P. pallida*, and *P. limensis*. Evidence for resolution of the "*P. pallida–P. juliflora* complex"

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Abstract The problems of delimitation of species of *Prosopis* originate from the few morphological discontinuities which exist among some of them; some, however, originated as a result of wide distribution of germplasm without proper knowledge of the species, in particular, much material catalogued as *P. juliflora*, but being of other species, was distributed for reforestation projects worldwide. This work tests the morphological results obtained for *P. pallida* and *P. limensis* of the Peruvian–Ecuadorian coast and for *P. juliflora* of the Caribbean Basin of Colombia and Venezuela utilizing a study of AFLPs and a study of the morphology of plantlets developed in a conventional garden study. The phenogram obtained for the AFLPs demonstrates each of the three species to be a well differentiated cluster and the molecular variance between them is significantly greater than the variance within each species. Study of the plantlets also indicates statistically significant differences for four morphological characters between *P. juliflora* and the other two species (*P. pallida* and *P. limensis*). These results, in addition to the morphological differentiation evident between adult plants of *P. pallida* and *P. limensis* and the clear separation of these two species from *P. juliflora*, corroborate the genetic identity of the three taxa analyzed.

Keywords Leguminosae · Prosopis · Taxonomy · AFLP · Morphology

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Introduction

The genus *Prosopis* L. emend Burkart (Leguminosae, Mimosoideae) contains 47 species that are distributed primarily in arid and semiarid zones of SW Asia, Africa, and, predominately, America (Burkart 1976). Some 43 of the 47 species that comprise the genus and which belong to three of the five sections proposed by Burkart (1976) (*Algarobia*, *Strombocarpa*, and *Monilicarpa*) inhabit dry ecosystems from the SW of North America down to Patagonia, Argentina. The problems with delimitation of the species in the genus occur principally within the section *Algarobia*, some of them originating from the small morphological discontinuities between some of the species that have been interpreted as a consequence of the high frequency of hybridization (Hunziker et al. 1975). However, many of the

problems were originated as a result of the broad distribution of germplasm without knowledge of the provenant species. One case in particular was a large quantity of material catalogued as *P. juliflora* (Sw.) DC, that was not that species but rather others, that was distributed for reforestation plans in various regions of the world, given that various species of the genus are known to be good producers of wood, food, and forage and because of their capacity to prosper in arid and semiarid zones.

There is general confusion about the delimitation of *P. juliflora* for various reasons. There is in the literature a lack of consensus in circumscribing the limits of *P. juliflora*, and because of this, its area of natural distribution and its potential are also controversial.

It should be indicated that the origin of the problem was the publication by Bentham (1875) who synonymized most of the species hitherto published in the section *Algarobia* with *P. juliflora*, founding in this treatment *P. limensis* Bentham as another distinct taxon.

The publication of Bentham (1875) synonymized various species of *Prosopis* under *P. juliflora*. Burkart (1940) restricted the limits of this species to the populations of Jamaica, Cuba, Santo Domingo, El Salvador, Nicaragua, Mexico, Colombia, and Venezuela. Benson (1941) amplified notably the concept of this species, placing the North American populations as subordinate varieties. Johnston (1962) in his excellent treatment of the genus for North America, restricted the limits of *P. juliflora* to the concept of Burkart (1940). Burkart (1976) later proposed two varieties of *P. juliflora* for the coasts of Ecuador and Peru, thus extending the area of distribution further to the south. Díaz Celis (1995) accepted *P. juliflora* and *P. pallida* for Northern Peru and published descriptions, but without citing herbarium specimens. Pasiecznik et al. (2001) in their extensive publication founded the "*P. juliflora*–*P. pallida* complex" and, like Diaz Celis, did not cite herbarium specimens. This "complex" derives from a taxonomic misidentification of the materials sent from Perú to Brazil, where the cultivations on which FAO based its recommendations occurred.

Various publications appeared after 2001, among them that of Harris et al. (2003), which established morphological and genetic differences among the species of the complex, principally on the basis of material that had been introduced to Africa. Mom et al. (2002), Alban et al. (2003), and Burghardt et al. (2010) analyzed the coastal populations of Peru and Ecuador, and pointed out that only *P. pallida* and *P. limensis* exist there, although the two species are considered synonymous under *P. pallida* by Burkart (1976).

Landeras et al. (2006) utilizing molecular markers (RAPDs), conducted comparative studies among accessions of *P. juliflora* and *P. pallida* along with other species of the section *Algarobia*, and although these authors did not then discriminate between *P. pallida* and *P. limensis*, they reported that those accessions they considered as *P. pallida* had differences at the genetic level from those accessioned as *P. juliflora*.

Landeras et al. (2006) presented RAPDs results that effectively differentiated provenances of, presumably, *P. pallida* and *P. juliflora*, yet, nevertheless, continued to recognize the *P. juliflora*–*P. pallida* species complex of Pasiecznik et al. (2001).

It is necessary to point out that FAO designated *P. juliflora* as a promising species to include in reforestation plans for arid zones worldwide, and under this name germplasm of different species was distributed to distinct arid and semiarid regions. This recommendation was made on the basis of experience in Brazil, where the cultivated species is, really, *P. pallida* from Peru.

After a field trip to study the Peruvian–Ecuadorian coast by Palacios et al., and analysis of pertinent herbarium deposits, the conclusion was reached that in the zone north of the coastal forests of Peru only two taxa are well differentiated: *P. pallida* and *P. limensis* (Mom et al. 2002) and the existence there of *P. juliflora* and *P. affinis* Sprengel should be discarded (Alban et al. 2003; Burghardt et al. 2010).

Palacios (2006) critically revised the Mexican species of *Prosopis*, restricting the area of distribution of *P. juliflora* to the coast of the Yucatan Peninsula, contributing one species and accepting a second for the Pacific Coast of Mexico and Central America. It can also be found in Venezuela and Colombia.

Several publications report the tetraploid character of *P. juliflora* (Hunziker et al. 1986; Saidman et al. 1997; Harris et al. 2003; Trenchard et al. 2008).

All the above information led to initiation of our testing all the results previously obtained for *P. pallida* and *P. limensis* on the Peruvian–Ecuadorian coast and for *P. juliflora* in the Caribbean Basin, Colombia, and Venezuela, utilizing the exomorphological characters of the seedlings and the molecular technique amplified fragment length polymorphism (AFLP) to clarify the correct identification of materials of Peru and solve definitively the "*P. juliflora*–*P. pallida* complex".

Materials and methods

Samples of *Prosopis pallida* and *P. limensis* in algarrobales of Peru and Ecuador, and of *P. juliflora* in Colombia (Cartagena) and Venezuela (Barquisimeto, State of Lara) were collected. It should be noted that *P. pallida* and *P. limensis* are partially sympatric and can be clearly differentiated by morphological characters in sympatric areas (Mom et al. 2002; Burghardt et al. 2010).

Herbarium voucher specimens were deposited in the herbaria of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (BAFC) in Argentina and of the Universidad Privada Antenor Orrega (HOA) of Trujillo, Peru.

In addition to branches with leaves, fruits were collected from each individual and labeled with provenance data and collection date.

The fruits were transferred to the biological laboratory of the Institute of Agricultural Science of the University of Guanajuato, Mexico, where they were treated by water scarification. Fruits were submerged in water at 80°C during 1 h after which the pulp was macerated, freeing the seed still enveloped in fragments of the endocarp. Subsequent to this procedure, the joints of the endocarp were cut with forceps and scissors to finally free the seeds.

These seeds were placed in black polyethylene bags of 1 kg capacity which held a substrate composed of slime and compost in 1:1 proportion. Two seeds were placed in each labeled bag to ensure that at least one germinated. In cases where both germinated, the seedling with less vigor was removed. Seeds were planted in 30 bags, 10 bags each for the three *Prosopis* species.

The bags were placed in a greenhouse located at the same institution and inspected every week to monitor the state of humidity and the incidence of pests and/or diseases and to verify the growth rate. They were irrigated with distilled water.

Seed was sown in March and growth in the greenhouse was for three months; at this stage pieces of leaves and tender branches were cut from the 10 pots of each species, mixed together for each species, and taken to the Laboratorio de Genetica Molecular del Centro de Investigaciones y de Estudios Avanzados (CINVESTAV), Unidad Irapuato, for subsequent treatment and application of AFLP techniques, which are described below.

AFLP analysis

DNA extraction

Frozen tissue from each individual plant was ground to a fine powder in a sterilized mortar and pestle. Extraction buffer (100 mM Tris–HCl pH 8.5, 20 mM NaCl, 20 mM EDTA pH 8.0, 1% *N*-lauroylsarcosine) was added, and, after mixing, samples were extracted at 3,000 rpm in an Eppendorf microcentrifuge for 15 min (Vos et al. 1995). The supernatant was extracted once with cold isopropanol. The resuspended pellet was treated with RNase 10 mg/ml (10 mM Tris–HCl pH 7.5, 15 mM NaCl) at 37°C, and the samples were again extracted with phenol–chloroform–isoamyl alcohol, precipitated, washed in 70% cold ethanol, and resuspended in TE (Tris 10 mM pH 7.0, EDTA 0.1 mM pH 8.0). DNA was visually quantified in an agarose gel using lambda DNA/HindIII fragments as molecular weight markers, and also checked in a Beckman DV-650 UV–visible spectrophotometer to be adjusted to a concentration of 100 ng/µl.

AFLP® procedure

The amplified fragment length polymorphism procedure was performed in accordance with the method reported by Vos et al. (1995). Genomic DNA (100 ng/µl) was cut by two restriction enzymes *Eco*RI (G/AATTC) and *Mse*I (T/TAA) to generate small DNA fragments. A ligation in which double-stranded DNA adapters are ligated to the ends of the restricted DNA fragments was performed. Oligonucleotide primers used for the preamplification were *Eco*RI (*Eco*RI+A) 5′-AGACTGCGTACCAATTC/A-3′ and *Mse*I (*Mse*I+C) 5′-GACGATGAGTCCTGAGTAA/C-3′. This step was followed by a second selective amplification with oligonucleotides having an additional three or four nucleotides at the ends of the pre-amplification primers. The primers used were E-ACC/M-CAT, E-ACT/M-CAT, E-ACA/M-CTC, and E-AGG/M-CTC, where the letter "E" stands for the *Eco*RI site and the letter "M" stands for the *Mse*I site. The *Eco*RI primers used were fluorescence labeled as suggested in the procedure of the LI-COR IRDye Fluorescent AFLP® kit (Myburg et al. 2001). Finally, selective amplification products were separated by denaturing polyacrylamide gel electrophoresis and the bands were captured and scored by use of LI-COR software.

Statistical analysis

AFLP data were coded as presence or absence of each band and a matrix of genetic dissimilarities was constructed using the simple matching coefficient (Skroch et al. 1992). From the dissimilarity matrix, a dendrogram was produced using the unweighted pair group method with arithmetic averages (UPGMA) by means of s-plus 2000 for Windows software (Mathsoft, Seattle, WA, USA). Confidence intervals were calculated for each node of the dendrogram using Felsenstein's bootstrap method (Felsenstein 1985).

Additionally, molecular analysis of variance (Excoffier et al. 1992) was performed on the AFLP data using Arlequin software (Schneider et al. 2000).

Morphological analysis

Besides molecular analysis, morphological data were obtained for five seedlings per species (five replicates)

taken at three months of age after being sown in pots. For all plants five measurements of each of the following characters was taken per plant and their average values calculated: length of petiole; number and length of pinnae; length and width of folioles; number of pairs of folioles; length between nodes on stem; angle between pinnae. Additionally, the height of the plant (measured for all seedlings from the base of the pinna with the most height) and stem diameter of seedlings 5 cm above ground level were measured.

The lengths of petioles, pinnae, folioles, and stem internodes, and width of folioles and stem diameter were measured with an electronic caliper. Plant height was measured in millimeters with a graduated ruler, and the angle between pinnae with a protractor.

The results of these measurements for each variable were analyzed under a design completely aleatory to detect if there was a significant difference among species in which case Tukey's multiple range test was used to determine whether there were significant differences between the three species or if only one of them was different from the others. For these analyses the program Statgraphics Plus was used.

Results

AFLP

Three individual samples from plants of each of the three species (*P. pallida*, *P. juliflora*, and *P. limensis*) were processed by the four oligonucleotide combinations by the AFLP procedure reported in the Materials and methods section. One of the samples from *P. limensis* did not amplify and thus only two samples for that species were obtained. For the remaining eight samples a total of 180 DNA fragments of within 58–491 bp were obtained. The presence or absence of each fragment in each one of the eight samples was recorded and processed as described in the Materials and methods section.

Table 1 presents the mean genetic dissimilarities within and between species resulting from the AFLP analysis.

Table 1 Genetic dissimilarities within and between species measured as average proportion of different AFLP bands within and between species and standard errors for the measurements

Species	*P. pallida*	*P. juliflora*	*P. limensis*
P. pallida	0.006 ± 0.006	0.465 ± 0.04	0.013 ± 0.008
P. juliflora		0[a]	0.456 ± 0.04
P. limensis			0[a]

[a] No differences among the AFLP band pattern of individuals from this species were found

From Table 1 it is possible to appreciate that the genetic polymorphism or dissimilarity within species is very small, being estimated as 0.006 in *P. pallida* and as 0 in the other two species. With regard to the genetic dissimilarity between species, *P. pallida* and *P. limensis* are closely related with an average dissimilarity of only 0.013, and these two species are very well differentiated from the individuals of *P. juliflora*, with genetic dissimilarities of 0.465 and 0.456, respectively.

Figure 1 presents the dendrogram resulting from processing of the genetic dissimilarity matrix obtained from the AFLP patterns by the UPGMA algorithm and including the Felsenstein's confidence coefficients for each node.

From Fig. 1 we can appreciate that each of the three species forms a well differentiated cluster. This is apparent from the 100% Felsenstein's confidence intervals in each of the nodes that aggregate the species. By far, the largest dissimilarity is between individuals of *P. juliflora* and the other two species (*P. pallida* and *P. limensis*).

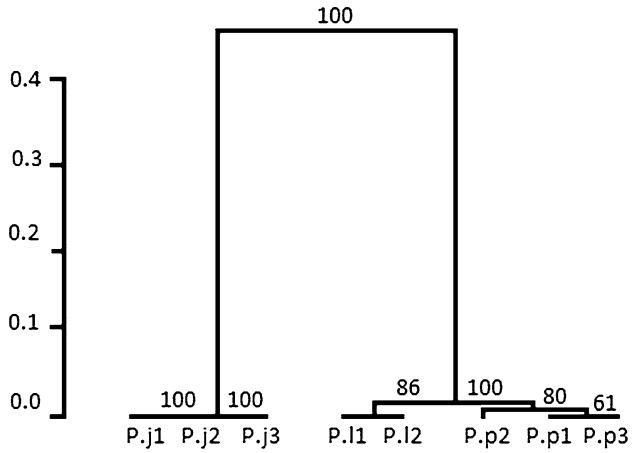

Fig. 1 Dendrogram of genetic dissimilarities between individuals of *Prosopis pallida* (P.p1, P.p2 and P.p3), *P. juliflora* (P.j1, P.j2 and P.j3), and *P. limensis* (P.l1 and P.l2)

Table 2 Analysis of molecular variance for the AFLP data

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation
Among species	2	156.958	29.84603 Va	99.56
Within species	5	0.667	0.13333 Vb	0.44
Total	7	157.625	29.97937	
Fixation index	FST		0.99555	

Significance tests (1023 permutations), Va and FST: *P* (rand. value > obs. value) = 0.00000, *P* (rand. value = obs. value) = 0.00489, *P* (rand. value ≥ obs. value) = 0.00489 ± 0.00203

Table 2 presents the results of the MANOVA performed with the AFLP data.

The results presented in Table 2 confirm the results apparent in the dendrogram, i.e. the molecular variance among the three species is significantly larger than the variance within species. More than 99% of the molecular variation present in the data is among populations.

Morphology of seedlings

Table 3 shows mean and standard deviation for nine phenological variables measured for seedlings of the three species of *Prosopis* studied.

The results from analysis of variance reveal highly significant differences ($P < 0.01$) for the variables: pairs of folioles, width of folioles, internode length, and significant differences ($P < 0.05$) for the variable angle between pinnae (Appendix Tables 5, 6, 7, 8); the rest of the variables were not significantly different, these results are summarized in Table 4. With regard to the Tukey test used for variables with highly significant and significant differences, it was observed that in the four cases, the statistical difference was detected between *P. juliflora* and the other two species (*P. pallida* and *P. limensis*), no statistically significant difference was detected between the last two species for the morphological characters analyzed under the conditions of this experiment for the seedling phase of the plant. See Appendix tables for results from ANOVA of the characters for which there are significant and highly significant differences between species.

Discussion

This study provides results from analysis of genetic similarity expressed by means of an AFLP analysis of accessions of *P. juliflora*, *P. pallida*, and *P. limensis* and by comparison of ten morphological variables measured on seedlings of the three species grown in a greenhouse.

This work, taking into account both the evident morphological differentiation previously reported between *P. pallida* and *P. limensis* (Mom et al. 2002; Alban et al. 2003; Burghardt et al. 2010) and the clear separation of these from *P. juliflora*, has corroborated the genetic identity of these three taxa using AFLP.

Figure 1 indicates with clarity the differentiation between the provenances of Colombia and Venezuela (of *P. juliflora*) and those of Ecuador and Peru (belonging to *P. pallida* and *P. limensis*). This result of AFLP is in accordance with those reported by Landeras et al. (2006) for other molecular markers. The measurements of the morphological characteristics also indicate the differentiation in characters that are considered crucial to differentiate species of the genus, for example pairs of folioles, their width, and the form in which the pinnae are disposed (angle between pinnae and internode length) that already have been successfully used to differentiate the species of the genus (Brizuela et al. 2000; Burghardt et al. 2000).

Table 4 Results from analysis of variance of ten morphological variables measured for seedlings of three species of *Prosopis* (*P. juliflora*, *P. pallida*, *P. limensis*) grown in greenhouse conditions

Variables	HS ($P < 0.01$)	S ($P < 0.05$)	NS
Petiole length			X
Pairs of pinnae			X
Pinnae width			X
Angle between pinnae		X	
Pairs of leaflets	X		
Leaflet width	X		
Leaflet length			X
Length of internodes	X		
Stem diameter			X
Plant height			X

Table 3 Mean and standard deviation of nine phenological variables measured for seedlings of three species of *Prosopis*

Variable	*P. juliflora*		*P. pallida*		*P. limensis*	
	Mean	SD	Mean	SD	Mean	SD
Petiole length	39.15	1.97	41.32	3.15	37.39	3.08
Pinnae width	25.36	2.55	25.38	3.58	22.57	3.10
Leaflet length	6.14	0.46	6.39	1.24	6.11	1.14
Leaflet width**	2.62	0.23	2.01	0.29	1.78	0.34
Pairs of leaflets**	6.78	0.32	9.52	0.60	8.40	0.97
Pairs of pinnae	11.80	1.79	11.60	3.13	11.50	3.84
Length of internodes**	10.05	1.82	20.32	4.12	17.59	4.49
Angle between pinnae*	66.10	3.91	85.10	4.13	64.12	4.59
Plant height	8.44	1.94	12.36	3.58	13.22	5.08
Stem diameter	1.28	0.02	1.50	0.24	1.39	0.04

* Significant differences among species ($p < 0.05$)

** Highly significant differences among species ($p < 0.01$)

These results provide additional support to that proposed earlier over the nonexistence of *P. juliflora* on the coasts of Ecuador and Peru. On the other hand, they provide evidence for the differentiation of, and a high grade of affinity, between *P. pallida* and *P. limensis*.

In our opinion, these results suggest a first separation into two groups which occurred at the moment of a substantial marine ingression in the early Miocene (Burnham and Graham 1999; Fig. 10) and in this manner two disjunct populations were generated, one on the coasts of the Caribbean and the other on the Pacific coast. After the uplift of the Andes, speciation occurred with differentiation at the chromosomal level (probably autopolyploidy) in the area of the Caribbean, whereas in Ecuador and Peru there originated, probably mediated by some type of homoploid speciation, two species with good morphological differentiation but with little variation at the genetic level shown by AFLP.

It should be noted here that many of the species of the section *Algarobia* have great tolerance to soils and water with a high salt content (Felker et al. 1981), so it is reasonable to suppose that, in most cases, speciation occurred in coastal areas at the time part of the South American surface was occupied by the sea. The Andean uplift would have displaced the sea but the salt resistance of the plants would have been conserved.

Conclusions

The results obtained in this work, both the analysis with molecular techniques (AFLPs) and the study of morphological characteristics of the seedlings, clearly show a substantial distance between the species of *Prosopis* of the Peruvian–Ecuadorian region (*P. pallida* and *P. limensis*) and *P. juliflora*, such that it demonstrates in a conclusive manner that *P. juliflora* does not naturally inhabit that region and that the Peruvian native material distributed and promoted in diverse parts of the world in previous decades would have been *P. pallida* and/or *P. limensis*, and not *P. juliflora* as it was called then. This clarifies the confusion created by this situation.

Although there was greater similarity between *P. pallida* and *P. limensis*, some genetic differentiation between these two species is evident. Their existence as separate entities on the basis of morphological differences has previously been reported by Mom et al. (2002).

Appendix

Tables 5, 6, 7, and 8

Table 5 Analysis of variance for the character "angle between pinnae"

Source	Sum of squares	*df*	Mean squares	*F*	*P*
Among groups	1341.8	2	670.901	678.873	0.0107
Within groups	1185.91	12	98.8257		
Total	2527.71	14			

Table 6 Analysis of variance for the character "pairs of leaflets"

Source	Sum of squares	*df*	Mean squares	*F*	*P*
Among groups	18.9773	2	9.48867	20.42	0.0001
Within groups	5.576	12	0.464667		
Total	24.5533	14			

Table 7 Analysis of variance for the character "leaflet width"

Source	Sum of squares	*df*	Mean squares	*F*	*P*
Among groups	1.90836	2	0.95418	11.41	0.0017
Within groups	1.0034	12	0.0836167		
Total	2.91176	14			

Table 8 Analysis of variance for the character "length of internodes"

Source	Sum of squares	*df*	Mean squares	*F*	*P*
Among groups	282.757	2	141.378	10.49	0.0023
Within groups	161.689	12	13.4741		
Total	444.446	14			

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