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ORIGINAL ARTICLE

Genetic and morphometric markers are able to differentiate three morphotypes belonging to Section Algarobia of genus *Prosopis* (Leguminosae, Mimosoideae)

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Abstract The section Algarobia of genus *Prosopis* includes promising species for reforestation and afforestation programmes in arid and semiarid regions, mainly of the Americas. Many interspecific natural hybrid combinations have been described in this group. In this paper we analysed a hybrid zone in Chaco biogeographical province in Argentina, where *P. ruscifolia* and *P. alba* overlap and hybridise producing intermediate fertile hybrid forms. Eleven morphological traits and 76 loci RAPD were analysed to determine the effect of hybridization between these species. The comparison of morphological traits among groups yielded significant or highly significant differences for all traits. Estimates of H_e in *P. alba* and *P. ruscifolia* did not differ from each other, but both showed significantly lower values than the hybrid group. The analysis of correlations between shared phenotypes and pair-wise relationships estimated from RAPD gave also strong support to the hypothesis that most of the phenotypic traits analysed have significant heritability. The analyses of population structure and clustering based on morphological and molecular data by DAPC and STRUCTURE were rather consistent and indicated that the three morphotypes studied here are differentiated with low overlapping. All results indicated that despite the occurrence of natural hybridization and introgression, interspecific gene flow would be limited by hybrid breakdown or natural selection favouring the maintenance of species integrity.

Keywords *Prosopis* · RAPD markers · Morphometry · Hybridization · DAPC · Structure

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Introduction

Natural interspecific hybridization has been recognised as playing an important role in plant evolution (Rieseberg 1997; Arnold 1997; Barton 2001; Soltis and Soltis 2009). When natural hybridization occurs one of the consequences is the generation of hybrid zones (Harrison 1990), where genetically distinct groups of individuals meet and mate, resulting in at least some offspring of mixed ancestry.

Many evolutionary biologists have viewed hybrid zones as active sites of evolutionary change where species reproductive isolation mechanisms are weak and constitute sources of new recombinant types. Nevertheless, as hybridization may promote plant diversity, it is associated with taxonomic confusion and the loss of differentiation of pure species because of continuous variation in morphological and genetic traits. In this context, the observation of great species diversity, slight morphological discontinuities and frequent transitional forms which sometimes blur

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species boundaries suggests that species integrity might be lost through time, through hybridization and recursive introgression. Alternatively, the continuous production of hybrids might favour the occurrence of evolutionary experiments through the production of novel genetic combinations, reinforcement of reproductive isolating mechanisms, possible increase in fitness of hybrid individuals and the origin of new species (Arnold 1997; Rieseberg and Carney 1998; Mallet 2007; Soltis and Soltis 2009).

The genus *Prosopis* includes highly valuable multipurpose species, which have an important ecological role contributing to the protection and improvement of soils (Dutton 1989). According to morphological criteria, mainly shape and size of leaves, pods and/or thorns, the genus was divided by Burkart (1976) into five sections, some of them subdivided into Series. The section Algarobia includes the most promising arboreal species for reforestation and afforestation programmes in arid and semiarid regions, mainly of the Americas.

The taxonomy of the section Algarobia is interesting because of the occurrence of many interspecific natural hybrid combinations in sympatry areas, usually in disturbed environments (Hunziker et al. 1977, 1986). Palacios and Bravo (1981) obtained evidence of natural hybridization between several species of section Algarobia based on morphological traits (measurements of leaflets, pods and thorns), also supported by the observation of chromatography of phenolic compounds. Hybrids in some cases showed addition of compounds "characteristics" of the putative parental species and in other cases they exhibit additional ("extra") compounds. Naranjo et al. (1984) were able to determine hybrids involving *P. alba*, *P. nigra* and *P. affinis* by morphological and chromatographic evidence. These hybrids showed a reduction of the proportion of fertile pollen respect to pure species, although they showed regular meiosis (Hunziker et al. 1975; Naranjo et al. 1984), what was interpreted as the occurrence of cryptic structural hybridism. Saidman (1990) found that the isoenzymatic system of glutamate oxalacetate aminotransferase (GOT) was useful in the identification of the possible origin of some hybrids between *P. caldenia* and *P. flexuosa*. The alcohol dehydrogenase system (ADH) was also used by Saidman (1986) to identify hybrids between *P. ruscifolia*, *P. alba* and *P. hassleri*, and by Verga (1995) to identify hybrids between *P. chilensis* and *P. flexuosa*. Vazquez Garcidueñaz et al. (2003) analysed *P. chilensis var. riojana*, a possible interspecific hybrid between *P. chilensis* var. *chilensis* and *P. flexuosa* var. *flexuosa*. The authors found no correlation between morphological traits and genetic distances generated by random amplified polymorphic DNA, although the taxon was well differentiated from its putative parents.

A question arises about the consequence of natural hybridization and possible introgression in the group of hybridising species of Algarobia. In fact, based on the frequent hybridization and the occurrence of intermediate forms, several authors describe the complex of related hybridising species of this section as a syngameon (Palacios and Bravo 1981), a hypothesis that seems to be also supported by the observation of low interspecific genetic differentiation (Saidman and Vilardi 1993). In order to analyse this issue, Saidman et al. (2000) and Ferreyra et al. (2007) used isoenzimatic and RAPD markers to characterise pure species and different combinations of hybrids of section Algarobia. These studies showed high genetic variability within species and low genetic differentiation between species. However, they also indicated that despite apparent weak barriers, the species seemed to be isolated from each other and the hybrids were not intermediate between their putative parents. Furthermore, hybridization in *Prosopis* seems to be induced under certain environmental conditions (Vega and Hernandez 2005), mainly naturally or anthropogenically induced environmental perturbations.

Direct evidence of the occurrence and consequence of hybridization in *Prosopis* could not be obtained because artificial crosses in species of genus *Prosopis* have not been successful so far. For this reason, morphological and molecular markers are needed to understand this important process. Morphological characters constitute the basic information to identify species and their putative hybrids. When interspecific hybridization occurs, it is expected that quantitatively inherited traits of hybrid individuals, mainly F1 individuals, will be intermediate between their putative parents. Those morphological traits that are controlled by several genes might exhibit intermediacy in the hybrid, while those controlled by one or a few genes may exhibit novel or parental character states (Rieseberg and Gerber 1995).

Some disadvantages of morphological characters are that they are assumed to be no neutral, and, more importantly, they are usually plastic, showing different expression in different environments. Phenotypic plasticity within plant taxa may make it difficult to discern between natural variation and processes of hybridization (Vázquez-Garcidueñas et al. 2003). Nevertheless, when combined with molecular techniques, these tools can be a powerful means of obtaining accurate identification of hybrid individuals and estimates of hybrid formation within populations.

Molecular markers have greatly improved the accuracy of hybrid identification and are now widely considered to be better suited for hybridization studies than morphological characters (Riesberg and Ellstrand 1993). In contrast to morphological markers, they have simple modes of inheritance and expression, they provide a random sample of the genome, and they are not subject to the effects of

environmentally induced plasticity. Since its introduction, random amplified polymorphic DNA (RAPD) method by Williams et al. (1990) has been one of the most widely used techniques in the characterisation of tree species and interspecific hybrid (González-Rodriguez et al. 2004; Tovar-Sánchez and Oyama 2004) due to its high mutation rate and suitability to distinguish genotypic variants, its low cost, ease and speed of the assay and lack of requirement of DNA sequence information of a species (Williams et al. 1990). Although RAPD is of dominant nature, several strategies have been put forward to minimise the dominance effects on genetic variation analyses (Lynch and Milligan 1994; Stewart and Excoffier 1996). In occasional cases RAPDs are poor in reproducibility, but this can usually be solved by the optimization of reaction conditions (Weising et al. 2005).

Recently, a powerful Bayesian statistical method implemented in the software STRUCTURE (Pritchard et al. 2000) has become available for hybrid identification. In particular, this is a model-based method that assumes that possible Hardy–Weinberg and/or gametic disequilibrium are attributable to population substructuration. It has become widely used due to its high efficiency for hybrid identification and because this method does not require prior information on reference population allele frequencies (Väha and Primmer 2006). Another recent approach is the discriminant analysis of principal components (DAPC) (Jombart et al. 2010), a multivariate exploratory method designed to identify and describe clusters of genetically related individuals. This approach is not based on any assumption and also allows extracting rich information from both genetic and morphological data, providing assignment of individuals to groups and a visual assessment of between-population differentiation and contribution of individual alleles to population structuring.

In this paper we analysed an area where *P. ruscifolia* and *P. alba* overlap and, based on morphological intermediacy, they apparently hybridise producing fertile hybrid forms, suggesting that the interspecific isolation barriers are weak. In order to determine if interspecific gene flow actually occurs between *P. alba* and *P. ruscifolia* through hybridization and introgression we investigated morphological traits and RAPD markers in individuals sampled in this area. The underlying hypothesis is that hybridization and introgression should lead to the homogenisation of allele frequencies between species and the generation of continuous morphological variation blurring interspecific boundaries. By contrast, if gene flow is somehow restricted, the result would be that the two species and the hybrid individuals would constitute three groups readily distinguished by both morphological and molecular markers.

Materials and methods

Description of the species

Burkart (1976) performed a detailed description of the species analysed in this study. *Prosopis alba* Grisebach, "algarrobo blanco" (white mesquite), is a heliophytic partially invasive tree which is very abundant in Northern and Central Argentina, mainly on sandy soils. It is much appreciated for its good quality wood and edible sweet pods. *P. ruscifolia* Grisebach the "vinal" is distributed from Bolivia and Paraguay to Northern-Central Argentina. It is highly invasive, mainly in unstable environments originated by fluvial action. Because of the huge spines (up to 15 cm long) and their canopy shape, vinal forests are frequently impenetrable and in some areas this species is considered a pest.

Sampling area

The material was exhaustively sampled throughout a transect from 63°23′W, 29°22′S to 63°10′W, 29°15′S near the locality of Sumampa, Santiago del Estero, Argentina (Fig. 1; Table 1). The transect runs from West to East over a zone characterised by an environmental gradient in Chaco biogeographical province (Cabrera and Willink 1973) from the foot of Sumampa hills and highlands of "quebrachal" (a forest dominated by "quebracho colorado", *Schinopsis balansae*), with *P. alba* as a secondary species, to the salty lands in the basin of lake Mar Chiquita, with halophytic species and *P. ruscifolia* as the dominant species.

Phenotypic assignment of individuals to a putative group

The sampled individuals were identified using the taxonomic key by Burkart (1976) and the criteria listed in Pasiecznic et al. (2004). The sample included some trees morphologically representative of both pure-bred species according to the morphological and botanical descriptors (Table 2; Fig. 2a–c). Those trees which showed intermediate phenotype or a combination of traits corresponding to different species were tentatively assigned to the hybrid group. Out of the 38 sampled individuals 14 fit the morphological characteristics of *P. ruscifolia*, 13 were identified as *P. alba*, and the remaining 11 trees were assumed as product of hybridization and/or introgression between the two species. A voucher specimen of each sampled tree is kept in the herbarium of IFFIVE, INTA, Córdoba, Argentina.

Morphometric methods and data analysis

All morphological traits were recorded after drying the voucher material collected in the field. A total of 11 leaf

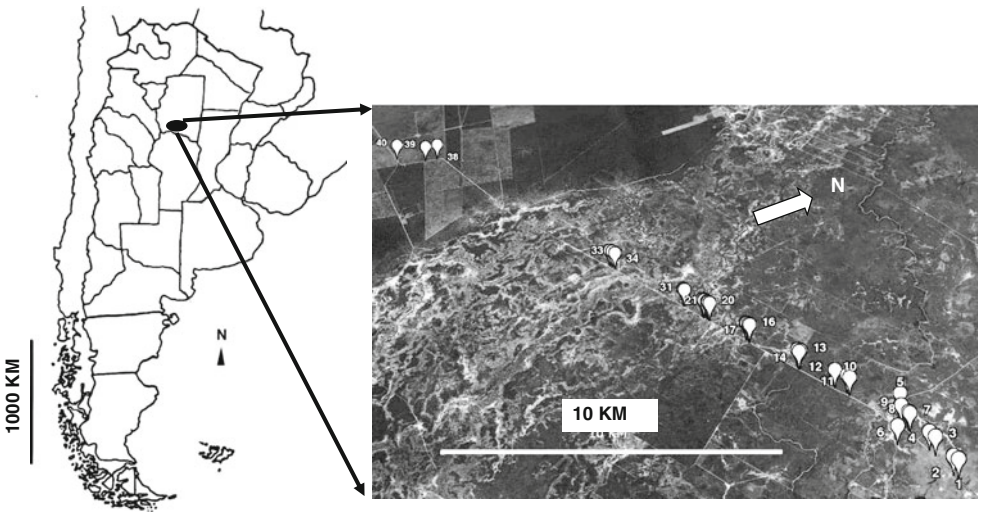

Fig. 1 Map of Argentina indicating collection site in Santiago del Estero Province, where individuals of *Prosopis alba*, *P. ruscifolia* and hybrid were sampled. Sampled trees are discriminated by specific group in Table 1

Table 1 Sampled trees (according to Fig. 1) discriminated by specific group

Species	Tree number
P. alba	10, 11, 13, 14, 15, 18, 19, 20, 33, 34, 36, 38, 39, 40
P. ruscifolia	1, 2, 3, 4, 5, 6, 7, 9, 12, 17, 28, 31, 32
Hybrid	8, 16, 21, 22, 23, 24, 25, 26, 27, 29, 30

morphology traits were measured and recorded for each individual plant: petiole length (PEL), number of pairs of leaflets per pinna (NLP), pinna length (PIL), number of pinnae (NPI), leaflet length (LEL), leaflet width (LEW), leaflet length/width (LEL/LEW), leaflet falcate (LEF), leaflet area (LEA), leaflet apex (LEX) and leaflet apex/total area (LEX/LEA). Some morphometric traits are shown in Fig. 3a. LEF is defined as the ratio *l*/*f*, where *l* is the length of a straight segment from the base to the tip of the leaflet, and *f* is the length from the same starting points but following the curve line that runs in the middle of the leaflet (Fig. 3b). LEX is the ratio *t*/*s*, where *t* is the area of the upper leaflet third and s is the area of a rectangle with the same dimensions (width and length) as the upper leaflet third (Fig. 3c).

In each individual, traits were measured over vouchers collected from different canopy regions, measuring ten leaves per tree. Statistical analyses were based on the individual averaged values. All leaflet measures (LEL, LEW, LEL/LEW, LEF, LEA, LEX, LEX/LEA) were obtained with the software HOJA1.1 (available from the author upon request: A. Verga, INTA-IFFIVE, arverga@yahoo.com.ar).

Differences among morphotypes for each morphological trait were evaluated by Kruskal–Wallis test. The differences in the dispersion of values of each trait around the mean, quantified as the standard deviation or the coefficient of variation, were compared among groups by Friedman test. Kruskal–Wallis and Friedman tests were chosen because they make no assumptions in reference to variable distribution. Friedman test was done with the package *coin* (Hothorn and Hornik 2007) and Kruskal–Wallis test with the package *stats* of the program R ver. 2.15.0 (R Development Core Team 2012).

Morphological pair-wise similarities between all individuals were quantified for each trait by the shared phenotypes (*Zij*) as defined by Ritland (1996), according to the following expression:

$$Z_{ij} = \frac{(Y_i - U)(Y_j - U)}{V}$$

where Y_i and Y_j are the values of the quantitative trait Y, respectively, for two individuals i and j, and U and V are, respectively, the sample mean and variance of Y in the whole sample. The structure of the matrices of phenotypic similarities was compared between traits by means of Mantel tests with 10,000 permutations.

The population structure was analysed from the morphological dataset by DAPC (Jombart et al. 2010). Trait measurements were averaged per individual. The analysis was conducted using the *adegenet* (Jombart 2008; Jombart and Ahmed 2011) and *ade4* (Dray and Dufour 2007) packages of the program R ver. 2.15.0 (R Development Core Team 2012). We applied two approaches: in the first one the number of clusters was assessed using the function *find.clusters*, which runs successive *K*-means clustering with increasing number of clusters (k). The choice of the optimal number of clusters was based on the lowest associated Bayesian information criterion (BIC). The prior groups in the subsequent DAPC analysis were those defined by the function *find.clusters*. In the second approach the prior clusters were defined by the groups obtained from the morphological determination, as described above (see phenotypic assignation of individuals to putative group).

Table 2 Leaf and pod characteristics of *Prosopis alba* and *P. ruscifolia*

		P. alba	*P. ruscifolia*
Tree shape	Height (m)	5–15	5–12
	Trunk	Short, to 1 m girth	70 to 90 cm
	Branches	Rounded crown	Arched downward
Spines	Thorn type and length	Thornless or scarce 2–4 cm	Spines always solitary large up to 33 cm
Flowers	Raceme length (cm)	7–11	8–15
	# Pairs of pinnae	1–3	1
	# Leaflet pairs per pinna	25–50	2–5
Leaf traits	Leaflet length and width (mm)	5–17 × 1.0–2.0	20–100 × 7–38
	Leaflet shape	Very narrowly linear	Lance-ovate more or less acute
Pod traits	Pod length and width (cm)	12–25 × 1.1–2.0	13–29 × 0.9–1.1
	Pod shape	Falcate to ring-shaped	Stipitate and acuminate, straight or S-shaped
		Compressed with parallel margins	Legume slender, subfalcate
			Compressed, submoniliform
	Pod colour	Straw yellow	Brown or yellow, violet-spotted

Adapted from Burkart (1976) and Pasiecznic et al. (2004)

RAPD methods and data analysis

DNA extraction and amplification

Leaves were collected from each tree in Mallet 2007 and were silica-gel preserved. DNA was extracted using DNA easy Plant mini kit (QIAGEN Inc., Valencia, California, USA) and samples were placed in a −20 °C freezer until analysis. DNA concentration was estimated by comparing electrophoretic patterns on 0.8 % agarose (in 1× TAE buffer) gels with standard DNA marker sets (phage λ double digested with *Eco*RI and *Hin*dIII). The PCR amplification involving arbitrary primers (Promega) was carried out in a 50-µl reaction volume containing 10–60 ng DNA, 0.6 µM each primer, 0.2 mM dNTPs, 0.3 U Taq DNA polymerase (Invitrogen) and 1.5 mM $MgCl_2$. A PROGENE Techne thermalcycler (Techne Cambridge LTD., Duxford, Cambridge, UK.) was used for amplifications, where the cycling profile was initial denaturation at 94 °C for 6 min followed by 45 cycles at 94° for 1 min denaturation, primer-specific annealing temperature (72°) for 2 min and at 72 °C for 45 s extension and a final extension step at 72° for 6 min. Re-amplification was performed routinely to ensure reproducibility of banding patterns. The usual cautions needed to prevent contamination of PCR experiments with previously amplified fragments were observed.

Reliability of PCR products was tested by several controls that were routinely used, one without primer, a second one with no Taq DNA polymerase and the third one with no genomic DNA. No amplification occurred in any of these negative controls. Each individual DNA sample was amplified three times and seeded in three different gels, and the same pattern was obtained. In each gel, we analysed individuals from different morphologically predefined groups in order to avoid bias in the comparisons among groups attributable to experimental error. Twenty primers were tested and three of them were selected on the basis of reproducibility of bands retrieved and their ability to show polymorphisms. The selected primers were A02 (5′ TCGAAGTCCT 3′), A03 (5′ CTAATGCCGT 3′) and A06 (5′ GAGTCTCAGG 3′). The RAPD products were separated by electrophoresis in a Model S2 cube (Gibco BRL Sequencing System, Life Technologies) through 4 % (w/v) polyacrylamide gel containing 8 M urea in 1× TBE buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA, pH 8). A 100-bp. DNA ladder (Invitrogen) size marker was included in each electrophoresis run. Gels were stained with silver nitrate (Bassam et al. 1991).

Genetic variability

Genetic variability and structure parameters of the three groups of *Prosopis* defined by morphological criteria were studied through RAPD markers. Binary matrix of band presence/absence was converted into allelic frequencies by a Bayesian method (Zhivotovsky 1999) with non-uniform prior distribution using the software AFLP-SURV 1.0 (Vekemans et al. 2002). Genetic variability was quantified by the unbiased expected heterozygosity (H_e) (Nei 1978) and percentage of polymorphic loci (P). Expected heterozygosities were compared among groups by means of the asymptotic Friedman test, taking groups and loci as factors. Pair-wise comparisons of H_e between groups were conducted by Wilcoxon test. Friedman test was done with the package *coin* (Hothorn et al. 2007) and Wilcoxon test with the package *exactRankTests* (Hothorn and Hornik 2007) of the program R ver. 2.15.0 (R Development Core Team 2012).

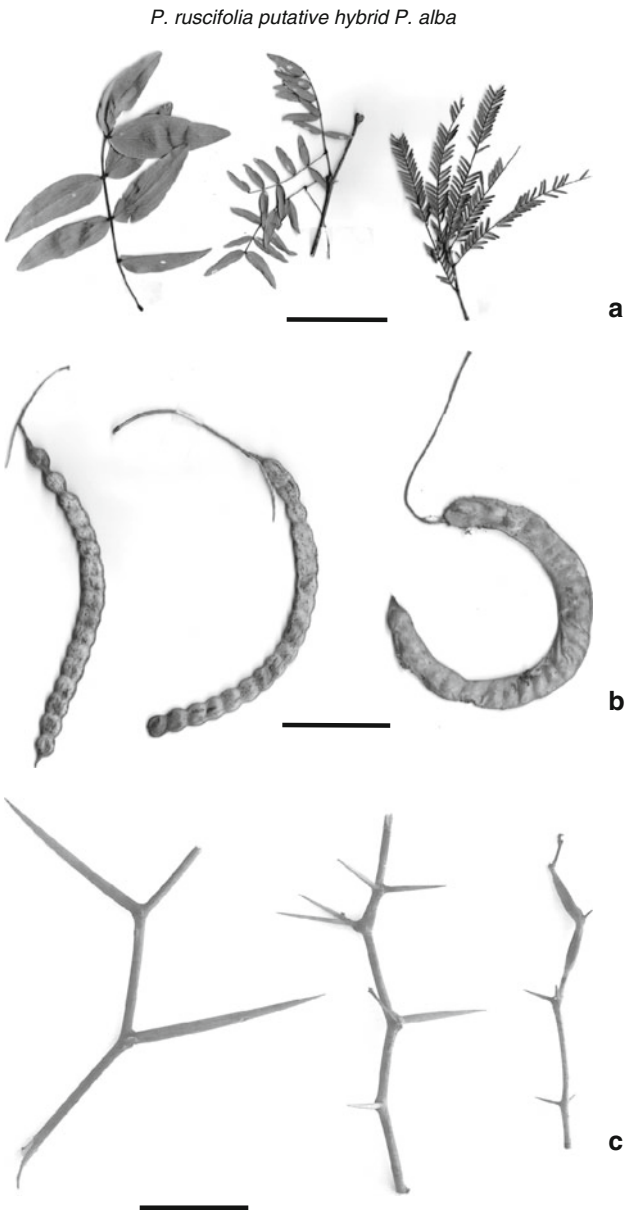

Fig. 2 Morphological characteristics of *Prosopis alba*, *P. ruscifolia* and putative hybrids in reference to leaf (**a**), pod (**b**), and spine (**c**) shape and size. *Bar* 5 cm

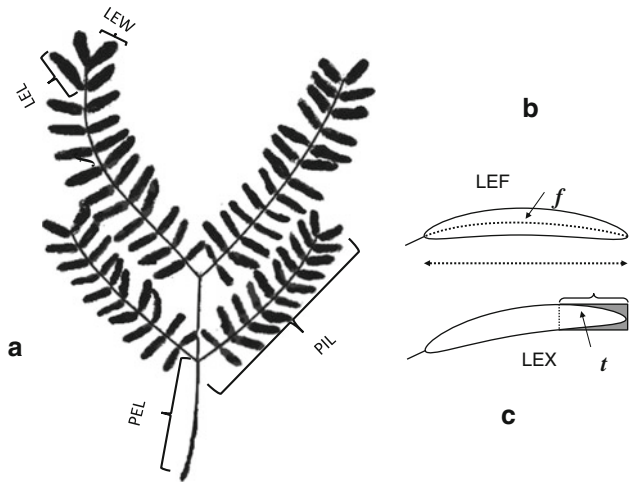

Fig. 3 Some leaf morphology traits measured and recorded for each individual plant. **a** Petiole length (PEL), pinna length (PIL), leaflet length (LEL), leaflet width (LEW). **b**, **c** Description of measurements to estimate leaflet falcate (LEF) (**b**) and leaflet apex (LEX) (**c**). l = distance from the base to the tip of the leaflet; f = length from the base to the tip the leaflet following a curved line running along the middle of the leaflet; t = area of the upper leaflet third; s = area of a rectangle with the same dimensions as t

Gametic disequilibrium

Gametic disequilibrium between RAPD loci was estimated by Otha's (1982) method using the program RAPDLD from the RAPDs package (Black 1996).

Genetic basis of morphological variation

We obtained a matrix of pair-wise relationships between individuals estimated from RAPD patterns (Lynch and Milligan 1994) using the software AFLP-SURV 1.0 (Vekemans et al. 2002). In order to determine if phenotypic variation has significant genetic basis, we applied a method based on Ritland (1996). The correlation between the matrix of pair-wise relationships (r_{ij}) estimated from RAPD data was compared with the matrices of shared phenotypes (Z_{ij}) for each morphological trait by means of Mantel tests with 10,000 permutations each. Similarity in the structure of both matrices constitutes an evidence of genetic basis of phenotypic variance (see Ritland 1996).

Clustering methods

Population structure was first analysed from molecular dataset by DAPC, to be compared with the clusterings based on morphological data obtained by the same statistical approach. As in the case of morphological data, two DAPC runs were performed. The first one determining prior clusters using the function *find.clusters* and selecting the optimal K according to the BIC. In the second run prior clusters were defined by the groups assigned by the morphological determination.

We also used the Bayesian model-based clustering method implemented in the program STRUCTURE version 2.1 (Pritchard et al. 2000) to assign individuals to K clusters on the basis of individual multilocus genotypes. This allowed us to analyse the correspondence between the morphologically based groups and inferred genetic structure. We conducted a series of independent runs for each value of K (the number of clusters) between 1 and 6. The results presented here are based on runs of 2×10^5 iterations, following an initial burn-in period of 50,000 iterations.

Performing a series of trial runs we found that using these parameters we obtained consistent estimates of posterior probabilities of K. The program was run without any information regarding group identification (USEPOPINFO = 0) and in the admixture mode in which the fraction of ancestry from each cluster is estimated for each individual. We used the correlated allele frequency model, which often improves clustering for closely related populations. As this method may increase the risk of over-estimating the number of clusters (Falush et al. 2003), we took into account the symmetry in individual assignment as a function of the number of clusters. Asymmetry (A) was quantified as the squared deviation of posterior individual assignment in comparison with the random assignment ($\sim 1/K$). Asymmetry was estimated as

$$A = \sum_{i=1}^{N} \sum_{j=1}^{K} (P_{i,j} - R)^2$$

where i represents the individual, j is the inferred cluster, $P_{i,j}$ is the posterior assignment of individual i to cluster j, and $R = 1/K$. The rationale to use the squared difference is to get in all case positive values.

We then continued our analysis to explore how well this structure corresponded to our morphological assignment of individuals to groups and to detect putative hybrids, using prior information (USEPOPINFO = 1). We report the posterior probabilities that the individual in question is correctly assigned to the given cluster or has ancestry in the other clusters. The results of STRUCTURE were edited with software CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) and Distruct 1.1 (Rosenberg 2004) to obtain the plot.

Results

Morphological data analysis

The comparison of morphological trait averages among groups (Table 3) yielded significant or highly significant differences for all traits. For four traits (LEF, NPI, PEL and LEX/LEA) the hybrid did not show average values intermediate between the putative parents. The comparisons of both standard deviation (SD) and coefficient of variation (CV) among groups yielded no significant differences according to Friedman tests ($\chi^2_2 = 1.3$ $P = 0.5292$ and $\chi^2_2 = 3.82$ $P = 0.15$, respectively, for SD and CV).

DAPC analysis of morphological data

The analysis of population structure was first conducted by DAPC combining all individuals (morphologically pure species and intermediate forms) in a single dataset without any a priori group assignment. To determine the optimal number of clusters using the function *find.clusters* of the package *adegenet* we retained 6 axes that represented more than 95 % of total variance. The program covered a range of possible clusters from 1 to 4. The corresponding BIC values were 90.19, 72.32, 63.41 and 60.43, respectively, for $K = 1$, 2, 3 and 4. The results indicated that the best number of clusters is $K = 4$. For DAPC analysis 6 axes of the PCA were retained (>99.9 % of total variance), and 2 (out of 3) discriminant functions were retained (95 % of variance). The scatterplot of individuals on the two principal components of DAPC showed the four clusters clearly differentiated without any overlapping (Fig. 4a). The consistency between prior and posterior assignment was 97.4 %. In the second analysis the clusters were defined a priori according to the morphological determination. In this case, for DAPC also six axes of the PCA were retained that corresponded to more than 99.9 % of variance, but only two discriminant functions were obtained. The scatterplot shows overlapping between morphologically a priori defined groups, and the consistency between prior and posterior assignment was 86.8 % (Fig. 4b).

The results obtained from the two approaches can be also compared from the posterior probability plots corresponding to the groups defined by the procedure *find.clusters* (Fig. 5a), the groups defined by morphological determination (Fig. 5b). The first group identified by *find.clusters* (red bars) is consistent with the group defined a priori in the second approach as *P. alba*. Individuals 5 and 6 show discrepancies between prior and posterior assignments. Group 3 shows high correspondence with the hybrids defined morphologically (yellow bars), whereas groups 2 (light blue bars) and 4 (lilac bars) would correspond to individuals classified as *P. ruscifolia.* Within *P. ruscifolia* individual 26 was misassigned in both plots and discrepancies were observed for individuals 15 and 18. For the hybrid group discrepancy between plots were observed in the assignment of individual 28. According to Fig. 5a it has a high probability of belonging to the lilac (*P. ruscifolia*) group, whereas in Fig. 5b is associated with the yellow (hybrid) group.

RAPD analysis

Genetic variability

With the three primers analysed we detected 94 bands from which we selected 76 for analysis on the basis of their reproducibility. The molecular weight of the bands ranged from 300 to 1,400 bp (Table 4; Figs. 6, 7, 8). After applying Otha's (1982) method none of 2,628 comparisons suggested epistasis disequilibrium between RAPD markers.

Table 3 Means and standard deviations (in parentheses) of the morphological traits analysed and Kruskal–Wallis statistics of mean comparisons among groups

Trait	Symbol	Means and SD of each group			Kruskal–Wallis test		
		P. ruscifolia	Hybrid	*P. alba*	χ^2	*df*	*P*
# Pairs leaflets/pinna	NLP	3.53 (1.14)	11.0 (4.59)	32.4 (6.60)	27.07	2	0.00
Falcate	LEF	0.67 (0.08)	0.92 (0.01)	0.94 (0.01)	23.36	2	0.00
# Pinna	NPI	1.50 (0.00)	1.00 (0.33)	1.15 (0.42)	28.81	2	0.00
Leaflet length/width	LEL/LEW	3.09 (0.7)	3.8 (0.5)	4.6 (0.7)	19.67	2	0.00
Leaflet width (mm)	LEW	1.30 (0.57)	0.73 (0.4)	0.3 (0.15)	24.00	2	0.00
Leaflet length (mm)	LEL	3.67 (1.17)	2.70 (1.32)	1.46 (0.78)	13.45	2	0.01
Leaflet apex	LEX	0.75 (0.19)	0.81 (0.05)	0.94 (0.04)	24.00	2	0.00
Leaflet area (mm^2)	LEA	3.61 (2.67)	1.94 (2.02)	0.47 (0.04)	17.20	2	0.00
Petiole length (mm)	PEL	2.69 (0.67)	1.89 (−0.56)	2.21 (0.65)	10.28	2	0.00
Pinna length (mm)	PIL	6.85 (1.67)	6.96 (2.10)	8.31 (1.72)	7.18	2	0.02
Leaflet apex/total area	LEX/LEA	0.24 (0.06)	0.21 (0.01)	0.21 (0.01)	29.00	2	0.00

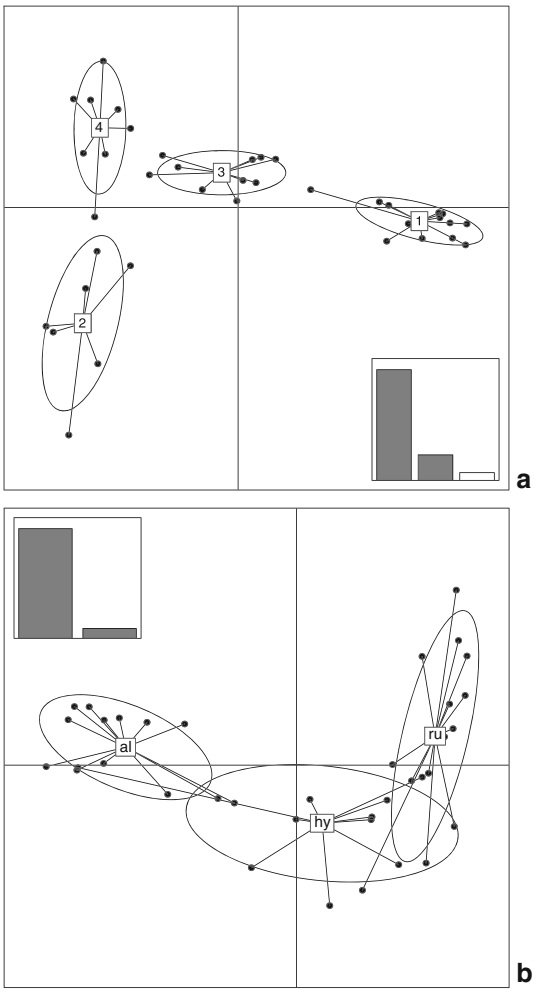

Fig. 4 Scatterplots showing the two principal components of DAPC based on morphological traits scored in individuals of *Prosopis alba*, *P. ruscifolia* and putative hybrids performed without prior information on group assignment (**a**) and prior information on group assignment (**b**)

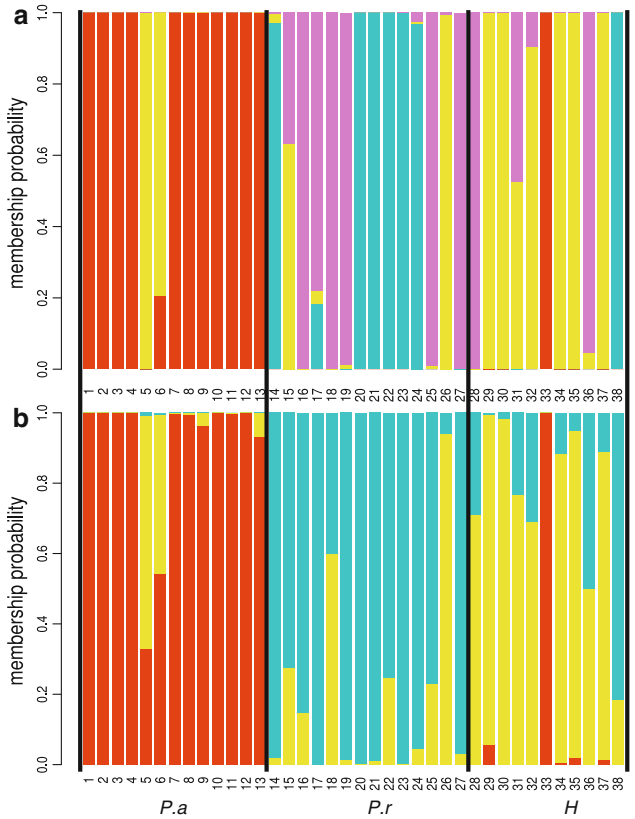

Fig. 5 Posterior probability plots based on the DAPC analysis from morphological traits corresponding to the groups defined by the procedure *find.clusters* (**a**) and to the groups defined a priori by morphological determination (**b**)

The comparison of band frequencies of RAPD loci (Table 5) showed that in more than half the cases (39 out of 73) band frequencies in hybrids were not intermediate. In 20 cases the hybrid group exhibits the lowest and in 19 the highest frequency value.

Table 4 RAPD primers used in this study, number of bands per primer and band size range

Primer	Base Sequence (5′ a 3′)	No of bands	Band size range (pb)
A-02	GGTGCGGGAA	25	290–1,470
A-03	AAGACCCCTC	27	200–1,450
A-06	GGAGTCTCAG	23	430–2,000

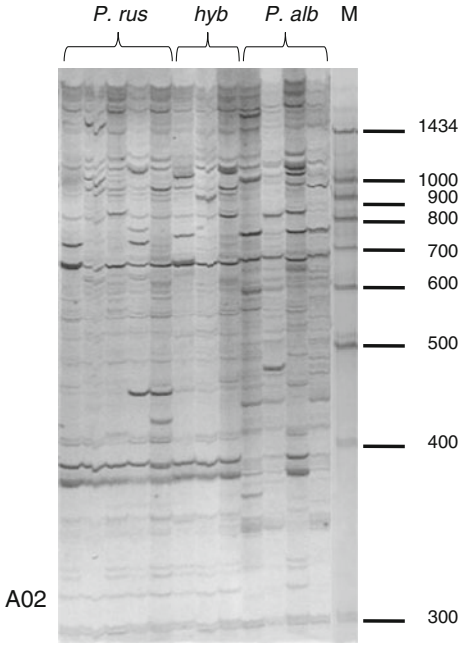

Fig. 6 RAPD profiles obtained with primer A02 in individuals analysed. *M* molecular weight marker

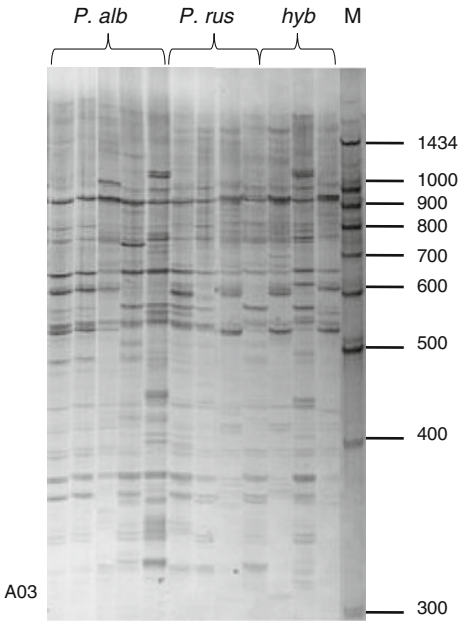

Fig. 7 RAPD profiles obtained with primer A03 in individuals analysed. *M* molecular weight marker

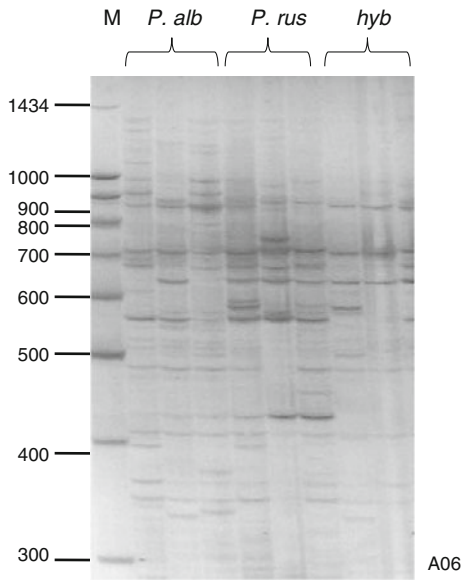

Fig. 8 RAPD profiles obtained with primer A06 in individuals analysed. *M* molecular weight marker

Genetic variability (H_e) estimated from RAPD (Table 6) showed significant differences among groups according to Friedman test ($\chi^2_2 = 7.589$, $P = 0.02$). Pair-wise comparisons of H_e between groups by Wilcoxon test showed highly significant differences between *P. alba* and hybrids ($W = 1842$, $P = 0.001$) and *P. ruscifolia* and hybrids ($W = 1830$, $P = 0.001$), whereas no significant differences were observed between *P. alba* and *P. ruscifolia* ($W = 3023$, $P = 0.16$).

DAPC analysis of molecular data

The analysis of population structure from molecular data was also conducted by DAPC applying the same approaches previously described, without and with prior information of groups. Using the function *find.clusters* 12 axes were chosen retaining about 70 % of total variance. The program covered a range of possible clusters from 1 to 4. The corresponding BIC values were 86.17, 78.10, 77.69 and 77.94, respectively, for $K = 1$, 2, 3 and 4. The results indicated that the best number of clusters is $K = 3$. For DAPC analysis 12 axes of the PCA were retained (70.44 % of total variance), and 2 discriminant functions were obtained. The scatterplot of individuals on the two principal components of DAPC showed the three clusters clearly differentiated without any overlapping (Fig. 9a). The consistency between prior and posterior assignment was 100 %. In the second analysis the

Table 5 Fragment frequencies of RAPD loci in *Prosopis ruscifolia* (*P. r*), *P. alba* (*P. a*) and the interspecific hybrids (*Hyb*)

Band	*P. r*	*P. a*	Hyb
A3-01	1.00	1.00	0.82
A3-02	0.93	0.85	0.82
A3-03	1.00	0.85	0.82
A3-04	0.86	0.69	0.55
A3-05	1.00	0.92	1.00
A3-07	0.43	0.85	0.36
A3-08	1.00	0.85	0.91
A3-09	0.93	1.00	1.00
A3-12	0.57	0.54	0.73
A3-13	0.79	0.85	0.91
A3-14	0.79	1.00	0.55
A3-17	0.43	1.00	0.91
A3-18	1.00	0.85	0.91
A3-19	0.21	0.62	0.73
A3-20	1.00	0.77	1.00
A3-21	0.71	0.92	0.73
A3-22	0.93	0.31	0.55
A3-23	0.79	0.31	0.91
A3-24	0.57	0.46	0.91
A3-25	0.86	0.46	0.45
A3-27	0.79	0.92	0.64
A3-28	0.64	1.00	0.55
A3-29	0.86	0.23	0.45
A3-30	0.36	0.31	0.18
A3-31	0.86	0.85	1.00
A3-33	0.71	0.85	0.91
A3-34	0.93	0.46	0.73
A2-03	0.71	0.23	0.18
A2-04	1.00	0.46	0.91
A2-06	0.71	0.92	1.00
A2-07	0.00	0.46	0.73
A2-10	0.71	1.00	0.82
A2-12	1.00	0.62	0.82
A2-13	1.00	0.92	0.82
A2-14	0.71	1.00	0.91
A2-15	0.43	0.85	0.55
A2-16	0.86	0.62	0.55
A2-17	0.43	1.00	0.91
A2-18	1.00	0.46	0.73
A2-22	0.57	0.46	0.55
A2-23	1.00	0.62	0.73
A2-24	0.93	0.62	1.00
A2-26	0.79	1.00	0.64
A2-27	1.00	0.62	0.91
A2-29	0.21	0.62	0.73
A2-30	0.21	0.69	0.36
A2-31	0.93	0.31	0.82
A2-33	1.00	0.92	0.73
A2-34	1.00	0.38	0.73
A2-35	0.86	0.23	0.55
A2-36	1.00	0.62	0.82
A2-37	0.93	0.92	0.91
A6-01	0.86	0.54	0.64
A6-02	0.93	0.08	0.73
A6-03	0.14	0.77	0.64
A6-04	0.64	0.62	0.36
A6-05	0.79	0.85	1.00
A6-06	1.00	0.15	0.82
A6-07	0.29	0.69	0.55
A6-08	0.50	0.23	0.73
A6-09	0.93	0.92	0.91
A6-10	0.00	0.69	0.36
A6-11	0.50	0.00	0.00
A6-12	0.50	0.00	0.18
A6-13	0.93	0.62	1.00
A6-14	0.43	1.00	0.73
A6-15	0.86	0.92	0.64
A6-16	0.07	0.46	0.18
A6-17	1.00	1.00	1.00
A6-18	0.50	0.00	0.27
A6-19	0.93	0.23	0.45
A6-20	1.00	1.00	1.00
A6-21	0.57	0.62	0.55

clusters were defined a priori according to the morphological determination. In this case, for DAPC also 12 axes of the PCA were retained and 2 discriminant functions were obtained. The scatterplot (Fig. 9b) shows the three groups well differentiated and the consistency between prior and posterior assignment was 97.4 %.

The probability plots corresponding to the groups defined by the procedure *find.clusters* (Fig. 10a) and to the groups defined by morphological determination (Fig. 10b) showed high consistency for individuals corresponding to group 2 (light blue) and *P. ruscifolia*. In both cases, all *P. ruscifolia* individuals belong to the same group with no misassignment. In both cases individual 28 is associated with the same group. In reference to group 1 (red bars), there is some correspondence with *P. alba*, but the first analysis assigns individuals 2, 5 and 6 to group 3 (yellow), whereas they correspond to *P. alba* in the analysis with prior information. Several individuals determined a priori as hybrids in the first analysis (Fig. 10a) are grouped with those belonging to *P. ruscifolia* (individuals 35, 36 and 38) or *P. alba* (33 and 34).

Table 6 Genetic variability in *Prosopis ruscifolia* (*P. r*), *P. alba* (*P. a*) and interspecific hybrids (*Hyb*)

Group	*N*	*P*	He	SE	VarI	VarL
P. r	14	97.30	0.38	0.01	60.70	39.30
P. a	13	95.90	0.40	0.01	56.00	44.00
Hyb	11	100.00	0.45	0.01	85.60	14.40

N number of trees analysed, *P* percentage of polymorphic loci, H_e mean expected heterozygosity, *SE* standard error of H_e, *VarI* percentage of variance of H_e due to individual sampling, *VarL* percentage of variance of H_e due to locus sampling

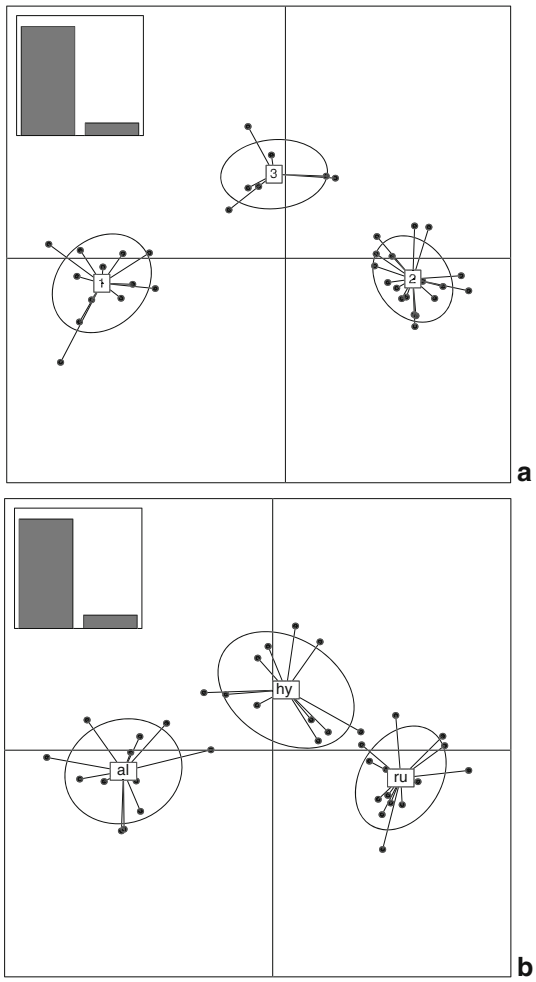

Fig. 9 Scatterplots showing the two principal components of DAPC based on RAPD patterns scored in individuals of *P. alba*, *P. ruscifolia* and putative hybrids performed without prior information on group assignment (**a**) and with prior information on group assignment (**b**)

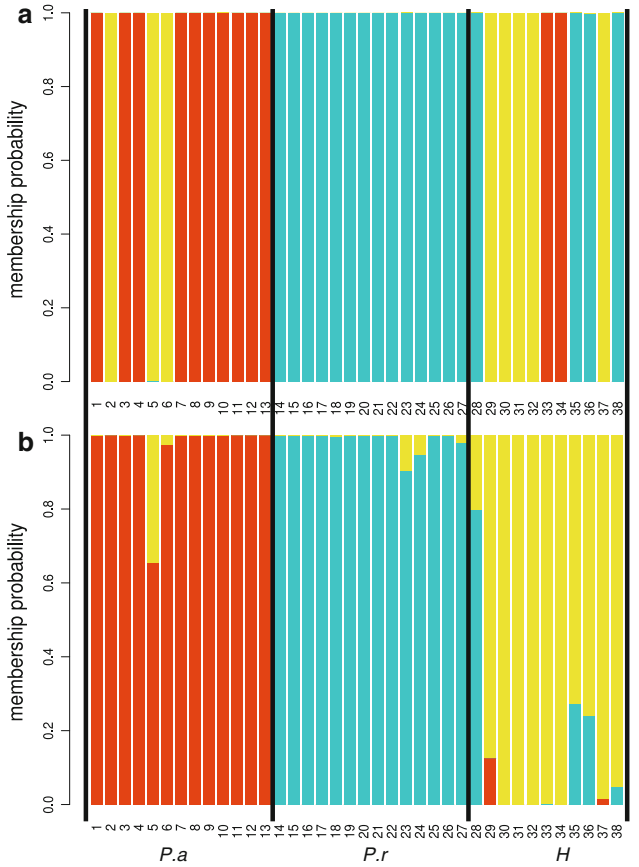

Fig. 10 Posterior probability plots based on the DAPC from RAPD patterns corresponding to the groups defined by the procedure *find.clusters* (**a**) and to the groups defined a priori by morphological determination (**b**)

Structure analysis

For the first modelling approach with the STRUCTURE program all individuals, morphologically pure species and intermediate forms were combined into one data set, without any a priori species assignment. Given *X*, the observed genotypes, the values of log likelihood of the multilocus genotype data, ln Pr(*X*|*K*), as a function of the number of clusters, *K* (Fig. 11a), showed that the maximum corresponded to $K = 6$, what might be interpreted as the best number of clusters. However, the differences are small for $K > 3$. Besides, the comparison of the asymmetry in individual assignment showed a maximum for $K = 3$ (Fig. 12). Taking into account Pritchard et al.'s (2010) recommendation to be sceptical about population structure inferred on the basis of small differences in Pr(*K*), we consider $K = 4$ as the best compromise between the maximum Pr(*K*) and asymmetry.

The plots produced by STRUCTURE (Fig. 11a) showed in all cases that all individuals determined by morphological criteria as *P. ruscifolia* are included in the same cluster. By contrast, evidence of admixture (that is, occurrence in a cluster of individuals proceeding from another cluster) is observed in *P. alba* consistent across plots based on different numbers of clusters (*K*). The highest evidence of admixture is observed in all cases in individuals determined a priori as interspecific hybrids.

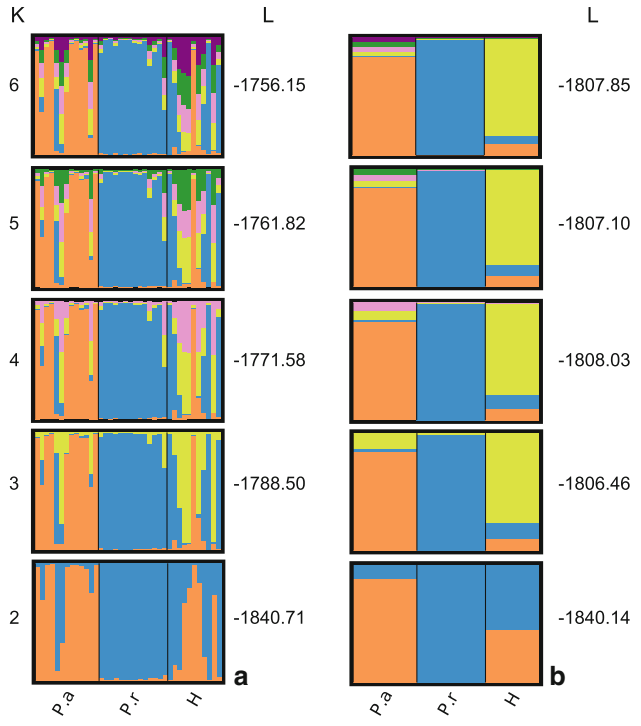

Fig. 11 Posterior probability plots based on STRUCTURE analysis on RAPD patternes for K between 2 to 6 for the first modelling approach without any a priori species assignment (**a**) and for the groups defined a priori by morphological determination (**b**). K = number of clusters, L = the values of log likelihood of the multilocus genotype data, ln Pr($X|K$), as a function of the number of clusters, K

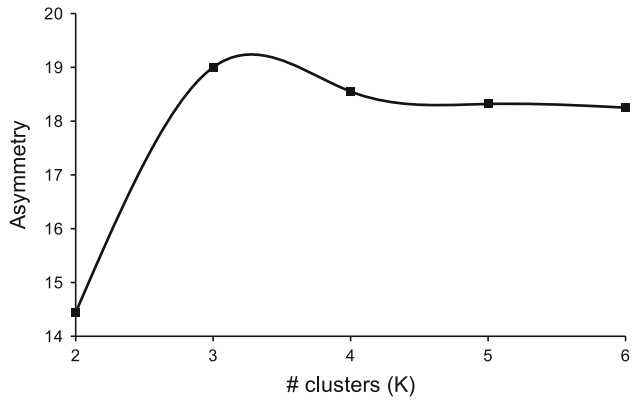

Fig. 12 Comparison of the asymmetry of individual assignment for the analysis shown in Fig. 11

Following Curtu et al. (2007), within each phenotypically pure species, an individual was considered to be assigned to the corresponding species cluster when it has an equal to or greater than 0.90 probability of belonging to that cluster. Hybrid forms are defined here as those showing less than 0.90 probability of belonging to their own species clustering. If we take the plot based on $K = 4$, out of 13 individuals morphologically determined as *P. alba*, 5 should be considered hybrid forms. In the case of *P. ruscifolia*, only 2/14 individuals had a probability less than 0.9 to belong their own species. Within the morphologically determined hybrid group two individuals had a probability higher than 0.9 to belong to *P. ruscifolia*, and one to belong to *P. alba*.

Next, we tested in STRUCTURE whether any individual in each species sample is misassigned, i.e. incongruence between morphology and molecular markers, or is a first or second generation hybrid between species. For this purpose, we incorporated group information into the inference procedure. According to this second approach all individuals of *P. ruscifolia* were assigned correctly. In the case of *P. alba*, 10 out of 13 individuals are assigned correctly, two individuals were missassigned, having a high probability (0.73–0.77) of being hybrids (Table 7), and a third individual had a low probability of being *P. alba* (0.46), with evidence of being second generation descendant of *P. ruscifolia* (0.30) or hybrid (0.15). For the hybrid group eight individuals were determined correctly: one should be considered *P. alba* ($P = 0.98$), and two individuals exhibit evidence of being hybrids of first introgressant generation with *P. ruscifolia* (0.66 and 0.59).

In summary, the number of misassigned individuals was small relative to the total sample size, being the majority of the individuals (84 %) assigned to the group they were classified based on morphology and *P. ruscifolia* the group with the highest consistency.

Genetic basis of morphological variation

The test of similarity in the structure of the matrices of pair-wise relationships (r_{ij}) estimated from RAPD data and shared phenotypes (Z_{ij}) indicated a highly significant correlation between relationship and phenotypic similarity for all traits but LEX/LEA and PEL (Table 8), suggesting a strong genetic determination of the measured traits. Table 8 also indicates that most of the measured morphological traits are correlated with each other. An exception is PEL, which is not significantly correlated with any other trait.

Discussion

Natural hybridization is the spontaneous crossing between populations that have undergone a divergent history to the level of generating disjoint races, semi-species or species and that are partially ecologically and/or reproductively isolated (Arnold 1997; Soltis and Soltis 2009). The effects of hybridization may be lasting or ephemeral. In some cases, hybridization may be considered as only an inversion of the evolutionary divergence process. In other cases,

Table 7 Morphological and genetic assignment of individuals that exhibited miss assignment according to the STRUCTURE analysis G_0, G_1 and G_2: probabilities of being admixed, first- or second-generation descendant of the corresponding group

Indiv	Morphological assignment	*P. a.*	*P. r.*			hyb		
			G_0	G_1	G_2	G_0	G_1	G_2
2	*P. alba*	0.46	0.00	0.01	0.30	0.00	0.09	0.15
5	*P. alba*	0.00	0.08	0.13	0.02	0.73	0.03	0.00
6	*P. alba*	0.00	0.00	0.05	0.02	0.77	0.14	0.01
Indiv	Morphological assignment	hyb	*P. a.*			*P. r.*		
			G_0	G_1	G_2	G_0	G_1	G_2
28	Hybrid	0.19	0.00	0.00	0.00	0.66	0.12	0.04
33	Hybrid	0.00	0.98	0.01	0.00	0.00	0.00	0.00
36	Hybrid	0.20	0.00	0.00	0.00	0.59	0.17	0.05

Table 8 Correlations (above diagonal) between morphological characters and RAPD distance matrices and their significance (below diagonal) according to Mantel tests based on (10,000 permutations)

	RAPD	LEW	LEA	LEX	LEX/LEA	LEF	LEL/LEW	LEL	PEL	PIL	NLP
RAPD		0.23	0.15	0.14	0.00	0.34	0.39	0.17	0.01	0.05	0.51
LEW	0.00		0.94	0.37	0.12	0.48	0.58	0.89	−0.01	0.17	0.40
LEA	0.00	0.00		0.27	0.07	0.28	0.42	0.87	−0.02	0.15	0.26
LEX	0.00	0.00	0.00		−0.02	0.15	0.40	0.29	−0.03	0.02	0.29
LEX/LEA	0.41	0.02	0.03	0.71		0.22	0.07	0.06	0.00	0.03	0.00
LEF	0.00	0.00	0.00	0.01	0.00		0.53	0.29	0.08	0.06	0.42
LEL/LEW	0.00	0.00	0.00	0.00	0.07	0.00		0.34	−0.04	0.26	0.65
LEL	0.00	0.00	0.00	0.00	0.07	0.00	0.00		−0.01	0.09	0.31
PEL	0.26	0.48	0.70	0.88	0.37	0.06	0.99	0.52		−0.03	−0.01
PIL	0.07	0.01	0.01	0.19	0.16	0.08	0.00	0.03	0.88		0.29
NLP	0.00	0.00	0.00	0.00	0.35	0.00	0.00	0.00	0.39	0.00	
NPI	0.00	0.00	0.00	0.00	0.54	0.00	0.00	0.00	0.51	0.01	0.00

ability to hybridise gives a group of related species particular evolutionary potentialities (Riesberg 1997; Arnold 1997; Soltis and Soltis 2009). Frequently hybrids display special capabilities to exploit open or hybrid habitats (Anderson 1949). Nevertheless, an unrestricted gene flow should reduce genetic differentiation among hybridising species and produce a continuous morphological variation that is expected to sustain species boundaries in more or less a diffuse state (Saidman et al. 1998b).

Natural hybridization has been reported between different species of section Algarobia of genus *Prosopis,* mainly in highly disturbed environments in the Chaco biogeographical region (Hunziker et al. 1986; Vega and Hernandez 2005). Evidence of natural hybridization in *Prosopis* comes from diverse methodologies including cytology, morphology, pollen fertility and biochemical and molecular markers (Palacios and Bravo 1981; Hunziker et al. 1986; Vega and Hernandez 2005; Ferreyra et al. 2007).

Another interesting result in our analysis was that the dispersion of phenotypic values around the average was not different between the hybrid group and the putative parents. This is not expected if we assume that the hybrid group is genetically more variable as a consequence of the addition of the gene pool of both parental species. A possible explanation would be that the phenotypic variation is caused by environmental rather than genetic factors. If this were the case, phenotypic variance would be the consequence of plasticity. An issue that was taken into account in our analysis is the extent at which the differences in quantitative traits may be due to only environmental causes. For evaluating this question we applied a method based on Ritland (1996) to determine if phenotypic variation has significant genetic basis. We analysed the correlation between the shared phenotypes and the pair-wise relatedness estimated from RAPD data. In almost all traits the correlations were highly significant suggesting that the corresponding heritabilities are also significant. Moreover,

most quantitative traits were correlated with each other, reflecting possible pleiotropic effects or linkage disequilibrium. An exception would be PEL, which was not significantly associated with any of the other phenotypic traits. The correlation of shared phenotype and relatedness for this trait was also not significant suggesting that most of its variation is environmentally determined. The analysis of correlations between shared phenotypes and pair-wise relatedness gave strong support to the hypothesis that most of the phenotypic traits analysed here have significant heritability, and, therefore, the phenotypic differences among groups cannot be explained solely on environmental causes.

As phenotypic plasticity within plant taxa may in some cases make it difficult to discern between natural variation and the process of hybridization (Vázquez-Garcidueñas et al. 2003), we used a combination of morphological and non-morphological markers. RAPDs are not influenced by the environment and provide data from diverse genomic regions giving information on the genome as a whole (Li et al. 2008; Joseph et al. 2008). For these reasons they are more appropriate for the study of hybrid zones and they usually provide better discrimination between closely related species (Coart et al. 2002; González-Rodriguez et al. 2004). Although other markers like chloroplast DNA markers or ITS sequences are more appropriate to study hybridization, previous studies have shown that these markers are not able to differentiate hybridising species of section Algarobia (Catalano et al. 2008). The RAPD technique proved to be useful to provide markers to discriminate groups of species in the genus *Prosopis* (Ramírez et al. 1999; Vázquez-Garcidueñas et al. 2003; Ferreyra et al. 2004, 2007; Vega and Hernandez 2005). In the present study we obtained a large number of markers suitable for genetic variation analysis. A quick overview of fragment frequencies in the three groups analysed indicated that hybrid frequencies are not intermediate between parental species for a high number of loci. The reason for this is that the case of species of the section Algarobia of genus *Prosopis* seems to correspond to the most complicated of the scenarios discussed by Soltis and Soltis (2009), *i.e.*, the species involved in hybridization episodes are highly polymorphic, closely related and share alleles in many loci. In fact, we observed in previous works that although molecular markers allowed differentiating some groups of species, there is a virtual lack of molecular markers useful for unequivocal species diagnose within each group (Saidman et al. 1998b; Ferreyra et al. 2004, 2007).

In this paper the genetic variability estimated by RAPD loci indicated that parent species did not differ from each other but both showed significantly lower H_e estimates than the hybrid group. This result is consistent with the expectations as in hybrids alternative allele tend to be evenly distributed, but contrast with the similar variance observed in hybrids and parent species for morphological traits.

The analyses of population structure in hybrid zones by morphological and molecular markers still represents an important challenge. Nevertheless, the availability of markers and powerful statistical procedures, for example Bayesian clustering methods, which do not relay on a priori morphological classification, has facilitated the detection of first-generation (F1) hybrids and backcrosses (Väha and Primmer 2006). The methodology and model implemented by Pritchard et al. (2000, 2010) that allows to assign individuals probabilistically to populations if their genotypes indicate that they are admixed has became widely used because their model can be applied to most of the common molecular markers and to its high efficiency for hybrid identification. Nevertheless, it has some shortcomings: the method relays in many assumptions, i.e. Hardy–Weinberg and linkage equilibrium, which not always are fulfilled, and the procedure requires considerable computational time when analysing large datasets (Jombart et al. 2010). DAPC (Jombart et al. 2010) is also an appealing approach that does not rely on a particular population genetics model and can also identify and describe clusters of genetically related individuals. Although the method is not able to identify migrant, first-, and second-generation admixed individuals, it has the advantages that computational time is negligible and it can be applied to both molecular and morphometric data. Taking into account the advantages of both methodologies the population structure was analysed from the morphological dataset by DAPC and the molecular dataset was analysed by DAPC and STRUCTURE.

The analysis of population structure by DAPC based on morphological traits indicated that, without prior information on group assignment, the sample is split into 4 clearly differentiated clusters: one corresponded to most individuals of *P. alba*, another integrated mostly by hybrids, and the remaining two clusters included most individuals of *P. ruscifolia*. When the information of taxonomic criteria assignment is given, the correspondence between prior and posterior assignment is 87 %.

The analyses of population structure based on molecular data, conducted by DAPC and STRUCTURE, were rather consistent in showing that *P. ruscifolia* individuals are included in a single cluster both without and with prior information on morphological assignment. The same individuals of *P. alba* (#2, #5 and #6) are misassigned according to both analyses when no prior information is given. In both analyses the hybrids #28 and #36 showed a high probability of belonging to the same cluster as those of *P. ruscifolia*. Similarly, the hybrid #33 in both cases is assigned to the same cluster of most *P. alba* individuals. There are, however, some discrepancies, related with the

assignment of individuals #34, #35 and #38. The per cent of misassignment in respect to morphologically pre assigned groups is only 3 % for the DAPC approach and 16 % according to STRUCTURE. The latter analysis indicated that five of the misassigned individuals involve different degrees of introgression and one case may represent an individual of *P. alba* misclassified morphologically as hybrid probably due to phenotypic plasticity. In summary, the different approaches used, DAPC and STRUCTURE, detected $K = 3$ or $K = 4$, respectively, as the optimal number of clusters in the genetic assignment analysis. Despite this slight discrepancy, which can be attributed to the different assumptions associated with the software used, the results are highly consistent.

The comparison of clusterings based on morphological and molecular datasets showed similar structure, as the per cent of correct assignment to species and hybrid groups varied from 86 to 97 %, suggesting that the discontinuities among such groups are significant. Molecular data show that individuals identified taxonomically as *P. ruscifolia* show almost no admixture with any of the other groups. By contrast, hybrids and introgressants can be found among individuals determined a priori as *P. alba*. This result suggests that introgression may be asymmetric favouring the transmission of genes of *P. ruscifolia* into the *P. alba* gene pool. A similar trend was proposed to occur in the case of a hybrid swarm involving *P. caldenia* and *P. flexuosa*, where Saidman (1986) using isozymes observed that some seeds collected from *P. flexuosa* mother plants bore alleles characteristic of *P. caldenia*, but no seed collected from *P. caldenia* mother plants had alleles characteristic of *P. flexuosa*. In a previous study (Ferreyra et al. 2007), where the operative taxonomic units were populations rather than individuals, we noticed that in the phenograms obtained from different datasets (isozymes or RAPDs), the associations of hybrids between species of *Prosopis* were shifting and they were more tightly associated with one or the other putative parent. These shifting associations may be explained if backcrosses frequently occur between hybrids and their parental species, determining that hybrids are a mosaic of parental and intermediate characters (Allendorf et al. 2001; González-Pérez et al. 2004; Yuzbasioglu et al. 2008).

The evidence of hybridization in this analysis stems from combining the morphological and molecular analysis. The consistency between morphological and molecular-based clustering can only be interpreted as the result of interspecific hybridization. In fact, three different scenarios should be differentiated:

1. Two non hybridising species. The expectation is that only two clusters will be retrieved.
2. A single highly polymorphic species. The expectation is a morphological continuous variation for quantitative traits together with e single cluster based on molecular data.
3. Two hybridising species with post mating reproductive barriers. The expectation is three clusters corresponding to the two pure-bred species and the hybrid group should be recognised.

Our results are clearly more compatible with the third scenario, as more than two clusters are identified, and $K = 3$ or 4 is a discrete number that does not fit the expectation for scenario 2.

In sum, all analyses conducted indicated that the three groups studied here are differentiated with low overlapping. The conclusions from both molecular and morphological analyses are consistent and indicate that *P. ruscifolia* in the sampled area would remain none contaminated by *P. alba* gene pool. On the other hand, some gene flow might occur from *P. ruscifolia* to *P. alba* through hybridization and introgression although it would be low or too recent as it was not able to yield a morphological and genetic continuum. Similarly, hybridising oak species are capable of remaining morphological or ecologically different in the face of considerable introgression. This situation has been explained by several authors (Wu 2001), who claim that natural selection operates against the exchange of genes that constitute the basis of functional divergence between species. A possible cause that produces an increase of interspecific gene flow is the occurrence of environmental disturbance. The sampled area is altered by human activities, what may have favoured hybridization events that would not occur in non-disturbed areas.

Previous works (Saidman et al. 1998a, b) have shown that despite the frequent occurrence of fertile interspecific hybrids between species of section Algarobia of *Prosopis* considered as member of a syngameon, the effective gene flow is not significant among nominal species: Therefore, they may be considered "true species" maintained by demographic and/or ecological cohesive mechanisms (Templeton 1989) rather than only genetic cohesive mechanisms (Saidman et al. 1998a, b).

In conclusion, although the reproductive barriers between the species seem to be weak, interspecific gene flow through introgression may be prevented by hybrid breakdown or natural selection favouring the maintenance of species integrity.

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