The LINNEAN SOCIETY of London BOTANICAL Journal of the Linnean Society

Botanical Journal of the Linnean Society, 2015, **177**, 593–606. With 3 figures

Genetic diversity within and among two Argentinean and one Mexican species of *Acacia* (Fabaceae)

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Received 18 June 2014; revised 7 November 2014; accepted for publication 7 January 2015

Acacia is a pantropical genus comprising > 1450 species. Following Vassal's treatment *Acacia* is considered as a single genus with three subgenera (*Acacia*, *Aculeiferum* and *Phyllodineae*). *Acacia caven*, *A. curvifructa* and *A. farnesiana* belong to subgenus *Acacia* and the relationship between them is controversial. The aim of this study was to elucidate the relationship between the three species using amplified fragment length polymorphism, analysing 15 populations of these species, and to compare the results obtained with those from a morphological analysis. Genetic diversity indices (percentage of polymorphic loci, genetic diversity) showed that genetic variation in *A. caven* is higher than that in *A. curvifructa* and *A. farnesiana*. Of the total genetic diversity in *A. caven* and *A. farnesiana*, most is found within populations (~70%). Analysis with STRUCTURE showed that the optimal number of clusters (*K*) was ten, and in all cases where populations were grouped they were geographically close and/or belong to the same variety. The morphological canonical discriminant analysis did not result in a separation between all individuals, indicating that they do not harbour consistent morphological discontinuities. Altogether, the results of our molecular analyses showed the existence of significant differences between *A. caven*, *A. curvifructa* and *A. farnesiana*, which argues for recognizing them as different species. *Botanical Journal of the Linnean Society*, 2015, **177**, 593–606.

ADDITIONAL KEYWORDS: AFLP – genetic differentiation – genetic structure – Argentina – Mexico – morphological differentiation – morphological variation.

INTRODUCTION

Acacia Mill. (*sensu lato*) is the second largest genus in Fabaceae. It is a pantropical genus comprising > 1450 species. This genus is a predominant vegetation component in Australia and also important over large areas of Africa and the Americas. Additionally, in many dryland areas, it is the dominant shrub or tree on which humans and animals depend (Rico-Arce, 2007).

The circumscription of *Acacia* is currently controversial, and it may be treated as a single genus or as multiple genera. Discussions on this subject can be found in Orchard & Maslin (2005), Smith *et al*. (2006), Van Rijckevorsel (2006), Moore *et al*. (2010) and Kyalangalilwa *et al*. (2013).

Following Vassal's treatment (1972; Polhill, Raven & Stirton, 1981), *Acacia* is considered as a single genus with three subgenera [*Acacia*, *Aculeiferum* Vassal and Phyllodineae (DC.) Ser.]. The native American *Acacia* spp. belong to subgenera *Acacia* and *Aculeiferum*. However, other authors (Seigler *et al*., 2008) included American species of the subgenus *Acacia* in *Vachellia* Wight & Arn. In the present work, we acknowledge this last classification, but we will use the name *Acacia s.l*.

Acacia caven (Mol.) Mol., *A. curvifructa* Burkart and *A. farnesiana* (L.) Willd. belong to subgenus *Acacia* (or *Vachellia*). The first species includes six varieties

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based on morphological traits (Aronson, 1992; Pometti *et al*., 2007), random amplification of polymorphic DNA (RAPD) (Pometti *et al*., 2010) and amplified fragment length polymorphism (AFLP) (Pometti *et al*., 2012) markers: *A. caven* var. *caven*, *A. caven* var. *dehiscens* Burkart ex Ciald., *A. caven* var. *sphaerocarpa* Burkart ex Aronson, *A. caven* var. *stenocarpa* (Speg.) Burkart ex Ciald., *A. caven* var. *microcarpa* (Speg.) Burkart ex Ciald. and *A. caven* var. *macrocarpa* Aronson. Argentina is the only country where all six varieties are present (Aronson, 1992). Molina (1810) described *A. caven* var. *caven* from specimens found in central Chile and considered it to be closely related to *A. farnesiana*. These two species are superficially similar, and some subsequent authors have merged the two taxa (Hassler, 1909; Spegazzini, 1924). However, according to Ebinger, Seigler & Clarke (2000), these taxa are distinct, and they do not occur sympatrically, with a possible exception of south-eastern Brazil. They have similar inflated pods, short shoots with numerous small leaves, flowers and inflorescences, but differ in other traits. In *A. farnesiana*, the petiole has a small, circular gland that is raised above the petiolar groove, leaves with two to six pinna pairs and leaflets with obvious lateral veins. *Acacia caven*, by contrast, has larger, elongated petiolar glands that are sessile in the petiolar groove, many of the leaves have more than six pinna pairs and the leaflets lack obvious lateral veins (Ebinger *et al*., 2000).

Aronson & Nash (1989) treated *A. curvifructa* as a putative hybrid between *A. caven* and *A. farnesiana*. This treatment is doubtful because the ranges of *A. caven* and *A. farnesiana* virtually do not overlap (fig. 4 in Ebinger *et al*., 2000). According to Ebinger *et al*. (2000), *A. farnesiana* is not common in Argentina or Paraguay; it is present as scattered introductions, giving little opportunity for hybridization to occur. The relationships among these three species are thus controversial. *Acacia curvifructa* is similar to *A. farnesiana*, in that it has relatively large leaflets with obvious secondary venation; the structure of the petiolar gland, the puberulent petioles, rachis and the short peduncles, however, suggest a close relationship to *A. caven*. The strongly curved fruits of *A. curvifructa* that are flattened to slightly elliptical in cross-section are quite different from the mostly straight, inflated fruits of *A. caven* (Ebinger *et al*., 2000). In a more recent work, Pometti *et al*. (2007) suggested little morphological differentiation between *A. caven* var. *stenocarpa*, *A. caven* var. *microcarpa* and *A. curvifructa*.

In this context, molecular markers could be useful to provide evidence on the actual genetic relationships between *A. caven*, *A. curvifructa* and *A. farnesiana*. Therefore, the aim of this study is to elucidate the relationships between the three species by means of AFLP and to investigate the genetic diversity within and divergence between the studied populations. The results obtained by this method were compared with those from a morphological analysis.

MATERIAL AND METHODS

Species and populations sampled for AFLP analysis

Acacia caven

Here we used 11 Argentinean populations of *A. caven* (four of var. *caven*, two of var. *stenocarpa*, one of var. *microcarpa* and four of var. *dehiscens*). On the basis of the studies cited above, we choose *A. caven* vars. *microcarpa* and *stenocarpa*, because of their similarity to *A. curvifructa*, and *A. caven* var. *caven* because it is similar to *A. farnesiana* and this variety is the most widespread. Also, *A. caven* var. *dehiscens* was chosen because it is similar to *A. caven* var. *caven* except for the dehiscence of its pods (Table 1).

Acacia curvifructa

For this species, only one population was sampled (in 1991). Since then, several collection trips were made in all Argentinean regions, with no success in finding the species again (Table 1).

Collection methods for these two species were as described by Vilardi, Saidman & Palacios (1988) and Saidman & Vilardi (1993). Approximately 50 pods were collected from six to ten mother shrubs that were separated from each other by > 50 m. The varieties of *A. caven* were identified following the key for fruiting specimens proposed by Aronson (1992). Representative vouchers of each population were deposited at the herbarium SI, Instituto de Botánica Darwinion, San Isidro, Buenos Aires, Argentina.

Acacia farnesiana

For this species, we used three accessions coming from the Danida Forest Seeds Center (DFSC). All sampled sites corresponding to the accessions were from Mexico (Table 1).

AFLP methods and data analysis

DNA extraction. Cotyledons were ground to a fine powder in liquid nitrogen and then placed in a microtube. The DNeasy Plant kit (Qiagen) was used for DNA extraction following the manufacturer's instructions. DNA was stored at –20 °C.

The AFLP assay was performed as described by Vos *et al*. (1995), but with a slight modification. Four selective primers were combined as follows: E + ACA/M + CTT (C1), E + AGG/M + CAG (C3), E + AAC/M + CAA (C4) and E + AAG/M + CAA (C5). Other

Table 1. Populations of *Acacia* used in this study

Species	Country of provenance	Population	Population code	Latitude	Longitude	No. of individuals analysed	Accession number/seed bank
A. caven var *caven*	Argentina	Campo Quijano	CQ	24°55′12.00″S	65°39′0.00″W	13	–
A. caven var *caven*	Argentina	Ruta Nueve	RN	24°39′48.00″S	65°22′49.00″W	14	–
A. caven var *caven*	Argentina	Costanera Sur	CS	34°38′10.71″S	58°42′44.08″W	16	–
A. caven var *caven*	Argentina	Gualeguaychú	GY	33°22′4.00″S	58°44′3.00″W	22	–
A. caven var *dehiscens*	Argentina	Las Gemelas	LG	30°53′26.10″S	64°30′13.50″W	13	–
A. caven var *dehiscens*	Argentina	Pan de Azúcar	PA	31°15′58.90″S	64°20′28.60″W	11	–
A. caven var *dehiscens*	Argentina	Vaquerías	VA	31°23′38.93″S	63°51′30.87″W	12	–
A. caven var *dehiscens*	Argentina	Valle Hermoso	VH	31° 7′1.20″S	64°28′58.80″W	22	–
A. caven var *microcarpa*	Argentina	Vivero Forestal	VF	26°16′0.00″S	58°17′41.64″W	12	–
A. caven var *stenocarpa*	Argentina	Formosa	FS	26°16′13.20″S	58°17′7.92″W	12	–
A. caven var *stenocarpa*	Argentina	YPF	YP	26°11′26.76″S	58° 9′23.82″W	14	–
A. curvifructa	Argentina	Ingeniero Juárez	IJ	23°53′60.00″S	61°51′0.00″W	35	–
A. farnesiana	Mexico	Parras de la Fuente	PR	25°26′25.00″N	102°10′45.00″W	18	1270/84-DSFC
A. farnesiana	Mexico	Pailla	PL	25°33′22.03″N	102°27′32.04″W	18	1470/84-DSFC
A. farnesiana	Mexico	La Sauceda	LC	28°25′33.61″N	100°39′44.38″W	18	1465/84-DSFC

primer combinations assayed were discarded because they generated profiles in which the amplification products were too dense to allow reliable scoring or generated too few amplification products. Selective amplification products were mixed with an equal volume of dye reagent [98% (v/v) formamide, 10 mM EDTA, 0.025% (w/v) bromophenol blue and 0.025% (w/v) xylene cyanol]. Seven microlitres was separated by electrophoresis in a Model S2 apparatus (Gibco BRL Sequencing System, Life Technologies) through 6% (w/v) polyacrylamide gels containing 5 M urea, in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8). A 30- to 330-bp AFLP DNA Ladder (Gibco BRL, Life Technologies) size marker was included twice or three times in each electrophoresis run. The size of AFLP bands scored ranged from 50 to 330 bp. Gels were stained with silver nitrate (Bassam, Caetano-Anolle & Greshoff, 1991).

Data scoring and analysis. Each AFLP band was considered as a single bi-allelic locus with an amplifiable and a null allele. Bands with the same relative migration distance were considered homologous. Data were scored manually as band presence (1) or absence (0), double-checked and performed by two different people, together or separately.

The informativeness of AFLP primer combinations was assessed using the following parameters. The polymorphic information content (PIC) averaged over the fragments for each primer combination was calculated with the software PICcalc (Nagy *et al*., 2012). The marker index (MI) for each primer combination was determined as proposed by Varshney *et al*. (2007). The resolving power (RP) of each primer combination was measured according to Prevost & Wilkinson (1999).

To check for outlier–F_{ST} loci, the program BayeScan v2.1 (Foll & Gaggiotti, 2008) was used with a burn-in period of 50 000, a thinning interval of 10, 100 000 iterations, 20 pilot runs of and a length of each pilot run of 5000. The dataset was divided into two subgroups, neutral and 'selective' (outlier) AFLP loci, as suggested by Luikart *et al*. (2003).

For neutral loci, allele frequencies were estimated using the Bayesian method with non-uniform prior distribution of allele frequencies, as described by Zhivotovsky (1999) by means of the software AFLP-SURV (Vekemans, 2002), following the approach of Lynch & Milligan (1994). Non-hierarchical Wright's (1978) F_{ST}, variability measures and Nei's (1973) genetic diversity H were also estimated using the software AFLP-SURV (Vekemans, 2002). As the whole sample involves populations of different species, F_{ST} is interpreted as a raw measure of genetic distance with no implications for relative gene flow between populations.

The distribution of total genetic diversity was estimated in *A. caven* and *A. farnesiana* separately at different hierarchical levels by analysis of molecular variance (AMOVA) considering varieties (in *A. caven* only), populations and individuals as nested levels. The decomposition of variance by AMOVA was conducted following Excoffier, Smouse & Quattro (1992), using the matrix approximations from Dyer *et al*. (2004) with the software GeneticStudio (Dyer, 2008).

To identify population structure in the three species, a Bayesian model-based cluster analysis was performed using the STRUCTURE program version 2.3.4 (Pritchard, Wen & Falush, 2009). The burn-in period and the number of Monte Carlo Markov chain (MCMC) repetitions were set respectively to 50 000 and 100 000. An admixture model was used, with correlated allele frequencies. K was set at two to 15, and the highest K value was identified as the run with the highest likelihood value, following the recommendations of Pritchard, Stephens & Donnelly (2000). In addition, K values were averaged across ten iterations.

Levels of differentiation among populations were studied further with an assignment test with a Bayesian-based approach (Rannala & Mountain, 1997) using GENECLASS 2 (Piry *et al*., 2004). This test calculates the most likely population of origin of each individual. The additional parameters chosen included: (1) the simulation algorithm for population assignment described by Paetkau *et al*. (2004); (2) simulation of 10 000 genotypes for each population; and (3) an arbitrary threshold probability value of 0.01 for the assignment.

Canonical discriminant analysis was applied to AFLP data to summarize variation between predefined classes (species/varieties) for classification variables (band presence/absence). These analyses were carried out with the software Statistica 5.5 (StatSoft & Inc, 2000).

Morphological analysis

To compare the results obtained with the AFLP technique, morphological differentiation was evaluated in the three species and varieties. In total, 124 herbarium specimens were selected for statistical analyses and seven quantitative traits and one qualitative trait of fruits and leaves were measured. Flower characters were not included, as they are uniform in the genus. The quantitative traits were: rachis length (cm) (RAL), pairs of leaflets on the apical pinna (PLA), pairs of leaflets on the basal pinna (PLB), stipular spine length (cm) (SSL), fruit peduncle length (cm) (FPL), fruit length (cm) (FRL) and fruit width (cm) (FRW). The qualitative trait, position of the petiolar gland (PPG), showed three alternatives in the sampled individuals: base of rachis; centre of petiole; or base of petiole. These alternatives were coded as 1, 2 and 3, respectively. Rachis length, stipular spine length, fruit peduncle length, fruit length and fruit width were measured with a ruler to the nearest millimetre. All the measurements were made by the same person (C.P.). All the specimens were from the herbarium of the Instituto de Botánica Darwinion (SI) (San Isidro, Buenos Aires, Argentina) (see the list of material used in Appendix 1).

Canonical discriminant analysis was applied to morphometric data to summarize variation between predefined classes (species/varieties) for classification variables. The basic data matrix included discrete and continuous variables (mixed matrix). Data were transformed to standard deviation units. These analyses were carried out with the software Statistica 5.5 (StatSoft & Inc, 2000). The data matrix is available from the corresponding author.

RESULTS

Genetic diversity and population structure

The four primer pair combinations used for AFLP analysis generated 228 bands between 80 and 330 bp, with an average of 57 bands per primer combination. There were 225 bands in *A. caven* (221 polymorphic), 183 in *A. curvifructa* (130 polymorphic) and 144 in *A. farnesiana* (121 polymorphic) (Table 2). The unique bands produced by different primer combinations were specific to single species. Thirty-one unique bands with a range of one to 12 per primer combination per species were observed (Table 2). PIC, MI and RP estimates were higher for *A. caven* for all primer combinations (Table 2) than for *A. curvifructa* and *A. farnesiana*. Each of the 250 individuals analysed showed a distinctive banding pattern.

Analysis of presence of outlier–F_{ST} AFLP loci with a chosen q-value threshold of 10%, showed that *c*. 7% of loci (17 out of 228) appear to be under diversifying or purifying selection. According to these results the 17 outlier loci were removed and further analyses were based on the 211 loci assumed as neutral. All of these loci were polymorphic across populations.

In *A. caven*, the percentage of polymorphic loci (PLP) ranged among populations from 64.9 to 91.5% (Table 3). Average heterozygosity (H) varied from 0.21 in Formosa to 0.35 in Pan de Azúcar, Las Gemelas and Vaquerías. In *A. farnesiana*, PLP ranged from 18.0% in Pailla to 35.1% in Parras de la Fuente. H varied from 0.10 to 0.12 (Table 3).

The population of *A. curvifructa* presented a PLP of 49.8% and H of 0.21 (Table 3).

The analysis of population structure performed with the software AFLPsurv indicated that the component of variability within populations (Hw = 0.24) is higher

Table 2. Polymorphism and primer informativeness of four AFLP primer combinations analysed in 15 populations of three *Acacia* spp.

	Total bands	Monomorphic bands	Polymorphic bands	Unique bands	Fragment polymorphism (%)	PIC	MI	RP
Primer combination								
Acacia caven								
E-ACA/M-CTT (C1)	74	0	74	12	100	0.3	22.2	44.9
E- AGG/M-CAG (C3)	52	0	52	4	100	0.3	16.6	36.0
E- AAC/M-CAA (C4)	46	4	42	0	91.3	0.3	13.4	28.8
E- AAG/M-CAA (C5)	53	0	53	12	100	0.3	14.8	30.6
Total	225	4	221	28		1.2	67.1	140.3
Average	56.3	1	55.3	7	97.8	0.3	16.8	35.1
Acacia curvifructa								
E-ACA/M-CTT (C1)	59	13	46	2	78.0	0.2	6.9	15.3
E- AGG/M-CAG (C3)	44	15	29	0	65.9	0.2	4.4	7.9
E- AAC/M-CAA (C4)	40	8	32	1	80	0.2	7.4	11.0
E- AAG/M-CAA (C5)	40	17	23	0	57.5	0.2	3.9	15.4
Total	183	53	130	3		0.7	22.5	49.7
Average	45.8	13.3	32.5	0.8	70.3	0.2	5.6	12.4
Acacia farnesiana								
E-ACA/M-CTT (C1)	43	5	38	0	88.4	0.2	6.4	26.1
E- AGG/M-CAG (C3)	42	7	35	0	83.3	0.2	6.6	19.0
E- AAC/M-CAA (C4)	26	5	21	0	80.8	0.1	2.5	11.6
E- AAG/M-CAA (C5)	33	6	27	0	81.8	0.1	1.6	5.2
Total	144	23	121	0		0.5	17.2	61.9
Average	36	5.8	30.3	0	83.6	0.1	4.3	15.5

PIC, polymorphism information content; MI, marker index; RP, resolving power.

Table 3. Summary of genetic diversity based on 211 neutral AFLP loci analysed in 15 populations of three *Acacia* spp.

Species	Population	NL	PPL	*H*	SE (*H*)
A. caven var. *caven*	CQ	211	64.9	0.25	0.01
A. caven var. *caven*	RN	211	72.0	0.26	0.01
A. caven var. *caven*	CS	211	76.3	0.27	0.01
A. caven var. *caven*	GY	211	84.8	0.31	0.01
A. caven var. *dehiscens*	LG	211	86.3	0.35	0.01
A. caven var. *dehiscens*	PA	211	88.2	0.35	0.01
A. caven var. *dehiscens*	VA	211	91.5	0.35	0.01
A. caven var. *dehiscens*	VH	211	76.3	0.27	0.01
A. caven var. *microcarpa*	VF	211	80.6	0.25	0.01
A. caven var. *stenocarpa*	FS	211	68.7	0.21	0.01
A. caven var. *stenocarpa*	YP	211	67.8	0.24	0.01
A. farnesiana	PR	211	35.1	0.13	0.01
A. farnesiana	PL	211	18.0	0.10	0.01
A. farnesiana	LC	211	20.4	0.11	0.01
A. curvifructa	IJ	211	49.8	0.21	0.01

NL, number of loci; PPL, percentage of polymorphic loci; *H*, Nei's (1973) genetic diversity; SE (*H*), standard error of *H*.

than among populations ($Hb = 0.14$). However, the global F_{ST} estimate (0.36) is highly significant ($P < 0.001$), indicating genetic differentiation among populations.

Analysis of population structure was performed within species for *A. caven* and *A. farnesiana*, in which several population samples were available (Table 4). The estimates of global F_{ST} for *A. caven* (0.29) and for *A. farnesiana* (0.47) among populations were in both cases highly significant ($P < 0.001$), providing evidence of genetic structure within species.

The results from hierarchical AMOVA showed highly significant ($P < 0.001$) genetic differentiation among populations and within populations, indicating the presence of genetic structure in *A. caven* and *A. farnesiana* (Table 5). Moreover, in *A. caven*, the differentiation among varieties, although lower (10%), was highly significant (Table 5). Of the total genetic diversity, most resided within populations in both species (67.1% in *A. caven* and 77.6% in *A. farnesiana*). The estimated population differentiation Φ_{ST} value was 0.33 and 0.22 for the species, respectively, for this phenetic treatment of the data, and was similar to Wright's (1978) fixation index ($F_{ST} = 0.29$) for *A. caven*, but was lower for *A. farnesiana* ($F_{ST} = 0.47$).

Analysis of data using STRUCTURE revealed that $K = 10$ had the highest mean probability of density (ln $P(D) = -16229.85$), after which this value reached a plateau, which suggested that the optimal number of K was 10 (Fig. 1). In this analysis, individuals of populations of Formosa, YPF and Vivero Forestal, belonging to vars. *stenocarpa* and *microcarpa*, respectively, are grouped together (cluster 9); the same occurs with individuals of populations of Campo Quijano and Ruta Nueve, which belong to var. *caven* (cluster 6), and individuals of populations of Pan de Azúcar and Vaquerías, which belong to var. *dehiscens* (cluster 2) (Fig. 2). The remaining populations of vars. *caven* and *dehiscens* do not show any association between them or with any other population. In *A. farnesiana*, individuals of populations Pailla and La Sauceda are grouped together (cluster 7) (Fig. 2), but there is no association with Parras de la Fuente. The individuals of the population of *A. curvifructa* sampled are grouped together and appear well differentiated from the remaining populations (Fig. 2). Moreover, pairwise F_{ST} values showed that populations within these four clusters are more similar to each other than to other populations (Table 4). STRUCTURE detected some admixture individuals in all populations, although *A. curvifructa* showed the lowest amount of admixture.

In the test conducted with GENECLASS 2 the assignment of individuals to their respective population varied from 0.56 (YP) to 0.94 (PR) (Table 6). The highest assignment value of one population to another corresponded to GY (0.32 ± 0.01). For *A. caven* and

Table 4. Estimates of Wright's (1978) F_{ST} values among all pairs of populations on the three *Acacia* spp. separately

Species		*A. caven*										*A. curvifructa*		*A. farnesiana*	
A. caven	Population	CQ	CS	FS	GY	LG	PA	RN	VA	VF	VH	YP	IJ	PR	PL
	CQ														
	CS	0.35													
	FS	0.42	0.47												
	GY	0.28	0.23	0.37											
	LG	0.28	0.26	0.35	0.23										
	PA	0.30	0.30	0.33	0.20	0.17									
	RN	**0.16**	0.32	0.40	0.28	0.28	0.27								
	VA	0.31	0.25	0.34	0.20	0.18	**0.05**	0.29							
	VF	0.40	0.43	**0.11**	0.33	0.33	0.29	0.39	0.30						
	VH	0.20	0.31	0.34	0.20	0.23	0.20	0.20	0.20	0.32					
	YP	0.38	0.42	**0.12**	0.33	0.34	0.31	0.35	0.31	**0.08**	0.30				
A. curvifructa	IJ	0.45	0.50	0.41	0.42	0.36	0.34	0.45	0.36	0.39	0.41	0.43			
A. farnesiana	PR	0.47	0.48	0.49	0.35	0.42	0.39	0.47	0.42	0.43	0.41	0.43	0.58		
	PL	0.54	0.54	0.46	0.46	0.46	0.41	0.52	0.42	0.45	0.43	0.45	0.56	0.49	
	LC	0.54	0.54	0.48	0.44	0.44	0.40	0.52	0.41	0.46	0.43	0.46	0.55	0.53	**0.33**

Bold type: F_{ST} values for sampling site pairs grouped by STRUCTURE.

Table 5. Population structure estimated by AMOVA in 11 populations of *Acacia caven* and three populations of *A. farnesiana*

Source	d.f.	SSD	Variance component	% Variance	Φ	*P*
Acacia caven						
Among all populations (Φ_{ST})	10	1389.17	8.9	32.8	0.33	0.0010
Among varieties (Φ_{RT})	3	617.46	2.7	10.0	0.10	0.0010
Among populations within varieties (Φ_{SR})	7	771.71	6.2	22.8	0.25	0.0010
Within populations	150	2753	18.3	67.1	–	–
Total	160	4142.17	27.3	100	–	–
Acacia farnesiana						
Among populations (Φ_{ST})	2	261.68	6.1	22.4	0.22	0.0010
Within populations	51	1075.72	21.1	77.6	–	–
Total	53	1337.41	27.2	100	–	–

Phi (Φ), fixation indices; *P*, significance level.

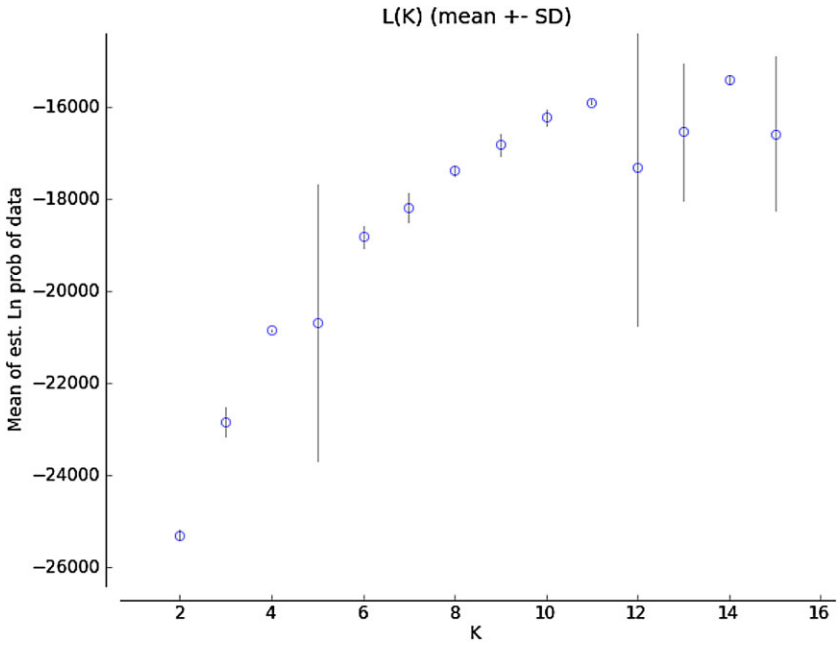

Figure 1. Plot of mean probability of density values obtained with STRUCTURE versus *K*. Vertical bars indicate the confidence interval.

A. farnesiana, the assignment rate of individuals of one species to populations of the same species was higher than to populations of different species. For *A. caven* the values were, respectively, 0.16 ± 0.21 and 0.10 ± 0.09, and for *A. farnesiana* 0.58 ± 0.27 and 0.10 ± 0.08.

Canonical discriminant analysis based on allele frequencies succeeded in differentiating all the species and varieties studied. Sixty of 211 neutral loci were sufficient to differentiate species and varieties. The correct classification of individuals into their respective species or varieties based on AFLP profiles was 100% in all cases. The first and second canonical roots accounted for 42.6 and 28.6% of the variation, respectively, explaining a cumulative 71.2% of the molecular variation. The plot shows roughly six groups that correspond to *A. caven* var. *stenocarpa*, *A. caven* var. *microcarpa*, *A. caven* var. *caven*, *A. caven* var. *dehiscens*, *A. curvifructa* and *A. farnesiana* (Fig. 3A).

Morphological analysis

According to canonical discriminant analysis (CDA), the traits that contributed most to the morphometric

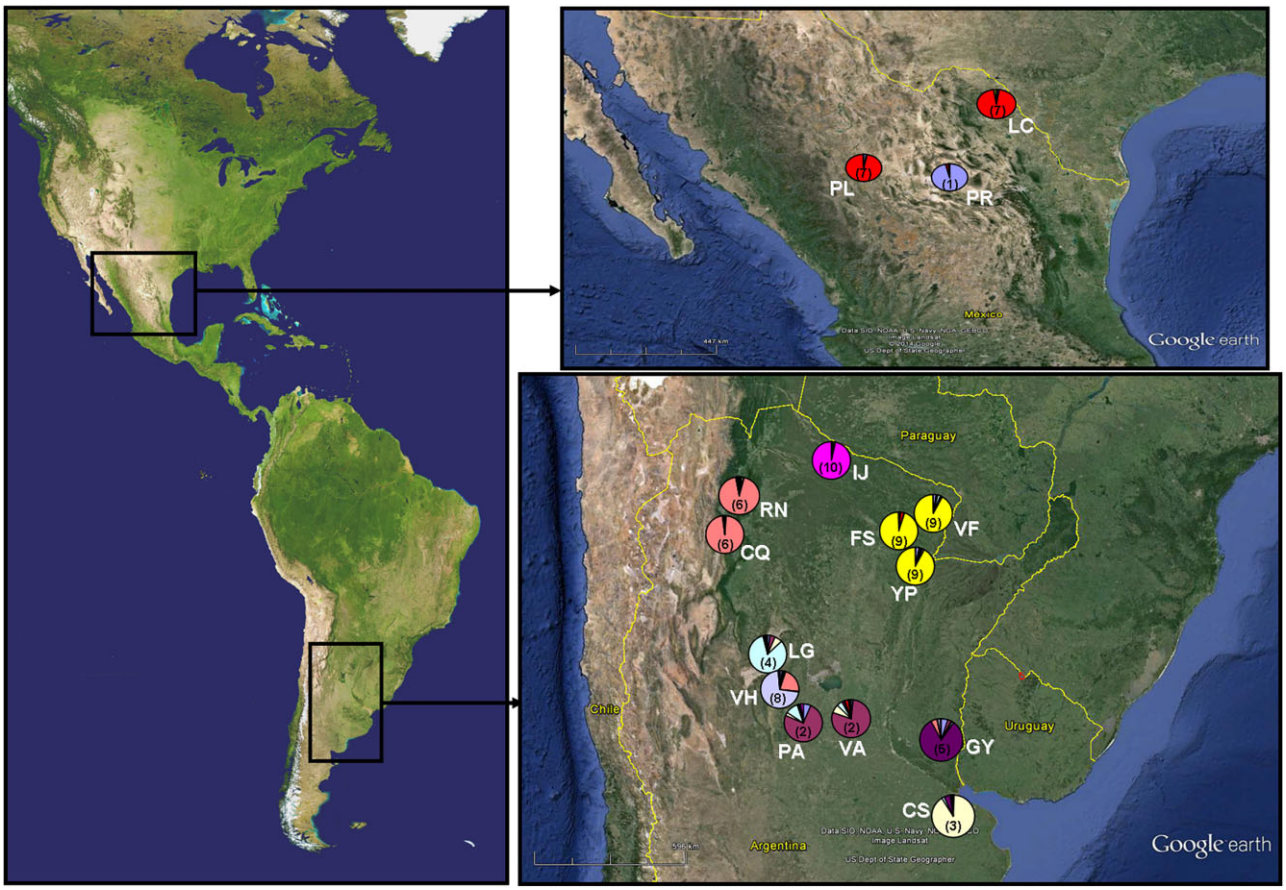

Figure 2. Geographical origin of the samples of three *Acacia* spp. Analysed. Pie charts represent combined genetic ancestries of all individuals sampled in each population, as obtained from STRUCTURE. The ten different colours correspond to ten different genetic clusters. Population codes are as in Table 1. Genetic cluster numbers determined by STRUCTURE are in parentheses.

differentiation were FRL, FRW, PPG, PLB, FPL ($P < 10^{-5}$) and SSL ($P = 0.017$) (Table 7). The correct classification of individuals into their respective species and varieties ranged between 37.5% in *A. curvifructa* and 77.4% in *A. caven* var. *caven*, with an average of 65.7%. The first and second canonical axes accounted for 62.8 and 30.4% of the variation, respectively, explaining a cumulative 93.2% of the morphological variation. The projection of individuals onto these canonical roots gave a picture different from that observed for AFLPs. The best discriminated group were individuals of *A. farnesiana*, and there was overlapping of *A. caven* var. *stenocarpa*, *A. caven* var. *microcarpa* and *A. curvifructa* individuals, and individuals of *A. caven* var. *caven* and *A. caven* var. *dehiscens* (Fig. 3B).

DISCUSSION

The population genetic diversity and structure of a species reflect the interaction of various factors, including the long-term evolutionary history of the species, genetic drift, gene flow, mating system, seed dispersal and geographical range (Hogbin & Peakall, 1999). A powerful tool for genetic variability and population structure studies is the analysis of AFLPs due to their ability to provide high numbers of polymorphic markers (Mueller & Wolfenbarger, 1999; Bensch & Akesson, 2005). In this study, we assessed the population genetic diversity and structure of three related species of *Acacia* subgenus *Acacia* (*A. caven*, *A. curvifructa* and *A. farnesiana*) using AFLP markers. None of the individuals studied here was genetically identical to any other based on its band pattern, indicating that this level of resolution was sufficient to distinguish all multilocus genotypes.

The 31 unique bands identified in this study provided baseline data for identifying individuals belonging to these three species for tree improvement programs. The primer combinations C1 (E-ACA/M-CTT) and C5 (E-AAG/M-CAA) for *A. caven*, and C1 (E-ACA/M-CTT) for *A. curvifructa* were the most reli-

Table 6. Average assignment rate of individuals into each population as calculated by GENECLASS 2 in 250 samples from 15 *Acacia* populations based on AFLP markers

Population	CQ	CS	FS	GY	LG	PA	RN	VA	VF	VH	YP	IJ	PR	PL	LC
CQ	**0.60**	0.00	0.00	0.32	0.08	0.04	0.16	0.10	0.03	0.25	0.00	0.03	0.06	0.00	0.11
CS	0.00	**0.74**	0.00	0.31	0.08	0.04	0.14	0.11	0.01	0.23	0.00	0.01	0.06	0.02	0.11
FS	0.00	0.00	**0.67**	0.31	0.08	0.12	0.14	0.11	0.36	0.23	0.10	0.07	0.11	0.39	0.13
GY	0.01	0.04	0.00	**0.85**	0.07	0.10	0.13	0.12	0.04	0.21	0.00	0.03	0.09	0.05	0.10
LG	0.00	0.02	0.00	0.31	**0.68**	0.13	0.13	0.12	0.02	0.21	0.00	0.03	0.06	0.00	0.10
PA	0.00	0.00	0.00	0.32	0.09	**0.69**	0.14	0.33	0.07	0.23	0.00	0.04	0.06	0.03	0.11
RN	0.01	0.03	0.00	0.31	0.07	0.08	**0.75**	0.10	0.04	0.22	0.00	0.03	0.06	0.00	0.09
VA	0.00	0.04	0.01	0.34	0.09	0.34	0.13	**0.69**	0.07	0.23	0.00	0.03	0.07	0.03	0.12
VF	0.00	0.00	0.14	0.31	0.07	0.11	0.13	0.11	**0.78**	0.21	0.11	0.06	0.10	0.26	0.12
VH	0.01	0.04	0.01	0.35	0.06	0.14	0.11	0.13	0.10	**0.79**	0.00	0.03	0.08	0.15	0.10
YP	0.00	0.00	0.14	0.32	0.08	0.09	0.14	0.11	0.38	0.23	**0.56**	0.04	0.09	0.31	0.12
IJ	0.00	0.00	0.00	0.31	0.08	0.13	0.14	0.11	0.14	0.22	0.00	**0.70**	0.06	0.06	0.11
PR	0.00	0.00	0.04	0.33	0.07	0.06	0.14	0.09	0.15	0.22	0.00	0.03	**0.94**	0.38	0.24
PL	0.00	0.07	0.08	0.31	0.05	0.11	0.09	0.10	0.13	0.16	0.00	0.06	0.40	**0.85**	0.60
LC	0.00	0.01	0.05	0.32	0.07	0.13	0.13	0.11	0.15	0.22	0.00	0.05	0.36	0.53	**0.92**
Ave.	0.002	0.017	0.034	0.320	0.075	0.115	0.132	0.125	0.121	0.220	0.015	0.039	0.119	0.158	0.155
SD	0.004	0.021	0.051	0.013	0.011	0.071	0.017	0.059	0.117	0.020	0.039	0.016	0.112	0.179	0.134

Correct assignments are marked in bold. Ave., average assignment value from one population to the others. SD, standard deviation.

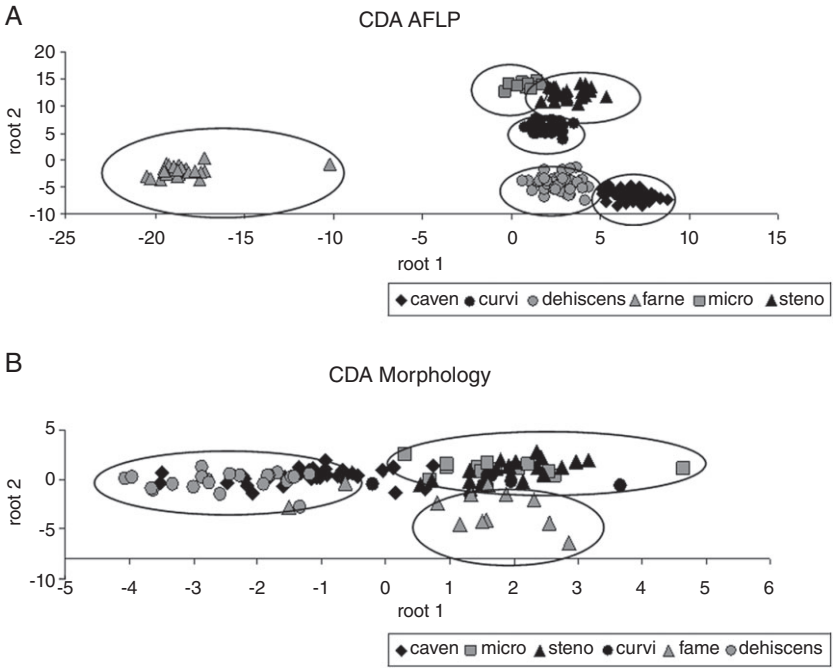

Figure 3. Plot of canonical discriminant functions 1 and 2 of *A. caven* varieties, *A. curvifructa* and *A. farnesiana*. A, from AFLP data; B, from morphological data.

Table 7. Morphological traits and standardized canonical coefficients (CV1 and CV2) from a discriminant analysis used to separate three *Acacia* spp. and varieties of *A. caven*

Trait	CV1	CV2
Rachis length	0.00	0.00
Pairs of leaflets on the apical pinna	0.00	0.00
Pairs of leaflets on the basal pinna	0.40	0.44
Stipular spine length	0.24	0.39
Fruit peduncle length	0.47	–0.66
Position of the petiolar gland	0.55	–0.46
Fruit width	–0.52	0.16
Fruit length	–0.55	–0.75

able and efficient in detecting unique bands in the species studied. *Acacia caven* presented the highest numbers of polymorphic (97.8%) and unique bands (28 bands). By contrast, *A. farnesiana* did not have any unique band for any primer combination.

The informativeness of the AFLP primer combinations, as revealed by PIC and MI, has been widely used in many genetic diversity studies (Varshney *et al*., 2007; Shen *et al*., 2010; Pavithra *et al*., 2014; Khadivi-Khub *et al*., 2015). Prevost & Wilkinson (1999) used a new concept to assess the discriminatory power of primers quantified by means of the RP coefficient. The MI was used to study the overall usefulness of a primer combination. The maximum MI (22.2) was recorded for C1 (E + ACA/M + CTT) in *A. caven*; this index was much lower in the other two species for the four combinations of primers used here. The PIC values obtained here were similar for the four primer combinations in the three species, although they were slightly lower in *A. curvifructa* and *A. farnesiana*. The values obtained for these two species (range 0.1–0.2) were in accordance with those found in *Pongamia pinnata* (L.) Pierre (Pavithra *et al*., 2014) and *Ulmus minor* Mill. and *U. glabra* Huds. (Cox *et al*., 2014), and those obtained in *A. caven* (= 0.3) were in accordance with those recorded in Satureja bachtiarica Bunge (Khadivi-Khub *et al*., 2015). The RP always showed the highest value for primer combination C1 (E + ACA/M + CTT) in the three species. However, RP reached its maximum values for all primer combinations in *A. caven*, and these values showed the same trend as those found with AFLP in *Pongamia pinnata* by Pavithra *et al*. (2014). In summary, the PIC, MI and RP values recorded for the four primer combinations may be considered relatively high in *A. caven* in comparison with the other two species, indicating that they can be effectively used to discriminate the individuals from different populations and varieties.

Studies on genetic diversity in other species of *Acacia* are mostly based on different markers including isozymes in *A. melanoxylon* R.Br. (Playford, Bell & Moran, 1993), *A. nilotica* (L.) Delile (Varghese,

Edwards & Hamrick, 1999) and *A. albida* Delile (Joly *et al*., 1992), RAPD plus inter-simple sequence repeats (ISSRs) in *A. senegal* Willd. (Chiveu Chemulanga *et al*., 2008) and RAPD in *A. raddiana* Savi (Shrestha, Golan-Goldhirsh & Ward, 2002). The variability recorded in the present work is within the range of values observed in those studies, despite the fact that the ability to detect diversity usually varies among markers (partially due to differences in mutation rates). The genetic variation in *A. caven* was higher than that found in *A. curvifructa* and *A. farnesiana*. The genetic diversity within populations of *A. farnesiana* was much lower (about half) than those observed in the other two species studied in the present work. Within-population variation is dependent on the properties of the sampled populations, including both environmental and demographic aspects. In isolated trees or shrubs sampled from farmlands and mixed forests, considerable selfing and regeneration of related individuals close to the parent trees might be expected. Differences could also occur when seed collections are made from natural modified environments (Varghese *et al*., 1999). The low PLP and *H* estimates for *A. farnesiana* may be due to sampling of only a few populations from a limited region of the natural species range. However, the lower variability within these populations may be the result of lower effective population sizes or higher inbreeding rates in comparison with the other two species.

The genetic structure of the group of species was assessed by several approaches in this work. A significant amount of genetic differentiation among populations was observed using Wright's approach (F_{ST} = 0.36). In *A. caven* the AMOVA showed that the genetic differentiation among varieties and among populations within varieties is significant. In *A. farnesiana*, the differentiation among populations was also significant. However, in both species most of the genetic diversity is represented within populations (~70%), in agreement with previous results for long-lived and outcrossing species (Hamrick & Godt, 1989) and for other South American and African *Acacia* spp. (Chiveu Chemulanga *et al*., 2008; Omondi *et al*., 2010; Pometti *et al*., 2013).

The analysis with STRUCTURE showed that the optimal number of clusters (*K*) was 10. Within *A. caven*, three groups of populations were observed: the first comprised Formosa, YPF and Vivero Forestal, the second group included Campo Quijano and Ruta Nueve, and the third involved Pan de Azúcar and Vaquerías. The remaining four populations of *A. caven* represented independent clusters. Two populations of *A. farnesiana* (Pailla and La Sauceda) were grouped in a single cluster, whereas Parras de la Fuente was separated. Finally, the *A. curvifructa* population (Ingeniero Juárez) was not associated with any other population. In all cases where populations were grouped they were geographically close and/or belong to the same variety. Moreover, this model-based analysis showed that all the individuals of the *A. curvifructa* population grouped together and showed the lowest amount of admixture. Consistent with these results, the other Bayesian approach conducted with GENECLASS 2 showed that the population of *A. curvifructa* has a high assignment rate to the same population (0.7) and low average rate of assignment to other populations (0.04), suggesting that it was well differentiated from the other species. In *A. farnesiana*, the assignation rate to populations of the same species was much higher to that of different species. In *A. caven*, the among-population diversity observed from F_{ST} values and STRUCTURE was also supported by the relatively small difference between the assignment rate to the same and to different species.

The joint analysis of molecular markers provided evidence to support that the species *A. curvifructa* is well differentiated from both *A. caven* and *A. farnesiana*, in contrast to previous evidence from morphological and biochemical studies (Aronson & Nash, 1989; Pometti *et al*., 2007).

One of the clusters of the STRUCTURE analysis within *A. caven* joined two populations of *A. caven* var. *dehiscens*. However, the other two populations of *A. caven* var. *dehiscens* that are situated at a greater geographical distance from each other (Las Gemelas and Valle Hermoso) appeared as separate entities in these analyses. Another group joined *A. caven* vars. *stenocarpa* and *microcarpa*. A close relationship between these varieties has been pointed out previously in some studies (Aronson, 1992; Pometti *et al*., 2010, 2012). A possible explanation for the relationship between populations of *A. caven* vars. *stenocarpa* and *microcarpa* could be found in morphology, as in these two varieties shrubs tend to be weaker and more spindly compared with other varieties. Moreover, Aronson (1992) hypothesized that some introgression may take place between these two varieties. However, when the CDA was done with AFLP data, it succeeded in differentiating all species and varieties. Unlike the AFLP CDA, the morphological CDA did not result in a separation between *A. caven* var. *stenocarpa*, *A.caven* var. *microcarpa* and *A. curvifructa* individuals, and *A. caven* var. *caven* and *A. caven* var. *dehiscens* individuals, indicating that they do not harbour consistent morphological discontinuities. This could be due to the fact that for *A. caven*, and many related species, most pinnae, leaflet and spinescent stipule parameters show such considerable infraspecific and even within-population variation that they gain taxonomic value only when substantiated by greenhouse or common-garden experiments

or when they are shown to be genetically fixed traits (Aronson, 1992).

The differences between morphological and molecular analyses can be explained by the constancy of DNA markers that are not influenced by the environment throughout ontogeny. These markers are usually considered to be selectively neutral (Strauss *et al*., 1992) and thus do not necessarily reflect the diversity in functional characters (Karhu *et al*., 1996; van Hintum & van Treuren, 2002). Consequently, it is extremely important to detect loci under selection to reliably infer population–demography history (Luikart *et al*., 2003). In this work, only 7% of the loci produced by the AFLP technique appear to be under selection. Also, many loci from diverse genomic regions are examined at the same time, giving information on the genome as a whole (Stammers *et al*., 1995). Therefore, some authors have suggested that molecular characterization of germplasm should be considered in addition to morphological criteria (Artyukova *et al*., 2000; Li *et al*., 2002, 2008).

Altogether, the results of our molecular analyses showed the existence of significant differences between *A. caven*, *A. curvifructa* and *A. farnesiana*. This argues for recognizing them as different species.

ACKNOWLEDGEMENTS

This work was supported by grants from the Universidad de Buenos Aires (EX201 and 20020100100008 to B.O.S.) and the Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 11220090100147 to J.C.V.).

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APPENDIX 1

Examined material of *Acacia*. Each specimen is cited by the last name of the first collector when there is more than one collector. Species number is indicated in parentheses. An asterisk (*) after the collection number is used for type material.

1. *Acacia caven* (Mol.) Mol. var. caven
2. *Acacia caven* (Mol.) Mol. var. *dehiscens* Ciald.
3. *Acacia caven* (Mol.) Mol. var. *microcarpa* (Speg.) Ciald.
4. *Acacia caven* (Mol.) Mol. var. *stenocarpa* (Speg.) Ciald.
5. *Acacia curvifructa* Burkart
6. *Acacia farnesiana* (L.) Willd.

Ahumada 4284 (1); Allen 5806 (6); Boelcke 178 (3), 1509 (3); Burkart 5496 (4), 14734* (4), 14984 (3), 15665 (1), 17722 (3), 21201 (1); Cabrera 3789 (1); Charpin 20311 (5); Deginani 202 (1), 270 (1); Guaglianone 2286 (3); Hatschbach 3960 (6); Hunziker 5749 (4), 9469 (6); Job 5287 (1); Jolly s/n (6); Jörgensen 960 (1), 2117 (4); Killip 42501 (6); Krapovickas 943 (3), 983 (4), 1239 (3), 1283* (3), Maradona s/n (5); Marchiori 1410 (5); Nicora 3151 (1); Oliveira s/n (6); Pedersen 4079 (5); Pometti 20 (4), 21 (4), 22 (4), 23 (4), 24 (4), 25 (4), 50 (4), 51 (4), 52 (4), 53 (4), 54 (4), 55 (4), 56 (4), 60 (3), 61 (3), 63 (3), 64 (3), 65 (3), 66 (3), 889 (1), 890 (1), 891 (1), 892 (1); Quarín 146 (3), Ragonese s.n. (3), Rojas 1346 (6), 2138* (5), 5066 (1), 6979 (3), 7056 (3), 7697 (3), 8456 (5), 9448 (6), 13746 (5); Saidman 594 (1), 595 (1), 596 (1), 597 (1), 598 (1), 599 (1), 600 (1), 601 (1), 602 (1), 776 (2), 777 (2), 778 (2), 779 (2), 780 (2) 781 (2), 796 (2), 797 (2), 799 (2), 803 (2), 805 (2), 806 (2), 807 (2), 808 (2), 809 (2), 810 (2), 811(1), 812 (1), 813 (1), 814 (1), 815 (1), 816 (1), 817 (1), 818 (1), 819 (1), 820 (1), 821 (1), 822 (1), 823 (1); Solbrig 4256 (5); Sousa 3896 (6); Tucker 552 (6), 879 (6); White 786 (6), 1887 (6); Anonymous s.n. SI 9463 (1), 14734 (4), 16024 (6), 32200 (1).