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Morphological and molecular characterization of *Bituminaria bituminosa* accessions from South-East Spain and the Canary Islands

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Summary - 14 *B. bituminosa* accessions from South-East Spain and 3 from the Canary Islands were established in 1998 at La Alberca (Murcia). Molecular and morphological characterization tests were carried out to screen accessions with largest genetic diversity. Morphological traits of stems, leaves, inflorescences, flowers and seeds, correlated well with variability shown by PCR molecular analysis. Dendrogrames based on morphological traits and PCR analysis divided accessions into 6 homogeneous groups: 3 canarian (the more diverse) and 3 peninsular.

Key-words: *Bituminaria bituminosa*, genetic diversity, morphological differences

Résumé - *14 accessions de* B. bituminosa *du Sud-Est de l'Espagne et 3 des îles Canaries ont été cultivées à la Alberca (Murcia) en 1998. La caractérisation de la diversité génétique du matérial, basée sur des caractères morphologiques et des marqueurs moléculaires (RAPD), donne des résultants similaires. L'analyse des données et l'établissement de dendogramme permettent de diviser les accessions en six groupes homogènes: 3 Canariens (les plus variables) et 3 péninsulaires.*

Mots-clés: Bituminaria bituminosa, *variabilité génétique, différences morphologiques*

Introduction

Bituminaria bituminosa (L.) Stirt. is a perennial pasture legume, widely distributed in the Mediterranean Basin and Macaronesia, but of low palatability in its natural form. It has been cultivated in the Canary Islands and Morocco, but not domesticated yet, despite its high intraspecific variability and good adaptation to different Mediterranean habitats (Muñoz and Correal, 1998).

In the Iberian Peninsula, the species has been considered of low pastoral value (Buendía, 1965) because its low palatability – its foliage contains oil glands and when leaves are crushed, a smell of "bitumen" emits from the plant -, and low forage yield – a rosette of leaves in winter and long stems with poor foliage in spring -; however, it presents a large ecological plasticity, with good adaptation to alcaline soils and cold semiarid environments, ranging in altitude from sea level to 1500 metres.

In the Canary Islands, the specie presents a larger diversity (Méndez *et al.*, 1990), with three varieties ranging from coastal semiarid habitats (var. *albomarginata* in Lanzarote, 150-300 mm) to high elevation subhumid areas (var. *crassiuscula* in Tenerife, 500 mm, 1700-2200 m altitud). The cultivar used in Tenerife, ("tedera") has good forage yield and palatability (Otal *et al.*, 1991), but low tolerance to cold (freeze injuries at –2 to –5°C), that

hampers its use in cold Mediterranean environments; however, var. *crassiuscula* accessions have shown a moderate cold tolerance (Muñoz and Correal, 1999).

A project is being carried out in Murcia to select *B. bituminosa* for cold winter semiarid Mediterranean areas; its first objective, presented here, is the characterization of the species morphological and genetic diversity in Spain.

Materials and methods

Plant material

During 1998, seed samples of 14 *B.bituminosa* accessions from Murcia (11), Almería (1) and Málaga (2) were collected from native stands, starting in May with coastal accessions and ending in August with those from high elevation areas (Table 1). Seed samples of the three Canary Islands varieties were supplied by Pilar Mendez. In the autumn of 1998, 20 seedlings of 14 peninsular accessions and 3 canarian varieties were planted in a field plot at La Alberca (Murcia).

Table 1: Altitude; soil/parent rock; temperature and precipitation/moisture regime (Rivas-Martínez, 1987) of the accessions habitat.

LOCALITY	ALT(m)	SUBSTRATE	TEMPERATURE	MOISTURE
Calnegre	35	Siliceous, ruderal	Lower Thermomedit.	Semiarid
Atamaría	70	Margin of crops	Lower Thermomedit.	Semiarid
Llano Beal	90	Nitrified road margins	Upper Thermomedit.	Semiarid
Ctra. Aguil-Mazarr.	95	Siliceous, alluvial	Upper Thermomedit.	Semiarid
Carboneras	200	Metamorphic	Lower Thermomedit.	Semiarid
Marbella (Málaga)	350	Calcareous; limestone	Upper Thermomedit.	Dry
Mijas (Málaga)	400	Calcareous; limestone	Upper Thermomedit.	Dry
Srra. Pila	800	Nitrified matorral	Warm Mesomedit.	Dry
Srra Espuña-La Perdiz	850	Road margins, calcar.	Cold Mesomediterranean	Dry
Srra Ricote	940	Stony, ruderal	Cold Mesomediterranean	Dry
Alberquilla	1000	Margin of crops	Cold Mesomediterranean	Dry
Srra Espuña-Eva13	1200	Subruderal, nitrofilous	Supramediterranean	Subhumid
María	1200	Nitrified pastures	Supramediterranean	Subhumid
La Rogativa	1300	Stony, calcareous	Supramediterranean	Subhumid
var. Albomarginata	150	Fisures, gravel slopes	Upper Infracanarian	Semiarid
cv. Tenerife	350	Fodder crop	Lower Thermocanarian	Dry
Var. Crassiuscula	2000	Rocks, gravel slopes,	Upper Mesocanarian	Subhumid

Morphological analysis

During May-June of 1999, 18 quantitative and 7 qualitative morphological traits were measured on 5 plants per accession, and on 10 fresh samples per plant.

Quantitative traits were: a) stems; number at ground level, internode length; b) basal leaves; petiole length, width and lenght of terminal leaflet, petiol/leaflet length ratio, width/length leaflet ratio; c) upper leaves; same measurements as basal leaves; d) inflorescence; width, length, pedicel lenght, number of flowers; e) flower; calix length, standard length, keel length and wing length; and f) seed; length, peak´s length.

Qualitative traits were: stem´s color–dark or green-; degree of zig-zaging in stems; leaf color–pale to intense green-; leaf apex–tipped or low necked-; leaf border pilosity–glabrous or hairy-; flower color–white to intense violet blue-; and seed peak pilosity.

Molecular analysis

Sampling and extraction: 1cm long meristematic young leaf apex were sampled, sterilised by immersion during 5 minutes in 5% (v/v) sodium hypochlorite and washed three times in sterile distilled water; the extraction was carried out following the method proposed by Torres *et al.*, (1993).

Amplification reaction: following the protocol of Williams *et al.*, (1990), using 0,5 units of *Taq*DNA polymerase Dynazyme II (*Finzymes Oy*) and a Perkin Elmer thermocycle Gene Amp PCR System 9600.

Results and discussion

A principal component analysis of the 25 morphological variables was carried out 5 components accounted for 78,9% of the variability in the original data. In a scatter plot of all accessions, 6 groups were identified. With mean values for each accession, a dendrogram was mapped using the nearest neighbor method-squared euclidean distance, which also grouped accessions into 6 groups (Fig.1a).

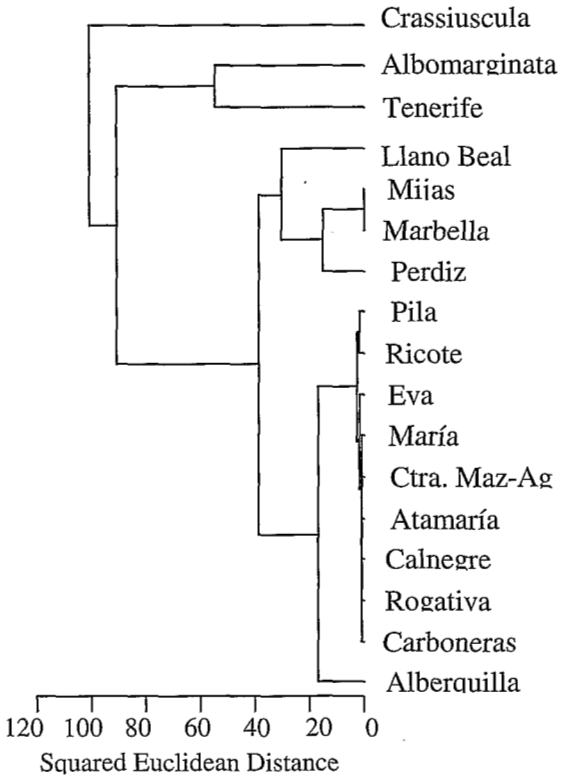

Fig. 1-a

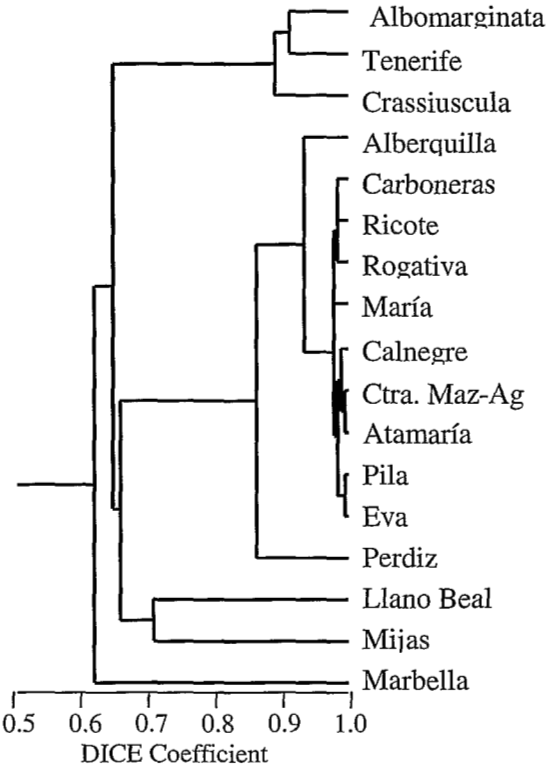

Fig. 1-b

Fig.1 Dendrogams of *Bituminaria bituminosa* accessions: 1-a) groups identified with morpholgical variables; 1-b) groups identified with RAPD molecular analysis.

Analysis of morphological traits separated *B. bituminosa* accessions into six groups, three for the Canary islands (*crassiuscula, albomarginata* and cv. Tenerife), and three for peninsular Spain (Llano Beal, Marbella/Mijas/Perdiz, and a general group with Alberquilla standing out) as ilustrated by the dendrogram of Figure 1-a.

RAPD molecular analysis was initiated by preselecting primers 10 bases in length from serie O (Operon Technologies), and finally were chosen O1, O3, O6, O7, O8, O9, O10, O11, O12, O13, O14, O15, O16, O17, O18 and O19, selected by its polymorphism and quality of bands. As a result, 135 bands were obtained and from the basic data matrix, a dendrogram was produced with the unweighted pair group method using DICE similarity coefficient.

RAPD molecular analysis also divided accessions into six groups (see dendrogram of Fig.1-b), which were very simmilar to those described before, but with little differences in a few peninsular accessions (Marbella, Llano Beal/Mijas, and a general group with Perdiz and Alberquilla standing out).

The analysis of variance of each morphological trait indicated the existence of significant diferences at the 95% confidence level in all cases. However, nine traits were selected to discriminate between canarian and peninsular accessions and to describe each group of accessions (see Table 2).

Table 2: Main morphological differences between *Bituminaria bituminosa* accessions from different origin and group.

		Stems			Leaves		Flowers	Seeds		
Origin	**Group**	**NS**	**IL**	**BS**	**PL/LLBL**	**LA**	**FC**	**SL**	**SPL**	**PP**
Canary Islands	*Crassiusc.*	25-30	35	No	low 100-130	tip	white pale blue	very long 5,7-6	long average long	hairless average
	Tenerife	1								
	Albomarg.	1								
Southeast Iberian Peninsula	*Llano Beal*	30-35	65	Yes	high 240-280	neck	i.v.blue viol.blue intense violet blue	long 4,2 average 3,4	average long 11-12 average 7-8	hairy
	Málaga	8-16	50							
	Perdiz		58							
	General Gr.		45-50	no	average 180					
	Alberquilla	25-30	65							

Legend: SN, number of stems at ground level; IL, internode length (mm); BS, black stems; LP/LLBS, petiol/leaflet length ratio of basal leaves; LA, leaf apex (tipped or low necked); FC, flower color; SL, seed length (mm); SPL, seed peak length (mm); PP, seed peak pilosity

Accessions origin (canarian v peninsular):

- stems: peninsular accessions start with a basal rosette of leaves from which numerous stems develop at ground level, whereas canarian accession present a main stem from which secundary branches develop (except var.*crassiuscula*).
- internode length: short in canarian accessions but long in peninsular ones.
- leaf apex pointed in canarians but low necked in peninsulars; petiole/leaflet length ratio, low in canarians but high in peninsulars.
- color of flowers: pale blue in canarians versus violet blue in peninsulars.
- seed size: canarian seeds larger than peninsulars.

Canarian groups:

Tenerife: develop woody stems; inflorescence with short pedicel and small number of flowers; large seeds but with short hairless peak.
Albomarginata: close to Tenerife, but seeds having long hairy peak and leaves with whitish hairy border.
Crassiuscula: grows taller; leaves large and pale green; inflorescences large, with long pedicel and big number of white flowers; seeds large and with long hairless peak.

Peninsular groups:

Llano Beal: all plant parts are larger size than other peninsulars (height, internode length, leaves, inflorescences, flowers and seeds); with black stems and flowers of intense violet blue.
Marbella/Mijas/Perdiz: with black zig-zaging stems and seeds with long peak; Perdiz grows taller and has dark green leaves with long pedicel.
General group/Alberquilla: stems are green and all plant parts have smaller size, except Alberquilla, which partly remembers Llano Beal.

Conclusions

Morphological and molecular analysis provided similar results to characterise *Bituminaria bituminosa* accessions into six homogenous groups. Easily identifiable morphological traits, like color of flowers, seeds length, leaf apex, etc, determined the accessions origin and group, that will facilitate future breeding selection of the species.

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