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The value of leaf cuticle characteristics in the identification and classification of Iberian Mediterranean members of the genus *Pinus*

SALVIA GARCÍA ÁLVAREZ*, IGNACIO GARCÍA-AMORENA, JUAN M. RUBIALES and CARLOS MORLA

Unidad Docente de Botánica, Escuela Técnica Superior de Ingenieros de Montes, Universidad Politécnica de Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

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This study reports the value of leaf cuticle characteristics in the identification and classification of Iberian Mediterranean species of the genus *Pinus* (*P. nigra* subsp. *salzmannii*, *P. pinaster*, *P. pinea* and *P. halepensis*), with the aim of using these characters to identify isolated cuticles and stomata in palynology slides. Preparations were made of the cuticles of pine needles belonging to one natural Iberian population of each of the above species. A number of epidermal morphological characteristics were then recorded with the aim of distinguishing these species from one another. The structure of the stomatal complex (the shape and arrangement of the subsidiary cells) was different in each species. The aperture of the epistomatal chamber was significantly smaller in *P. pinea* than in the other species examined, and the variables recorded for the thickening of the guard cells provided relationships that clearly distinguished all four taxa. The width and length of the stomata and the upper woody lamellae, the central distance between the external limits of the medial lamellae borders and the length of the stem were the most useful variables in this respect. The present results contribute to the ongoing discussion regarding the taxonomic classification of the members of *Pinus*, and provide valuable clues for the identification of Iberian Mediterranean pine species from small pine needle fragments or isolated stomata. After validation of the present results for multiple populations, these results could also be used to help identify fossil leaf macroremains and the scattered/isolated stomata commonly observed in palaeopalynological samples. © 2009 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2009, **161**, 436–448.

ADDITIONAL KEYWORDS: Iberian Peninsula – macrofossils – palaeobiogeography – palaeobotany – *Pinus halepensis* – *Pinus nigra* – *Pinus pinaster* – *Pinus pinea* – stomata.

INTRODUCTION

Improving our species-level knowledge of the less studied vegetative parts of plants, such as their cuticular and stomatal features, could provide information of great taxonomic and even palaeobotanical interest (Barclay *et al.*, 2007). This is true even for well-known taxa, such as members of the genus *Pinus* L.

The Iberian Peninsula is currently the natural home of six species of *Pinus* (Gaussen, Heywood & Chater, 1964). *Pinus sylvestris* L. and *P. uncinata* Ramond ex DC, both of typically Eurosiberian distribution, have been the subject of morphological studies at the level of the leaf epidermis, and, in some cases, the results have allowed the distinction of these species (Boratynska & Bobowicz, 2001; Stružková, 2002; García Álvarez *et al.*, 2009). However, little information is available for most of the *Pinus* species with Mediterranean distributions: *P. nigra* J.F.Arnold subsp. *salzmannii* (Dunal) Franco (Yoshie & Sakai, 1985), *P. pinaster* Aiton (Yoshie & Sakai, 1985) and *P. halepensis* Mill (Boddi, Bonzi & Calamassi, 2002). In addition, morphological details of the leaf cuticle of *P. pinea* L. remain unstudied.

In this article, we examine the differences and similarities of the cuticles and stomata of these four Iberian Mediterranean taxa. Morphological

*Corresponding author. E-mail: salvia.garcia@upm.es

differences in these features could allow the identification of these species when only fragments of pine needles are available, for instance, in the analysis of herbivore gut contents (e.g. Stewart, 1967). Epidermal information could also be used as a tool to identify palaeobotanical material. The resistance to degradation demonstrated by cutin allows cuticles to become fossilized (Kerp, 1990). The identification of these fragments would help reveal the role played by different forest species during the evolution of historical landscapes (Theobald, Krahulik & Rollins, 1979; Barrón & Buades, 2002). Finally, epidermal differences may also be important in systematic studies, as different authors have classified the six Iberian species of *Pinus* in different ways (Shaw, 1914, 1924; Pilger, 1926; Little & Critchfield, 1969; Price, Liston & Strauss, 1998; Liston *et al.*, 1999; Wang *et al.*, 1999; Gernandt *et al.*, 2005). Any taxonomic differences shown by the cuticles could provide new information for determining the phylogenetic relationships between them.

Stomatal analysis is commonly used in the examination of the dispersed stomata observed in pollen preparations (Hansen & Engstrom, 1996; Birks & Birks, 2000; Hicks, 2006). The assumption of the local presence of taxa based on their pollen record could be controversial in some instances, but the finding of stomata in a fossil pollen sample allows the local presence of a taxon to be confirmed (Dunwiddie, 1987; Ammann & Wick, 1993). The taxonomic identification of pine stomata has been successful at the genus level (Hansen, 1995; Sweeney, 2004), but, to date, the classification of these dispersed stomata has been possible at the species' level only in contexts in which a single known species is thought to have been present. For example, stomata belonging to *P. sylvestris* L. have thus been identified in material from recent Quaternary Scottish and Scandinavian settings (Gervais *et al.*, 2002; Froyd, 2005). The ability to identify Iberian Mediterranean pines properly via the remains of their stomata and cuticles would also be of great help in determining the influence of each species on the Quaternary evolution of Iberian *Pinus* forests. Currently, this is well understood at the genus level, but only a few works have contributed to the history of *Pinus* spp. (Franco Mújica *et al.* 2000; García-Amorena *et al.*, 2007; Rubiales *et al.*, 2007, 2009).

In this article, we report the taxonomic value of different leaf epidermal characteristics in single populations of the four Iberian Mediterranean *Pinus* species. Three of these taxa, which are native to south-western Europe, have been included in previous leaf epidermal studies (Yoshie & Sakai, 1985; Boddi *et al.*, 2002), but *P. pinea* has not been examined in this way.

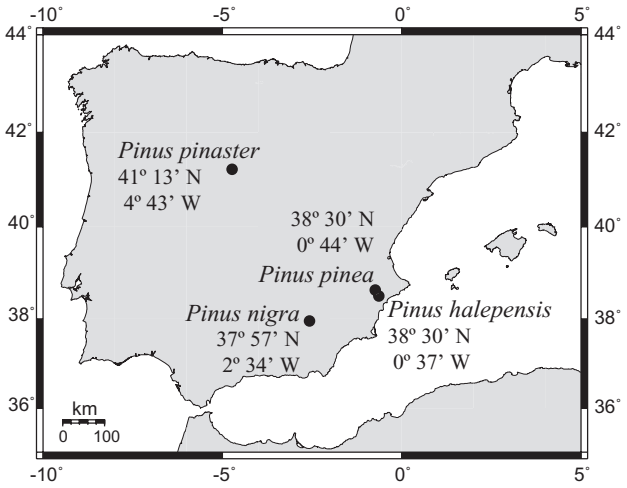

Figure 1. Map of the Iberian Peninsula showing the populations sampled.

MATERIAL AND METHODS

Pine needles were collected from four natural Iberian forests and catalogued according to the regions of origin defined for the main forest species of Spain (Martín Albertos, Díaz-Fernández & De Miguel y Del Ángel, 1998): (1) a population of *P. nigra* subsp. *salzmannii* from La Sagra (Granada), region of origin *Cordilleras Béticas* (Catalán Bachiller, 1991); (2) a population of *P. halepensis* from Maigmó (Alicante), region of origin *Levante Interior* (Gil Sánchez *et al.*, 1996); (3) a population of *P. pinea* from Biar (Alicante), region of origin *Biar* (Prada *et al.*, 1997); and (4) a population of *P. pinaster* from Ataquines (Valladolid), region of origin *Meseta Castellana* (Alía Miranda *et al.*, 1996) (Fig. 1).

Three adult trees of each population were sampled and three pine needles from each tree were analysed. For cuticle preparations, a section of approximately 5 mm in length was obtained from the middle third of each needle and placed in boiling water for 1 h to remove the epicuticular wax. These sections were then macerated in Schulze's reagent (Kerp, 1990). The remains of the mesophyll and part of the hypodermis were removed, and the samples were mounted on microscope slides and observed using transmission light microscopy. Measurements were obtained using digital photomicrographs (600× magnification) with the help of Image Pro Plus software (IPP4).

The analysis of the cuticle involved the observation and description of the epidermal cells. Special attention was paid to the stomatal complex (the pore and subsidiary cells). The maximum diameter (p) was recorded for 10 pores per needle on three needles per tree. Verification of the normality of each of the 30 counts (Kolmogorov–Smirnov test) was performed to

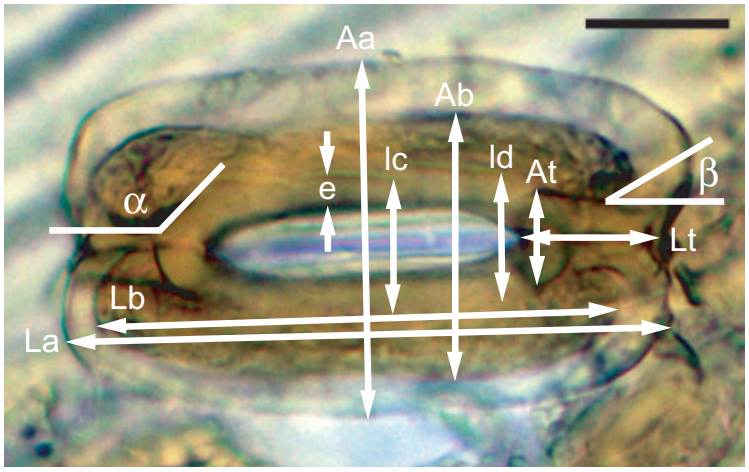

Figure 2. Stomatal variables: *Aa*, stomatal width; *La*, stomatal length; *Ab*, upper woody lamellar width; *Lb*, upper woody lamella length; *lc*, distance between the external limits of the medial lamellae borders measured at the centre; *ld*, distance between the external limits of the medial lamellae borders measured at the point at which both meet to form the stem; *e*, medial lamellae border width; *Lt*, stem length; *At*, stem width; *α*, angle of attachment of the upper woody lamella; *β*, angle between the stem and medial lamellae border; *coef_a* = *Aa*/*La*, stomatal width ratio. Terminology based on that of Florin (1931), Trautmann (1953) and Hansen (1995) (Appendix).

test for the suitability of the average estimations. Averages for each tree were subsequently calculated to perform a one-way analysis of variance (ANOVA) with the aim of determining the taxonomic value of this variable. The ANOVA assumptions were verified using the Kolmogorov–Smirnov and Levene tests for normality and homoscedasticity of residuals with 95% confidence. Tukey's Honest Significant Difference Test (HSD-Tukey) was performed to compare the means of the populations.

The analysis of the stomata was based on the characterization of the thickening of the guard cell walls in terms of 11 variables (Fig. 2, Fig. S1) (Hansen, 1995; Sweeney, 2004; García Álvarez *et al.*, 2009); the terminology used to define these variables was that of Florin (1931), Trautmann (1953), Stace (1965), Hansen (1995) and Sweeney (2004). In addition, five ratios were calculated from some of these variables. Table 1 shows all 16 stomatal variables. Ten stomata were examined on three needles per tree. Subsequent analysis of the suitability of the average estimates for each tree was tested by verifying the normality of every set of 30 counts. Average values for each tree were calculated to perform a descriptive analysis using the HSD-Tukey test, after validation of the ANOVA assumptions. In addition, the 360 total data points were subjected to stepwise discriminant analysis to describe the behaviour of the groups in relation to the variables analysed and to obtain functions capable of identifying the species to which new stomatal samples might belong (Fisher's classification functions). SPSS (16.0) software was used for statistical analysis.

RESULTS

Cuticular characteristics

The qualitative examination of the cuticles revealed that the four species possessed some common features. The epidermis fragments were composed of elongated cells, and the stomata, distributed in regular longitudinal rows, appeared to be sunken to the level of the hypodermis. The stomatal rows contained fewer elongated cells than those observed in the rest of the epidermis. The guard cells communicated with the leaf surface via the epistomatal chamber. Subsidiary cells surrounded the aperture of the epistomatal chamber, forming its boundary (Fig. 3, Fig. S2).

Each species also showed some individual features, mostly concerning the shape and position of the subsidiary cells. At the lateral edges of the pores of *P. nigra* subsp. *salzmannii*, two or three (occasionally four) small, elliptical-rounded isomorphic cells were visible. The poles of the epistomatal chamber made contact with larger, more elongated cells, similar to those of the rest of the stomatal row. The floor plan of the pores was polygonal, and of the same size and outline as that determined by the walls of the epistomatal chamber (Fig. 4, Fig. S3).

The stomatal rows of *P. pinaster* were numerous and close to one another, such that the area between the stomatal rows was much reduced. The cells of the stomatal rows were much shorter and more rounded than the rest of the epidermal cells. The lateral subsidiary cells were isomorphic and smaller; there

Table 1. Measured characters describing the variation in size and shape of stomatal cuticular thickenings

Variable		Mentioned in previous studies besides García Álvarez *et al.* (2009)
Stomatal width	*Aa*	Trautmann (1953)
Stomatal length	*La*	Trautmann (1953), Sweeney (2004)
Upper woody lamellar width	*Ab*	Trautmann (1953), Hansen (1995), Sweeney (2004)
Upper woody lamellar length	*Lb*	Trautmann (1953), Hansen (1995), Sweeney (2004)
Distance between the external limits of the medial lamellae borders measured at their centre	*lc*	Yu (1997)
Distance between the external limits of the medial lamellae borders measured at the point at which both meet to form the stem (see Appendix for the use of this term)	*ld*	
Medial lamellae border width	*e*	Sweeney (2004), Yu (1997)
Stem length	*Lt*	Hansen (1995), Yu (1997)
Stem width	*At*	Hansen (1995), Yu (1997), Sweeney (2004)
Angle of attachment of upper woody lamella	α	Hansen (1995), Sweeney (2004)
Angle between the stem and medial lamella border	β	Sweeney (2004)
Stomatal width ratio*†	$coef\_a = Aa/La$*†	
Upper woody lamellar width ratio*‡	$coef\_b = Ab/Lb$*‡	
Coefficient associated with the shape of the medial lamellae border*	$coef\_c = lc/ld$*	
Coefficient associated with the relative size of the medial lamellae border width of a guard cell with respect to the distance between the external limits of the medial lamellae border*	$coef\_e = lc/e$*	
Stem width ratio*§	$coef\_T = At/Lt$*§	

*Recalculated variables. †Coefficient of stomatal slimness according to García Álvarez *et al.* (2009). ‡Coefficient of slimness of the upper woody lamella according to García Álvarez *et al.* (2009). §Coefficient of slimness of the stem according to García Álvarez *et al.* (2009)

tended to be two (sometimes one or three) on each side. Commonly, two contiguous epistomatal chambers shared the same polar subsidiary cell. The outline of the pore coincided with the outline determined by the epistomatal chamber walls, which was similar in size and shape to the contiguous cells. This gave the stomatal rows a homogeneous appearance.

Pinus pinea showed two (occasionally one or three) isomorphic cells at the lateral sides of the epistomatal chamber. It was common for the polar subsidiary cells of the stomatal complex to be somewhat distinct from the lateral ones. The pore was small compared with the floor plan dimensions of the epistomatal chamber. The difference between the optimum focusing planes for each element showed the pore to be more elevated than the subsidiary cells (Fig. 5, Fig. S4).

Pinus halepensis showed a pattern similar to that described for *P. nigra* subsp. *salzmannii*. The main difference was the number of lateral subsidiary cells (three to four) and a slight widening of the stomatal rows in these areas, a consequence of the large number of subsidiary cells flanking the epistomatal chambers (Fig. 4, Fig. S3).

The maximum diameter of the pore (variable *p*) also showed differences between populations. Means and standard deviations of the variable *p* for each tree shown in Figure 6 could suggest smaller values for the *P. pinea* population than the others. In line with this intuitive approach, the ANOVA results for the factor 'species' rejected the hypothesis of equality between the means of the groups (99% confidence level), and the HSD-Tukey test identified the *P. pinea* population as the significantly different population. Examination of the homogeneous tree groups revealed similar values for the populations of *Pinus nigra* subsp. *salzmannii* (35.44 ± 1.06 μm), *P. halepensis* (36.73 ± 4.00 μm) and *P. pinaster* (36.30 ± 5.95 μm), whereas *P. pinea* had significantly

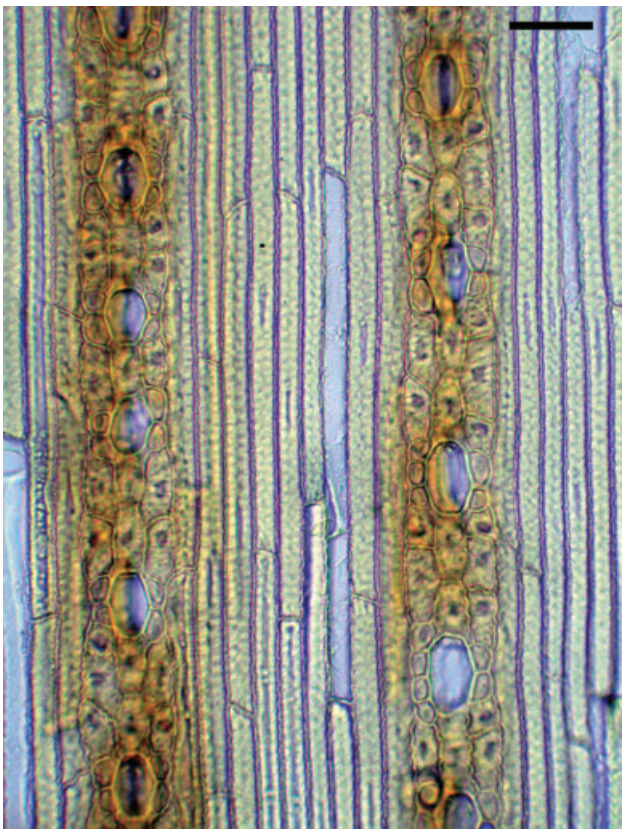

Figure 3. Stomatal rows of *Pinus nigra* subsp. *salzmannii*.

smaller pore diameters (23.37 ± 1.65 μm). The 95% confidence levels (mean ± two standard deviations) for the two groups showed no overlap between 26.64 μm and 28.81 μm. Thus, with a probability of 95%, values of p that are less than 26.64 μm can be attributed to *P. pinea*.

Stomatal features

Several stomatal features were common to all the taxa studied: the lower woody lamella covered the entire lower periclinal wall of the guard cells, the upper woody lamella was thicker and smaller than the lower, the stems joining the poles of the guard cells were the thickest elements, the medial lamellae border ran longitudinally from stem to stem, and a less thickened zone was visible in the central region of the pair of guard cells (Fig. 7, Fig. S5). Although no qualitative differences were observed between the stomata of the four species, significant differences were detected during statistical analysis of the stomatal variables.

The HSD-Tukey test (Table 2) revealed that the distance between the external limits of the medial lamellae borders measured near the stem (*ld*), the coefficient associated with the shape of the medial lamellae border (*coef_c*), the medial lamellae border width (*e*), the stem width (*At*) and the angle of attachment of the upper woody lamella (α) were unable to discriminate between population averages. The stomatal length (*La*), upper woody lamellar length

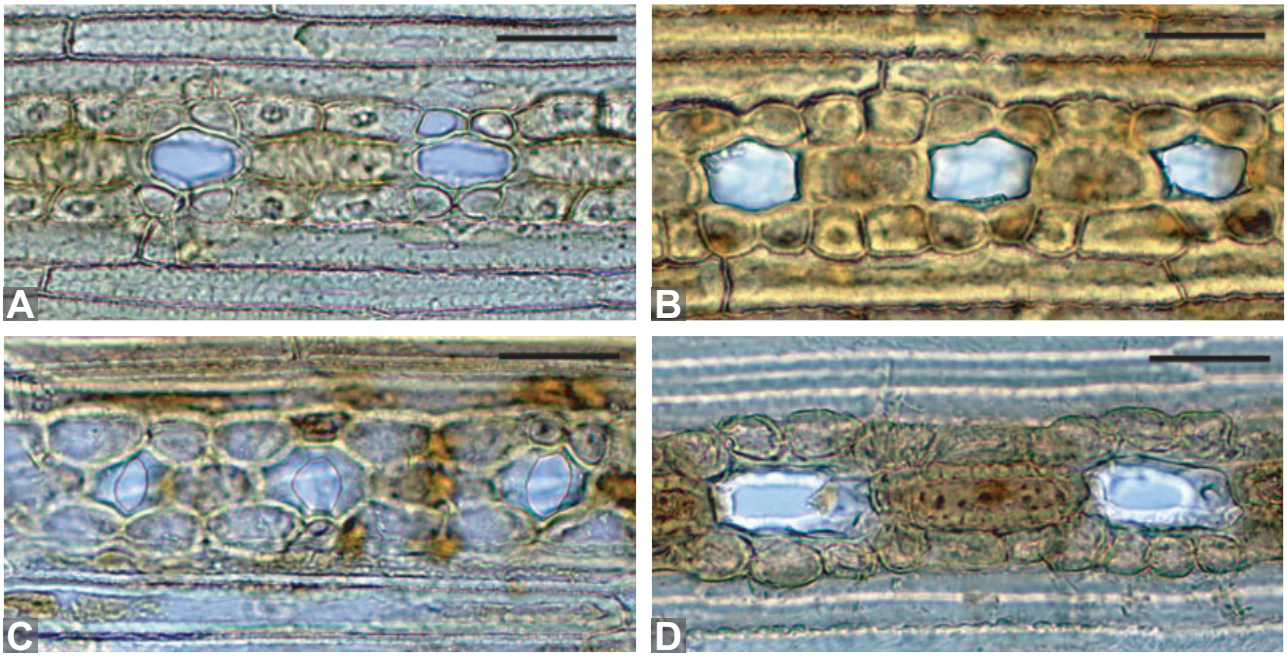

Figure 4. Stomatal complex of *Pinus nigra* subsp. *salzmannii* (A), *Pinus pinaster* (B), *Pinus pinea* (C) and *Pinus halepensis* (D). Scale bar, 50 μm.

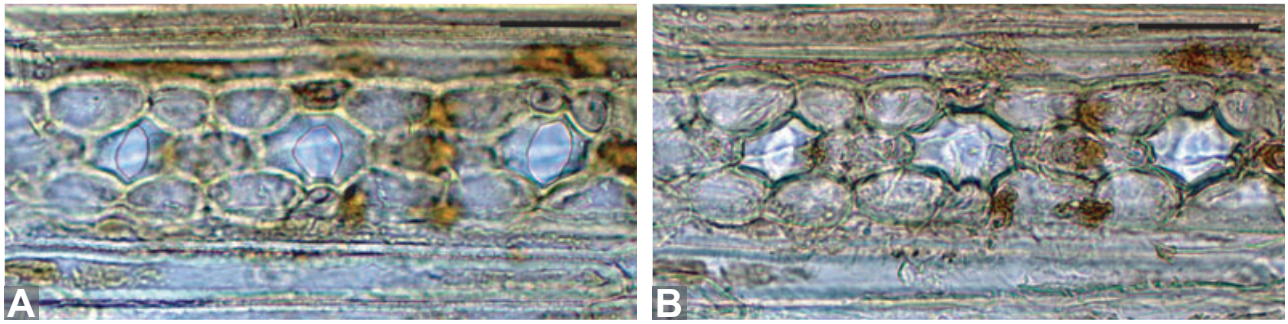

Figure 5. Stomatal complex of *Pinus pinea* showing the upper focus (A) and lower focus (B). Scale bar, 50 μm.

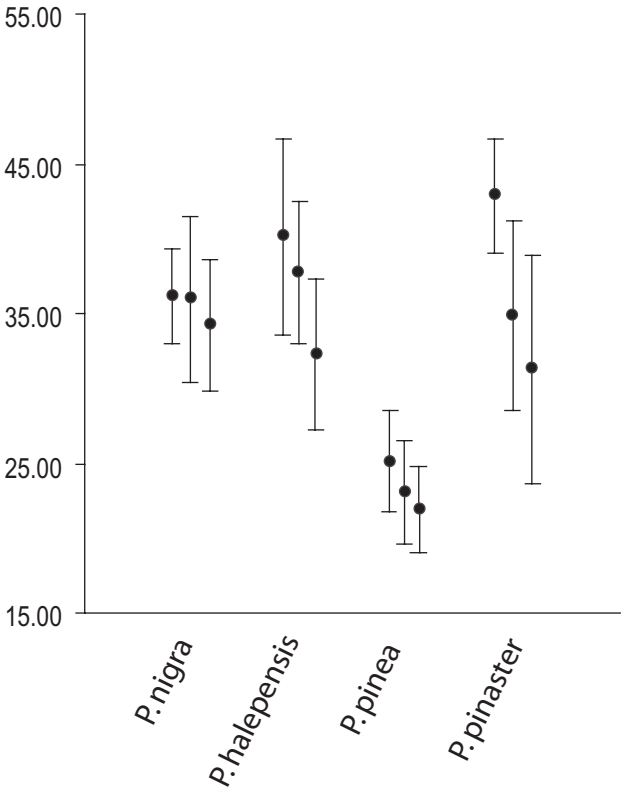

Figure 6. Maximum diameter of the pore (variable *p*) and tree averages ± standard deviations (μm) per population.

(*Lb*), stem length (*Lt*), stem width ratio (*coef_t*), upper woody lamellar width ratio (*coef_b*) and upper woody lamellar width (*Ab*) were suitable variables to discriminate between the population averages. *La* and *Ab* segregated *P. halepensis* and *P. nigra*, respectively, from the others. *Lt* and *coef_t* provided repetitive information for differentiating between two groups (*P. nigra–P. pinaster* and *P. halepensis–P. pinea*). Finally, *Lb* and *coef_b* separated the data into three groups (*P. pinaster*/*P. nigra–P. pinea*/*P. halepensis* and *P. nigra–P. halepensis*/*P. pinea*/*P. pinaster*). Accordingly, these variables had high weights in the intragroup correlations between variables and typified canonical discriminant functions (Table 3) and discriminant functions (Table 4, Fig. 8). However, *coef_t* and *Lb* were not selected for the discriminant functions, probably because of the repetitive information they provide.

The discriminant analysis provided three discriminant functions, which covered 100% of the variation (Table 5) and provided significant values for Wilk's lambda with 99% confidence (Table 6). The discriminant functions (Table 4) classified the stomata by the values of the stomatal width (*Aa*), the stomatal length (*La*), the stomatal width ratio (*coef_a*), the upper woody lamellar width (*Ab*), the upper woody lamellar width ratio (*coef_b*), the distance between the external limits of the medial lamellae borders measured at the centre (*lc*), the stem length (*Lt*), the stem width (*At*) and the angle between the stem and medial lamellae border (β).

On the plane formed by the first two discriminant functions, stomatal data formed four clouds corresponding to the four populations studied (Fig. 8). The first discriminant function separated two subgroups formed by *P. nigra* + *P. pinaster* and *P. pinea* + *P. halepensis*. The second discriminant function also discriminated two subgroups from the clouds of points, although this time they corresponded to *P. nigra* + *P. halepensis* and *P. pinea* + *P. pinaster*. The third discriminant function contributed little in the discrimination of the groups in this way and explained just 11.4% of the variation (Fig. 8, Table 5).

The discriminant analysis also provided a numerical rule to classify new stomata on the basis of this model. Introducing their measures on Fisher's classification functions (Table 7), the group of the function with the highest value will be the group to which each new stoma is assigned. In a simple validation of that rule, 85.8% of the original data were correctly classified.

DISCUSSION

The cuticular features attributed to the genus *Pinus* and the subgenus *Pinus* (Mirov, 1967; Yoshie & Sakai,

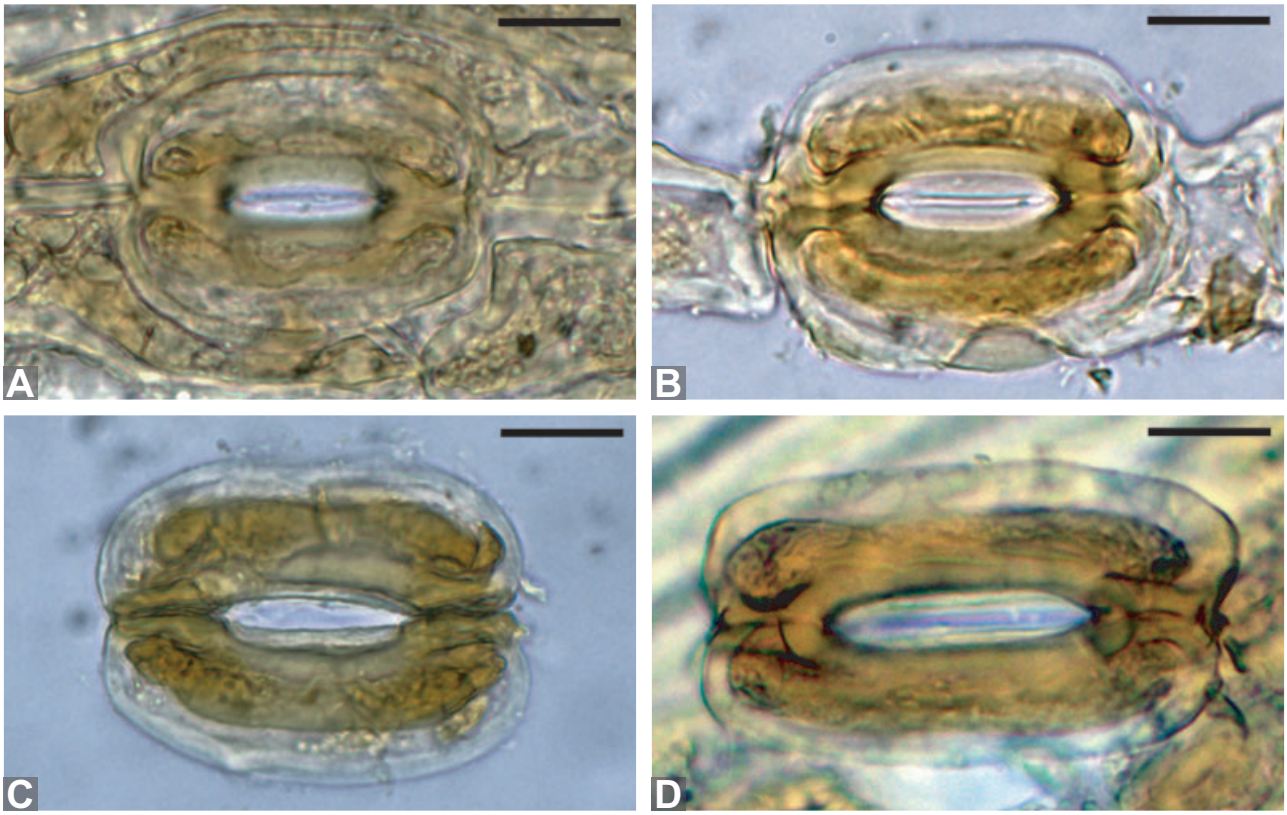

Figure 7. Stomata of *Pinus nigra* subsp. *salzmannii* (A), *Pinus pinaster* (B), *Pinus pinea* (C) and *Pinus halepensis* (D). Scale bar, 20 μm.

Table 2. HSD-Tukey test for differences between populations

	P. nigra		*P. pinaster*		*P. pinea*		*P. halepensis*	
	m ± σ	T	m ± σ	T	m ± σ	T	m ± σ	T
Aa	47.34 ± 2.82	a	51.90 ± 2.10	ab	58.02 ± 1.18	b	54.26 ± 3.20	b
La	68.34 ± 5.53	ab	64.01 ± 1.94	a	73.21 ± 1.38	b	84.11 ± 1.54	c
coef_a	0.70 ± 0.09	ab	0.81 ± 0.04	b	0.80 ± 0.01	b	0.65 ± 0.03	a
Ab	30.55 ± 1.27	a	35.57 ± 1.95	b	37.84 ± 0.45	b	35.11 ± 1.95	b
Lb	57.12 ± 4.53	b	47.21 ± 1.04	a	58.44 ± 0.63	b	67.40 ± 2.93	c
coef_b	0.54 ± 0.06	a	0.76 ± 0.02	c	0.66 ± 0.01	b	0.53 ± 0.04	a
lc	14.72 ± 1.55	a	18.00 ± 0.79	b	17.39 ± 0.52	b	15.69 ± 0.62	ab
ld	13.62 ± 0.94	a	15.28 ± 0.97	a	14.84 ± 0.86	a	14.21 ± 0.92	a
coef_c	1.09 ± 0.07	a	1.18 ± 0.05	a	1.18 ± 0.05	a	1.11 ± 0.03	a
e	3.06 ± 0.12	a	3.20 ± 0.06	a	3.21 ± 0.06	a	3.08 ± 0.16	a
coef_e	4.87 ± 0.35	a	5.78 ± 0.31	b	5.55 ± 0.14	b	5.23 ± 0.12	ab
At	11.88 ± 0.31	a	12.98 ± 1.02	a	12.64 ± 0.77	a	11.92 ± 0.61	a
Lt	16.10 ± 0.54	a	17.01 ± 0.43	a	19.79 ± 0.10	b	19.57 ± 1.13	b
coef_t	0.74 ± 0.04	b	0.77 ± 0.05	b	0.64 ± 0.04	a	0.61 ± 0.01	a
α	33.70 ± 1.50	a	39.64 ± 2.34	a	36.41 ± 3.64	a	35.31 ± 4.66	a
β	147.97 ± 0.45	b	149.32 ± 2.09	b	143.78 ± 1.99	a	146.45 ± 0.82	ab

m, mean (μm); σ, standard deviation (μm); T, letter designating homogeneous groups.

Table 3. Structure matrix. Pooled within-group correlations between discriminating variables and standardized canonical discriminant functions

	Function		
	1	2	3
La	0.685*	–0.341	0.368
Lb†	0.578*	–0.540	0.003
Lt	0.520*	0.271	–0.047
coef_t†	–0.427*	0.038	0.059
α†	0.072*	–0.023	–0.061
coef_b	–0.264	0.754*	0.150
Ab	0.268	0.621*	0.002
coef_a	–0.203	0.605*	–0.303
lc	–0.029	0.582*	0.102
Aa	0.433	0.552*	–0.193
ld†	0.059	0.415*	0.097
At	–0.064	0.282*	0.014
coef_e†	0.017	0.217*	–0.058
coef_c†	–0.098	0.205*	0.001
e†	–0.045	0.205*	0.152
β	–0.110	–0.038	0.195*

Variables ordered by absolute size of the correlation within function.
*Largest absolute correlation between each variable and any discriminant function.
†Variables not used for the analysis.

Table 4. Standardized canonical discriminant function coefficients

	Discriminant 1	Discriminant 2	Discriminant 3
Aa	–0.017	0.060	–2.478
La	0.996	–0.128	3.271
coef_a	0.763	0.113	3.103
Ab	0.610	–0.106	–0.381
coef_b	–0.552	0.734	1.014
lc	0.109	0.342	0.267
At	–0.392	0.018	–0.008
Lt	0.255	0.585	–0.232
β	–0.169	–0.127	0.356

1985; Farjon & Styles, 1997; Kim, Whang & Hill, 1999; Whang *et al.*, 2001; Whang, Kim & Hill, 2004) were visualized in the examined specimens. The classic stomatal structures described for *Pinus* (Trautmann, 1953; Esau, 1982; Hansen, 1995; Sweeney, 2004) were also observed. Nevertheless, morphological and statistical analyses revealed that significant differences existed among the four species examined.

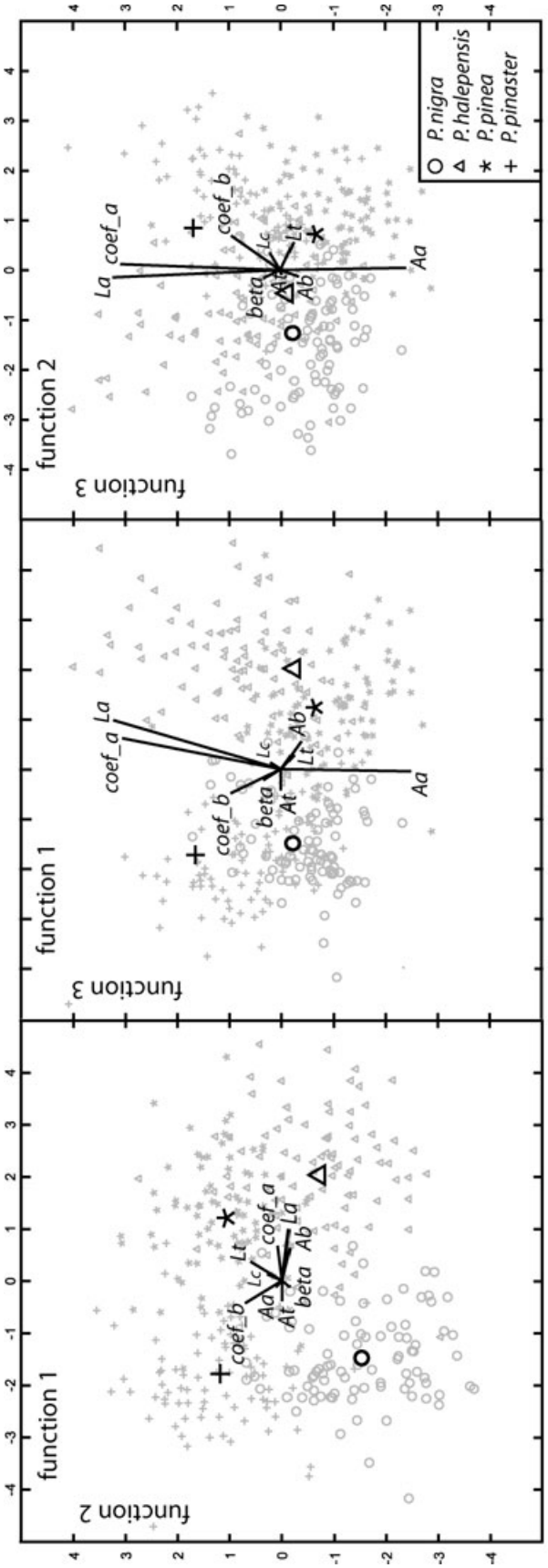

Figure 8. Plot of the analysed stomata represented by their values for the tree discriminant functions. Bold, group centroids. Lines, contribution of the variables to the discriminant functions derived from the canonical coefficients.

Table 5. Eigenvalues of the discriminant analysis

Function	Eigenvalue	% of variance	% cumulative	Canonical correlation
1	2.771†	59.6	59.6	0.857
2	1.350†	29.0	88.6	0.758
3	0.531†	11.4	100.0	0.589

†The first three canonical discriminant functions were used in the analysis.

Table 6. Wilks' lambda of the canonical discriminant functions

Test of functions	Wilks' lambda	Chi-square	d.f.	Sig.
1 to 3	0.074	919.237	27	0.000
2 to 3	0.278	451.351	16	0.000
3	0.653	150.207	7	0.000

Table 7. Fisher's classification function coefficients

	P. nigra	*P. halepensis*	*P. pinea*	*P. pinaster*
Aa	–52.955	–53.735	–52.658	–53.622
La	42.270	43.499	42.421	42.776
coef_a	3923.718	4001.781	3935.383	3966.123
Ab	1.271	1.775	1.759	0.982
coef_b	77.339	77.770	77.936	111.619
lc	0.984	1.105	1.230	1.639
At	4.140	3.137	3.407	4.255
Lt	3.015	3.635	4.329	3.725
β	3.059	3.030	2.952	3.072
(Constant)	–1888.298	–2008.477	–1943.387	–1959.405

Cuticular features

The shape and arrangement of the subsidiary cells of the stomatal complex appear to be valid features for the differentiation of the four species in the studied populations. They are therefore potentially useful when discussing the general taxonomy of *Pinus*.

The circular structure around the pore observed in many species of *Pinus*, the Florin ring (Appendix) (Florin, 1931; Farjon & Styles, 1997; Whang *et al.*, 2004), has been recorded in different populations of *P. sylvestris* L. (Yoshie & Sakai, 1985; Stružková, 2002; García Álvarez *et al.*, 2009), but was not seen in any of the taxa studied in the present work. Yoshie & Sakai (1985), who studied two of the present species by scanning electron microscopy, only reported small variations in the cuticle surface: *P. nigra* Arnold was described as having a type A Florin ring (absent or barely visible), and *P. pinaster* Ait. was described as having a type B Florin ring (slightly visible). The unremarkable nature of the Florin ring in these species could be caused by the fact that they are not supported by any cellular structure of circular shape.

In *P. pinaster*, the homogeneity observed for all elements of the stomatal row agrees with the results of anatomical studies analysing cross-sections of pine needles. The shape and size of the pore are similar to those of the cells of the stomatal row, and the perpendicular nature of the anticlinal walls of these cells and the epistomatal chamber is noticeable (Fieschi, 1932).

Pinus pinea showed the most anatomical differences among the species studied. The pore size (p) in this species was noticeably smaller than in the other taxa, allowing its numerical differentiation. Furthermore, the pore did not correspond, either in shape or in size, to the outline of the epistomatal chamber floor plan, and it was present in a different focal plane.

These features indicate a unique form of stomatal complex for this species. The substantial difference between *P. pinea* and the other three species supports the segregation of this taxon into a different group, as established by Price *et al.* (1998) – subgenus *Pinus* section *Pinus* subsection *Pineae*.

Stomatal characteristics

The statistical analyses performed using the stomatal variables highlighted differences among the four populations studied. This opens up the possibility of making taxonomic differentiations despite the apparent morphological similarity of the stomata of these taxa.

The participation of the coefficients, not just the direct measurements, in the stepwise discriminant analysis is notable. The stomatal and upper woody lamellar width ratios (*coef_a* and *coef_b*) were variables with great weight in the first discriminant function, which is associated with 59.6% of the variation. It could be argued that, as these coefficients reflect ratios of perpendicular direct measurements, they are less dependent on stomatal size and therefore less dependent on the influence of environmental conditions (Tichá, 1982; Jones, 1992; García-Amorena *et al.*, 2006).

The angle of attachment of the upper woody lamella (*angle a*) displayed similar values in all of the studied populations. This is a reflection of its stability within *Pinus*, as indicated by other authors (Florin, 1931; Trautmann, 1953; Hansen, 1995).

The classification of stomata into two subgroups, suggested by the first discriminant function, supports the older infrageneric classifications that position *P. nigra* and *P. pinaster* in the same section or subsection and leave *P. pinea* and *P. halepensis* in different groups, as suggested by Little & Critchfield (1969) and Price *et al.* (1998). However, in the light of modern phylogenetic studies, *P. pinaster* seems to be more closely related to *P. pinea* and *P. halepensis*, all in *Pinus* section *Pinus* subsection *Pinaster*, than to *P. nigra*, in *Pinus* section *Pinus* subsection *Pinus* (Gernandt *et al.*, 2005). The second discriminant function grouped the species into two different pairs: *P. nigra* + *P. halepensis* and *P. pinaster* + *P. pinea*. Although *P. pinea* has been found to be closely related to *P. pinaster* in some phylogenetic studies (Liston *et al.*, 1999; Wang *et al.*, 1999), the grouping of *P. nigra* and *P. halepensis* is not reflected in any current systematic classification. Therefore, this function is essential for the statistical separation of the four clouds of points, but has no systematic interpretation. Rather, it appears to respond to morphological differences with no phylogenetic importance.

CONCLUSIONS

The differences found in the arrangement of the stomatal complex subsidiary cells and pore size highlight the diagnostic capacity and potential taxonomic use of cuticular analysis in Iberian Mediterranean pines. The shape and arrangement of the subsidiary cells, their comparison with those of the rest of the stomatal row cells and the pore size allow the taxonomic differentiation of the studied populations. These features may therefore be useful in the development of a taxonomic key to distinguish between Iberian Mediterranean pines.

The stomatal complex of the *P. pinea* samples displays strong differences compared with the other individuals analysed, such as a narrower pore and the characteristic elevation of this opening. Further investigations of this poorly studied taxon will be useful for confirming the presence of the unique form of the *P. pinea* stomatal complex.

Despite the apparent morphological similarity of the stomata of the *Pinus* species, the present stomatal analysis detected significant differences between them. The variables related to the length and width of the stomata (*Aa*, *La*, *coef_a*) and woody lamellae (*Ab*, *Lb*, *coef_b*), the distance between the external limits of the medial lamellae borders measured at the centre (*lc*) and the stem length (*Lt*) had the greatest taxonomic weight. These findings will facilitate new studies that might establish the classification of dispersed *Pinus* stomata seen in fossil pollen preparations in an Iberian Mediterranean context. The generalization of the present results from individual populations to the species' level through the study of multiple populations is the necessary first step to achieve this goal.

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APPENDIX

GLOSSARY OF MORPHOLOGICAL TERMS BASED ON THE TERMINOLOGY OF FLORIN (1931), TRAUTMANN (1953), STACE (1965), HANSEN (1995) AND SWEENEY (2004)

Florin ring: A circular thickening formed by the cells surrounding the stomata of pine needles, first described by Florin (1931). Six different types of Florin rings have been described for the genus *Pinus*, four of which (types A, B, C and D) are seen in subgenus *Pinus* (Yoshie & Sakai, 1985; Farjon & Styles, 1997).

Lamella (woody lamella): Lignified portions of the upper and lower wall of the guard cells. The upper lamella is often thicker than the lower. The lower woody lamella is not often preserved in fossil pollen samples. In *Pinus*, the outline of the guard cells coincides with the shape of the lower woody lamella; the latter completely covers the lower wall of the cell.

Medial lamellae border: Portion of the lamellae bordering the stoma, often thickened; close to a line drawn through the stems.

Pore: The aperture of the epistomatal chamber. In many conifers, the guard cells are deeply sunken and are overarched by the subsidiary cells, such that, in a surface view, their position is marked by a ring of subsidiary cells around a nearly circular hole.

Stem: The portion of the lamellae borders beginning at their junction and extending towards the poles away from the stoma.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Stomatal variables: *Aa*, stomatal width; *La*, stomatal length; *Ab*, upper woody lamellar width; *Lb*, upper woody lamella length; *lc*, distance between the external limits of the medial lamellae borders measured at the centre; *ld*, distance between the external limits of the medial lamellae borders measured at the point at which both meet to form the stem; *e*, medial lamellae border width; *Lt*, stem length; *At*, stem width; α, angle of attachment of the upper woody lamella; β, angle between the stem and medial lamellae border. Terminology based on that of Florin (1931), Trautmann (1953) and Hansen (1995) (Appendix).
Figure S2. Stomatal rows of *Pinus nigra* subsp. *salzmannii*.
Figure S3. Stomatal complex of *Pinus nigra* subsp. *salzmannii* (A), *Pinus pinaster* (B), *Pinus pinea* (C) and *Pinus halepensis* (D). Scale bar, 50 μm.
Figure S4. Stomatal complex of *Pinus pinea* showing the upper focus (A) and lower focus (B). Scale bar, 50 μm.
Figure S5. Stomata of *Pinus nigra* subsp. *salzmannii* (A), *Pinus pinaster* (B), *Pinus pinea* (C) and *Pinus halepensis* (D). Scale bar, 20 μm.

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