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Taxonomic differences between *Pinus sylvestris* and *P. uncinata* revealed in the stomata and cuticle characters for use in the study of fossil material

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ABSTRACT

Taxonomic differences in the needle epidermis characteristics of *Pinus sylvestris* L. and *Pinus uncinata* Ramond ex DC. from two Iberian populations were sought; such information could help identify these species when pollen analysis and the inspection of wood anatomy fails. The features of the cuticle are commonly well preserved in the fossil record. Although the epidermal patterns of the examined taxa were similar, qualitative differences were seen in the subsidiary and guard cells. *P. sylvestris* showed small subsidiary cells homogenously arranged around the opening of the epistomatal chamber, while *P. uncinata* showed small, lateral subsidiary cells and non-differentiated subsidiary cells in the polar position. The aperture of the epistomatic chamber of *P. uncinata* was also larger in diameter (15.1 ± 1.8 μm *P. sylvestris*; 21.1 ± 2.8 μm *P. uncinata*). Principal components analysis and discriminant analysis was performed on the features of the guard cells characterising the size and shape of the cuticular thickenings — all the variables analysed can be measured in disperse stomata in microscope preparations for pollen analysis. Significant differences were found in the upper woody lamellae width and the coefficient associated with the shape of the medial lamellae borders (discriminant analysis weighting 0.739 and 0.826 respectively). Other significant parameters included the coefficient associated with the relative size of the medial lamellae border width of the guard cells with respect to the distance between the external limits of the medial lamellae borders, and the length of the upper woody lamella. Different light regimens appeared not to significantly affect the variability of the studied features.

1. Introduction

The cuticle preserves epidermal features of the leaf that are potentially important from a taxonomic point of view (Florin, 1939; Stace, 1965; Theobald et al., 1979; Kerp, 1990). The analysis of the shape and arrangement of the epidermal cells and other foliar structures such as trichomes and papillae has provided new evidence for phylogenetic and taxonomic discussion (Alvin et al., 1980; Barrón and Buades, 2002). Cuticle analysis has been used to revise the phylogeny of the genus *Pinus* L. and to classify it into sections, subsections and subgenera (Yoshie and Sakai, 1985; Kim et al., 1999; Whang et al., 2001, 2004).

Owing to the cuticle's strong resistance to degradation, its structures are frequently well conserved in samples from palaeobotanical sites (Florin, 1939; Stace, 1965; Boulter, 1971; Theobald et al., 1979; Kerp, 1990). This, has led to substantial interest in its study. The analysis of cuticles is of particular value in the case of *Pinus*, as pollen commonly does not allow identification at the species level in this genus (Huntley and Birks, 1983). In addition, wood anatomy often cannot be used to distinguish between *Pinus sylvestris* L. and *Pinus uncinata* Ramond ex DC. (Schweingruber, 1990). The importance of the genus *Pinus* in the forest dynamics of Iberia's vegetation history is relatively well known, but our knowledge of the actual species involved remains poor. Taxonomic precision with respect to fossil *Pinus* material, however, is of considerable palaeophytogeographic interest (Bennett and Parducci, 2006). The identification of species in the fossil record via the analysis of needle fragment cuticles and isolated stomata may help reveal forest history and the dynamics of species distribution in more detail.

Peat sediments used in palynological investigations commonly harbour the remains of cuticles belonging to trees of the genus *Pinus* (Del Río Merino, 2000; Rubiales et al., 2007). Analysis of these remains allows the identification of subsidiary cells of the stomatal complex, and reveals the structure and dimensions of the aperture of the epistomatic chamber (pore). Moreover, isolated stomata are frequently preserved in palynological preparations (Aubert et al., 2004; Franco Múgica et al., 2001). Fossil remains of both types have been found at palaeobotanical sites in the mountains of southwestern Europe (Del Río Merino, 2000; Aubert et al., 2004). In this area, *P. sylvestris* and/or *P. uncinata* were abundant throughout the Quaternary. Being able to identify these species from their stoma

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and/or cuticle characteristics would improve the interpretation of palynological and palaeobotanical datasets.

Any study on the morphological characteristics of widespread species, such as *P. sylvestris* (Gaussen et al., 1964; Farjon, 1984), will necessarily run into difficulties when it tries to be complete and rigorous. However, if the information provided by local or regional studies is integrated with that of studies from other areas of Europe that used the same methodology (e.g., those of Stružková, 2002; Sweeney, 2003), conclusions can be reached that are applicable to extensive territories. The selection of *P. sylvestris* and *P. uncinata* for the present study is the consequence of their being the only two *Pinus* species compatible with the environmental conditions prevailing in the high mountain areas of the Iberian Peninsula after the Wurmian glacial maximum (Costa et al., 1997). Palaeoremains that can be analysed are found in these areas (Rubiales et al., 2007; Turner and Hannon, 1988).

The aim of the present investigation was to examine the diagnostic value of the epidermal features of the needles of contemporary *P. sylvestris* and *P. uncinata*, such as the stomatal complex, the subsidiary cells, the epistomatic chamber and the cuticular thickening of the guard cells, and to assess the possibility of using these to distinguish the species represented in fossil materials.

2. Materials and methods

2.1. Material and pilot study

The studied material came from two natural Iberian populations (see Fig. 1 and Table 1). A pilot study was undertaken on 30 pine needles collected from one tree of each population to determine the number of needles required from each tree in the full study. In this pilot study the stability of variance was determined using the artificial sampling techniques of Efron (1982). The optimum sample size for the set of variables was set to a maximum error of 5%; significance was set at $P<0.01$ (Hansen et al., 1953). The pilot study also verified the possible influence of light conditions on the examined variables by comparing 15 needles from the sunny and 15 from the shady sides of the crowns of the two sampled trees. Analysis of variance (ANOVA) was performed for each variable taking into account the sun/shade factor.

Eighteen measurements for each variable were taken on one needle per population to determine the optimum number of measurements required; this was performed in a manner similar to that followed for calculating the number of needles required from each tree.

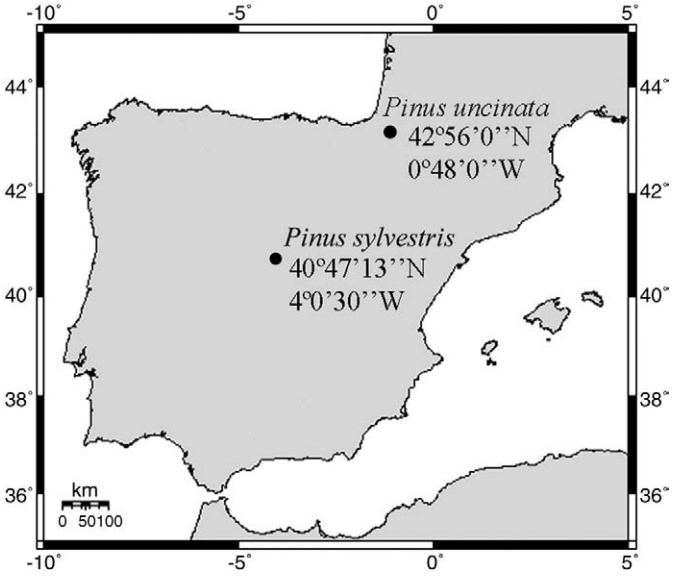

Fig. 1. Map of the Iberian Peninsula showing the populations sampled.

Table 1
Sampled populations. Region of Origin (Catalán Bachiller, 1991; Martín Albertos et al., 1998). DBH: Breast Hight Diameter of the sample individuals. C. Age: Calculated age (Madrigal Collazo et al., 1999; Serrada Hierro et al., 2008).

Species	Region of origin	Population	Geographic coordinates	DBH (cm)	C. Age (years)
Pinus sylvestris	Guadarrama Range	Navacerrada (Madrid–Segovia)	40°47′13″N 4°0′30″W	>30	>70
Pinus uncinata	Central Pyrenees	Larra (Navarra)	42°56′0″N 0°48′0″W	>30	>100

Six additional trees of the same characteristics as in the pilot study were eventually sampled in each population (Table 1).

2.2. Cuticle preparation

For the preparation of cuticles, a 5 mm-long fragment was cut from the middle third of each needle. These fragments were boiled in water for one hour to eliminate the epicuticular waxes; they were then macerated in Schultz's solution (Kerp, 1990). After manually removing the remains of the mesophyll and part of the hypodermis, the fragments' cuticles were mounted on microscopic slides for examination by transmitted light microscopy. To examine the influence of acetolysis (a common palynological technique) on the variables measured, this was performed on three needles from each population following the method of Faegri and Iversen (1989). Cuticular and stomatal analyses were performed by examining photographs at a magnification of ×600. Image Pro-Plus (IPP4) software was used to compare the variables examined.

2.3. Analysis of the cuticle

The cuticles were subjected to qualitative and quantitative analyses. The former involved the observation of the epidermal cells and the stomatal apparatus as a whole. The shape and arrangement of the subsidiary cells within each stomatal complex were examined, as was the shape of the pore. Quantitative analysis centred on measuring the diameter of this aperture (Fig. 2). The recorded values were subjected to ANOVA to determine the influence of the factor 'species'.

2.4. Analysis of stomata

This involved characterisation of the size and shape of the cuticular thickening of the guard cell walls. Based on the work of other authors (Hansen, 1995; Sweeney, 2003) and on preliminary observations of the collected samples, 11 variables were identified for measurement (the terminology employed is that of Florin (1931), Trautmann (1953) and Hansen (1995); see Appendix A for further information). Five coefficients were also calculated owing to their theoretical independence of stomatal size and environmental conditions (Tichá, 1982; Jones, 1992; García-Amorena et al., 2006). Table 2 shows all 16 stomatal variables measured.

The data obtained for each cuticle preparation in the final sample were subjected to principal components analysis (PCA) to determine the informative weight of each variable. Discriminant analysis was then performed to obtain a function capable of identifying needle fragments as either belonging to *P. sylvestris* or *P. uncinata*. All calculations were performed using SPSS v14.0.1 or STATGRAPHICS (Centurión XV) software.

3. Results

The analysis of the stability of variance performed in the pilot study showed that five needles per tree were sufficient to cover the variability of the data with a confidence level of 90%. For each pine