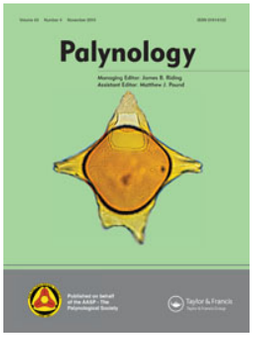

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Palynological analysis of soil in Portugal: potential for forensic science

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ABSTRACT

Forensic palynology is a discipline used in criminal cases, the importance of which has been increasing within the forensic sciences worldwide over the past three decades. Palynological analysis of surface soil samples collected from crime scenes, items and individuals has already been proven to provide important evidence linking suspects, victims and items to specific locations. A palynological study of surface soil obtained in two Portuguese districts, Coimbra and Setubal, was undertaken. The main aim was to determine the value of soil samples regarding the plant community diversity in a given area of the country, based on the evaluation of palynomorph assemblages, and to determine whether any variation could be useful in a forensic palynology context. Five surface soil samples were obtained and processed from three representative types of habitat (dunes, mixed forest and scrub) within the two districts, providing 30 samples. In total, 5434 palynomorphs were analysed and 62 taxa identified, representing nine families, 42 genera and 11 species. Results show that both districts were generally characterised by high pollen taxa frequencies, by composition diversity and by distinct palynological profiles for each district, area and collection site. In conclusion, this study shows that different locations varied in their pollen profiles, which may be of use to forensic palynologists.

KEYWORDS

Forensic palynology; palynomorphs; forensic evidence; crime scene; soil samples

1. Introduction

Palynology was defined by Hyde and Williams (1944) and originally used to denominate the study of plants spores and pollen grains (designated as palynomorphs) besides its practical applications. These microscopical structures appear in nature as a mass of yellow dust and their unique phenotypes, which can be taxonomically classified by a specialist, are only visualised and examined under the microscope, where thousands of individual pollen grains with several shapes and sizes can be found (Coyle 2005). Due to their unique and complex morphology, they are denominated as 'fingerprints of plants' (Bryant 1989). Currently, palynology is defined as the study of palynomorphs in general, including any microscopical entity dispersed away from its origin and able to be identified (Wiltshire 2016).

As a forensic methodology, palynology is used to acquire evidence in terms of a geographical location or to prove contact between objects or individuals, in contexts of criminal investigations (Bryant and Mildenhall 1998). Palynomorphs are very useful as forensic tools, being taxonomically classifiable and thus allowing their plant of origin to be identified in most cases. Other characteristics include: (1) being widely distributed in most environments and deposited in soil, vegetation, objects and surfaces (such as clothing, footwear, utensils, hair, feathers); (2) being produced and available for analyses in large numbers (about 100–100,000 per anther), which improves sampling counts and allows statistical data analysis; (3) being highly resistant to mechanical, chemical and biological degradation, which enables their accumulation and retention on surfaces in large numbers; and (4) being easily unnoticed by individuals involved in criminal acts since they have microscopical dimensions (generally less than 200 μm) (Bubert et al. 2002).

Furthermore, forensic palynology-based investigations are currently applied in court to solve legal proceedings in Australia, New Zealand and even in the United Kingdom (Mildenhall 1990; Coyle 2005; Mildenhall et al. 2006; Wiltshire 2006), although they have rarely or never been used in other countries such as Canada, the USA (Bryant and Jones 2006; Mathewes 2006) and Portugal. Nonetheless, several reported cases in the literature reveal palynology as an auxiliary tool for resolution of some legal cases (Mildenhall 1990; Coyle 2005; Mildenhall et al. 2006; Morgan et al. 2006; Wiltshire 2006). In this way, forensic palynology can be used to link a suspect to an item found at a crime scene or to correlate an item found elsewhere to a crime scene. These associations may help to determine the origin or route of an item (such as illicit drugs or objects), to calculate the deposition period of human remains (cadaveric or skeletonised), to differentiate crime scenes of human remains deposition sites, and even to

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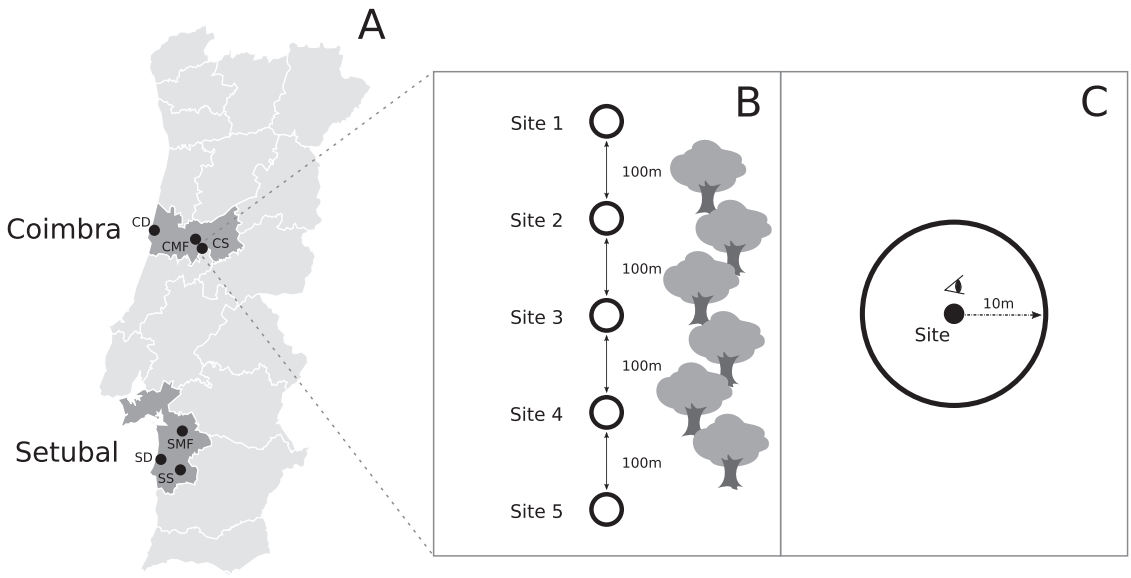

Figure 1. Selected sampling areas (A), sampling procedure (B) and surrounding vegetation in each studied site observed to a radius of 10 m (C). CD – Coimbra dunes, CMF – Coimbra mixed forest, CS – Coimbra scrub, SD – Setubal dunes, SMF – Setubal mixed forest, SS – Setubal scrub.

uncover what happened to a victim during the peri-mortem period (Wiltshire and Black 2006). Nevertheless, this technique is rarely applied in studies in Portugal (Guedes et al. 2011; Carvalho et al. 2014) and even less applied with legal purpose.

Palynology is therefore a very useful tool, and can be applied to several sub-disciplines, such as palaeoecology (Innes et al. 2013), melissopalynology (Jones and Bryant 1992), plant taxonomy (Harley and Ubera 2005), allergology (Singh and Mathur 2012), plant/animal interactions (Bińka 2003) and forensic palynology (Mathewes 2006; Morgan et al. 2006; Wiltshire and Black 2006; Wiltshire 2009).

The surface soil is an important substrate on which to focus research in crime cases (Adams-Groom et al. 2017). Control samples of a crime scene for comparative analyses are usually done in its most superficial layer, only a few millimetres deep, since it is the layer most likely to be collected on a suspect's footwear or clothing and therefore likely to offer the best match (Newsome and Adams-Groom 2017).

Surface soil analysis of samples collected from a wide range of places and objects/items (clothing, footwear, vehicles, human bodies, etc.), is therefore a potentially lucrative source of information for forensic reconstruction (Bull et al. 2006) and has been described by various authors (Horrocks et al. 1999; Bull et al. 2006; Riding et al. 2007; Adams-Groom 2017), reinforcing the idea that pollen analyses provide background information that can be used over time to help identify issues of interest and form the basis of a criminal investigation (Sandiford 2012).

The combination of palynomorph types and their percentage in an assemblage found in surface soil may be unique for a given location, a region, a larger geographical area or even a country (Milne et al. 2004). Every locality seemingly has a typical palynological profile due to the large variability in vegetation spectra and taphonomic factors (Riding et al. 2007). In a sample, the palynologist usually finds a group of palynomorph types (designated a pollen profile or pollen assemblage) and may then need to verify similarities with comparison samples (Adams-Groom 2015).

Few studies regarding the palynological analysis of surface soils for forensic purposes have been published in Portugal. These include studies by Carvalho et al. (2013), who characterised a portion of a river beach located in northern Portugal for forensic study (both criminal and environmental), and by Guedes et al. (2011), who revealed the utility of geobotanical techniques for forensic discrimination of soils from the Algarve region.

According to the Portuguese network of pollen sites that provides information on wind-pollinated taxa commonly found in the air during their flowering season, Betulaceae (*Betula*), Chenopodiaceae, Asteraceae (*Artemisia*), Cupressaceae, *Olea*, *Plantago*, *Platanus*, Poaceae and *Quercus* pollen occur most abundantly (www.rpaerobiologia.com). However, there is no reference in Portugal to the palynomorph types that can be found in surface soils, and there is no database of forensic palynological analysis.

In this context, a palynological study based on soil surface sample analyses was conducted in two regions of western Portugal. This research aimed to examine the frequency of occurrence and abundance of different pollen types in samples collected from surface soil in distinct locations to provide a directly relevant reference for forensic palynologists. All of the information collected in this study will be integrated in a database for future forensic palynology-based studies and legal purposes.

2. Materials and methods

2.1. Surface soil sampling

In this study, six sampling areas from two regions of Portugal were tested: (1) Coimbra district, with samples from Serra da Lousa (characterised as mixed forest [CMF] and scrub [CS] habitat types) and from Quiaios beach in Figueira

Table 1. Surrounding vegetation found in sampling sites (1–5) of Coimbra district.

Species	CD1	CD2	CD3	CD4	CD5	CMF1	CMF2	CMF3	CMF4	CMF5	CS1	CS2	CS3	CS4	CS5
Abies							x								
Acacia				x	x										
Ammophila arenaria		x	x	x	x										
Asteraceae	x	x	x	x	x						x	x	x		x
Baccharis trimera											x	x	x	x	
Betula							x								
Brassicaceae						x	x	x	x	x					
Calluna vulgaris									x	x	x	x	x	x	x
Calystegia soldanella	x	x	x	x	x										
Carex					x										
Carpobrotus edulis		x	x	x	x										
Castanea sativa						x	x	x	x	x					
Chamaespartium tridentatum											x	x	x		
Cistus			x		x										
Cistus psilosepalus															
Corema album			x	x											
Cupressus							x								
Cynara cardunculus															x
Digitalis purpurea						x	x			x					
Dryopteris													x	x	
Elymus farctus	x		x												
Ericaceae			x	x	x	x	x				x	x	x	x	x
Eryngium maritimum	x		x	x											
Euphorbia	x	x	x	x	x										
Fabaceae					x	x	x	x							
Halimium				x								x	x		x
Lavandula angustifolia			x												
Otanthus maritimum	x	x	x	x	x										
Pinus			x	x	x	x	x	x	x	x					
Pinus nigra						x	x								
Poaceae	x		x	x	x	x	x	x	x	x	x	x	x	x	x
Polypodium						x	x	x	x	x					
Quercus								x	x	x					
Quercus pyrenaica						x	x								
Quercus robur						x	x								
Rosaceae						x	x			x	x	x	x		x
Rubus fruticosus						x	x	x	x	x					
Ulex						x	x				x	x	x	x	x
Verbascum litigiosum			x		x										

CD – Coimbra dunes, CMF – Coimbra mixed forest, CS – Coimbra scrub.

da Foz (dunes [CD]); and (2) Setubal district, more specifically in Alcacer do Sal (mixed forest [SMF]), Santiago do Cacem (scrub [SS]), and Porto de Carretas beach in Santo Andre (dunes [SD]) (Figure 1A). These areas were chosen because they are typical habitats in Portugal and representative of potential crime scenes. Scrub and dune habitat varieties can be found in the Hill and Sweat descriptions (Hill and Sweat 2009). Mixed forest habitat identification was based on the World Wildlife Fund description (WWF 2017).

The sampling process included the following stages: (a) selection of the area by preliminary sampling and visual evaluation, aiming to choose the required habitat (mixed forest, scrub and dunes); (b) collection of approximately 1 cm^3 of surface soil (<5 cm depth) in five different sites (separated by 100 m) within six sampling areas (Figure 1B); and (c) observation of surrounding vegetation in each studied site to account for a radius of 10 m (Figure 1C, Tables 1 and 2).

Soil types were described due to their relevance to the types, amounts and conditions of the pollen, as well as to the variety of associated habitats. These included: sandy soils (dunes of Coimbra and Setubal districts) that are poor retainers of pollen; schist soils with different types of vegetation – shrub layer (scrub of Coimbra district), coniferous forest and deciduous forest (mixed forest of Coimbra district); soils of alluvial origin, resulting from podzolised sands or based on hard gravel and Miocene sandstones (mixed forest of Setubal district); and schist and granitic soils (scrub of Setubal district) where the vegetation cover is essentially dominated by scrub and bush (Costa et al. 1998).

To reduce contamination risks, all materials and containers used in each sampling procedure were sterilised prior to their use. In addition, sterile/clean implements were always used, records of the samples' travel history were registered and the laboratory work was always performed in sterile/clean conditions, using filtered air when possible and adequate. The laboratory was kept clean and the air was monitored for contaminant grains which were never found. Contamination was not an issue in this study.

2.2. Palynomorph isolation and identification

To proceed with the taxonomic identification of pollen grains collected in soil samples, palynomorph agglomerates were chemically disrupted using standard processes of hydroxide digestion, hydrochloric acid treatment and acetolysis (Adams-Groom et al. 2017). The treatments, described in detail by Erdtman (1960) and Jones (2014), allow dissolution

Table 2. Surrounding vegetation found in sampling sites (1–5) of Setubal district.

Species	SD1	SD2	SD3	SD4	SD5	SMF1	SMF2	SMF3	SMF4	SMF5	SS1	SS2	SS3	SS4	SS5
Acacia			x	x											
Asteraceae	x	x		x		x	x		x	x	x	x	x	x	x
Betula												x			x
Calluna vulgaris					x										
Chenopodiaceae						x	x	x	x	x	x	x	x	x	x
Cistus		x			x	x	x	x	x	x	x	x	x	x	x
Cistus ladanifer											x		x		
Cupressus					x										
Dryopteris													x	x	
Ericaceae													x		
Eryngium maritimum		x													
Euphorbia		x													
Fabaceae		x				x	x	x	x	x	x	x	x	x	x
Genista triacanthos						x	x					x			
Helianthemum										x					
Lagurus ovatus						x	x								
Pimpinella												x	x		x
Pinus	x	x	x	x		x	x	x	x	x				x	x
Poaceae		x	x	x		x	x	x	x	x	x	x	x		x
Polypodium															
Pteridaceae								x							
Quercus		x	x	x							x	x	x	x	x
Quercus ilex						x	x	x	x	x		x			
Quercus suber						x	x	x	x	x					
Rosaceae		x	x	x			x	x	x	x	x	x	x	x	x
Rubus fruticosus											x				
Rumex		x											x	x	x
Trifolium												x	x		x
Umbelliferae						x	x	x	x	x	x	x	x	x	x

SD – Setubal dunes, SMF – Setubal mixed forest, SS – Setubal scrub.

of unwanted components in soil samples except for palynomorphs, while acetolysis also removes the pollen grain contents, enhancing identification.

The palynomorphs were then mounted on slides, and the final preparations were examined by optical microscopy. To achieve a representative number of palynomorphs, based on the minimal amount of 100 pollen grains described in the literature (Horrocks 2004), 200 pollen grains per soil sample were counted and analysed. Nevertheless, in three samples (CD1 = 2, CD2 = 28, SD1 = 4) it was not possible to find that number of palynomorphs (Table 3). Taxonomic identification was performed based on existing literature (Reille 1992; Bruce and Dettmann 1996) or by comparison to reference samples obtained in the same collection sites from the surrounding plants.

In total, 5434 palynomorphs were analysed (2630 from Coimbra, 2804 from Setubal). Of 62 identified taxa, 55 were angiosperms, five were spores (pteridophytes) and two were gymnosperms. Eleven taxa were identified up to species level. Fifty-three palynomorphs taxa were only identified until the genus or to the family level. Forty-three palynomorphs were not identified (indicated as NI) due to their poor condition (mainly fractured or folded) or lack of distinctive morphological characteristics (Table 3).

2.3. Statistical analyses

Descriptive statistical analyses were performed with R software (R Development Core Team 2015). Bar and box plots were obtained by application of the 'ggplot2' package and the 'boxplot' and 'barplot' commands. To verify dataset distribution skewness, 1st, 2nd and 3rd quartile values obtained in all box plot analyses were compared (positively skewed distribution = [3rd quartile – 2nd quartile] > [2nd quartile – 1st quartile]; negatively skewed distribution = [3rd quartile – 2nd quartile] < [2nd quartile – 1st quartile]). In addition, the maximum range (MR = maximum whisker value – minimum whisker value) and the interquartile range (IQR = 3rd quartile – 1st quartile) values were also calculated in all box plot analyses.

3. Results

Surrounding vegetation observed at each studied site is presented in Tables 1 and 2, and Table 3 summarises the pollen count results in the 30 sampling sites, with some important data highlighted. In Coimbra district, a total of 51 taxa (CD = 17, CMF = 18, CS = 16) were found but only five were common to all locations (Asteraceae and Poaceae families; *Erica*, *Olea* and *Pinus* genera). On the other hand, 87 taxa were found in Setubal district (SD = 28, SMF = 26, SS = 33) and 10 were common to all locations (Asteraceae, Caryophyllaceae, Fabaceae, Poaceae and Rosaceae families; *Cistus*, *Erica*, *Pinus*, *Plantago* and *Quercus* genera). Regarding the habitat, CD and SD together contained 45 taxa and 13 of these were in common (Asteraceae, Caryophyllaceae, Fabaceae, Poaceae and Saxifragaceae families; *Acacia*, *Cistus*, *Erica*, *Olea*, *Pinus*, *Plantago* and *Quercus* genera; *Eryngium maritimum* species). CMF and SMF together had 44 taxa and 14 common taxa, which include seven families (Asteraceae, Brassicaceae, Chenopodiaceae, Fabaceae, Poaceae, Rosaceae and Umbelliferae families; *Cistus*, *Dryopteris*, *Erica*, *Olea*, *Pinus*

Table 3. Pollen count in sampling sites (1–5) of Coimbra and Setubal districts.

Taxon	CD1	CD2	CD3	CD4	CD5	SD1	SD2	SD3	SD4	SD5	CMF1	CMF2	CMF3	CMF4	CMF5	SMF1	SMF2	SMF3	SMF4	SMF5	CS1	CS2	CS3	CS4	CS5	SS1	SS2	SS3	SS4	SS5
Aegilops	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Acacia	0	0	4	9	6	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Allium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	27	6	13	2	3
Alnus glutinosa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Artemisia	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Asparagus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	8	2	2	3
Asteraceae	0	0	1	2	4	1	13	0	9	48	7	8	4	0	5	21	9	3	13	16	2	2	5	0	0	11	17	24	11	12
Avena sativa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	1	0	0
Betula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	2	2	0	0	0	0	0	1	2	1	0	2
Brassicaceae	0	0	0	0	0	0	0	0	0	0	11	21	7	6	11	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Calluna vulgaris	0	0	0	0	0	0	0	0	0	0	2	2	2	4	7	0	0	0	0	0	18	18	15	32	25	0	0	0	0	0
Calystegia	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Carex	0	0	0	0	34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Caryophyllaceae	0	0	1	1	1	0	5	2	0	0	0	0	0	0	0	0	0	0	1	6	0	0	0	0	0	3	8	9	7	7
Castanea sativa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chamaespartium tridentatum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	6	3	2	1	0	0	0	0	0
Chenopodiaceae	0	0	0	0	0	0	3	0	0	0	1	5	0	2	2	31	22	6	12	8	0	0	0	0	0	5	10	20	4	8
Cistus	0	0	5	1	1	0	8	0	2	0	0	4	1	0	0	3	11	10	9	11	0	0	0	0	0	7	7	22	14	7
Cistus psilosepalus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	1	3	1	0	0	0	0	0
Corylus avellana	0	0	0	1	0	0	0	0	0	0	3	0	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	2	1
Crytogramma	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Cytisus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	4	0	0	6
Dactylis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	1	0	0	0
Daphne	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	6	2	1	0
Dryopteris	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	3	5	0	0	0	7	3	0	0	0	2	4	0
Erica	0	1	20	17	4	0	0	3	14	31	8	8	2	8	1	2	0	0	1	0	73	45	24	70	67	0	0	4	2	3
Eucalyptus	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	2	0
Eryngium maritimum	0	0	0	0	3	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Euphorbia	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fabaceae	0	0	0	0	20	0	37	0	5	9	23	19	6	4	4	22	21	33	21	8	0	0	0	0	0	15	21	17	29	39
Halimium/ Helianthemum	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0
Halimium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	10	0	14	0	0	0	0	0
Helicrysum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
Hypericum	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Juniperus	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lagurus ovatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	5	6	2	0	0	0	0	0	0	0	0	0	0
Lavandula angustifolia	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lycopodium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	4	4	1	0	0	3	3	0	1	2	0	2
Malcolmia	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nymphaea	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Olea	1	1	4	2	3	0	0	0	8	0	2	2	0	0	0	1	2	0	0	0	0	0	1	0	0	0	0	0	0	0
Pancratium	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pimpinella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	3	0	3
Pinus	1	19	151	136	98	3	36	150	70	25	76	28	149	117	71	7	24	37	17	13	61	28	13	52	43	1	0	6	14	11
Plantago	0	0	0	0	2	0	0	0	1	1	0	0	0	0	0	1	0	2	5	0	0	0	0	0	0	2	0	0	2	1
Poaceae	0	1	7	21	17	0	15	5	13	19	32	40	24	21	65	47	48	32	43	54	21	42	70	20	34	53	37	18	4	17
Polypodium	0	0	0	0	0	0	0	0	0	0	0	8	0	5	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Pteris	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Quercus	0	0	3	6	2	0	19	11	3	0	20	37	2	25	14	51	46	43	44	34	0	0	0	0	0	12	20	19	58	32

(*continued*)

Table 3. Continued.

Taxon	CD1	CD2	CD3	CD4	CD5	SD1	SD2	SD3	SD4	SD5	CMF1	CMF2	CMF3	CMF4	CMF5	SMF1	SMF2	SMF3	SMF4	SMF5	CS1	CS2	CS3	CS4	CS5	SS1	SS2	SS3	SS4	SS5
Rosaceae	0	0	0	0	0	0	17	3	25	32	14	18	3	5	14	1	11	14	10	28	9	20	24	4	6	23	16	12	6	11
Rosmarinus officinalis	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rubus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	8	0	0	0	1	0	0	0	0
Rumex	0	0	0	0	0	0	17	2	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	17	8
Salix	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Saxifragaceae	0	0	3	1	3	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	1
Silene	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1
Trifolium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	11	1	4
Thymus	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ulex	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	19	0	11	6	0	0	0	0	0
Umbelliferae	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	2	3	3	6	5	0	0	0	0	0	15	13	7	10	15
Urtica	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Verbascum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	2	0	0	0
NI	0	6	0	2	2	0	4	8	9	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	4	2	0	0	3
Total for sample	2	28	200	200	200	4	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200

CD – Coimbra dunes, CMF – Coimbra mixed forest, CS – Coimbra scrub, SD – Setubal dunes, SMF – Setubal mixed forest, SS – Setubal scrub.

and *Quercus* genera; *Corylus avellana* species). CS and SS jointly had 49 taxa and share eight common taxa (Asteraceae, Poaceae and Rosaceae families; *Dryopteris*, *Erica*, *Lycopodium*, *Pinus* and *Rubus* genera).

3.1. Absolute frequencies on the common taxa presented in all six sampling areas

Acquired box plots based on the common taxa absolute frequencies presented by all six sampling areas are presented in Figure 2A.

In total, all six sampling areas included in this study (CD, CMF, CS, SD, SMF and SS) have four common taxa (Table 3), which include two families (Asteraceae and Poaceae) and two genera (*Erica* and *Pinus*). *Pinus* genus and the Poaceae family are the more frequent palynomorph taxa in all six sampling areas.

3.2. Palynomorph diversity – box plot analyses

Regarding the dunes sampling areas, 17 taxa were identified from a total of 630 observations in the CD sampling area, and 28 taxa were identified from 804 observations in the SD sampling area. For the remaining sampling areas, 18 taxa were identified in the CMF area, 16 taxa in the CS area, 26 taxa in the SMF and 33 taxa in the SS area; all counted from a total of 1000 observations per sampling area. Overall, 51 taxa were identified for the Coimbra District from a total of 2630 observations, and 87 taxa were found in the Setubal District from a total of 2804 observations (Table 3).

Box plots based on these taxa diversity frequencies found in the two sampling districts are presented in Figure 2B.

The Coimbra sampling district dataset is shown as positively skewed and the Setubal dataset as negatively skewed. Although the Setubal dataset showed a higher diversity (Setubal median = 0.033, Coimbra median = 0.018), the Coimbra dataset presents higher MR and IQR values (Coimbra MR = 0.011, Setubal MR = 0.009; Coimbra IQR = 0.005, Setubal IQR = 0.004), although the differences in these values are minimal. Nevertheless, these results show diverse profiles that are clearly from two different sampled locations (Coimbra and Setubal).

3.3 Different palynomorphs – bar plot representation

Bar plots presenting the different palynomorphs obtained by habitat pair regarding each sampling area are shown in Figure 3. The areas at Setubal show a much greater diversity compared to Coimbra for all three habitats (dunes, mixed forest and scrub) and very little similarity in the types and amounts of each taxon (Table 3).

4. Discussion

This study shows different palynomorph diversity between two Portuguese districts (Coimbra and Setubal), as well as associations between palynomorphs at each sampling site, which are typical of the vegetation communities present in

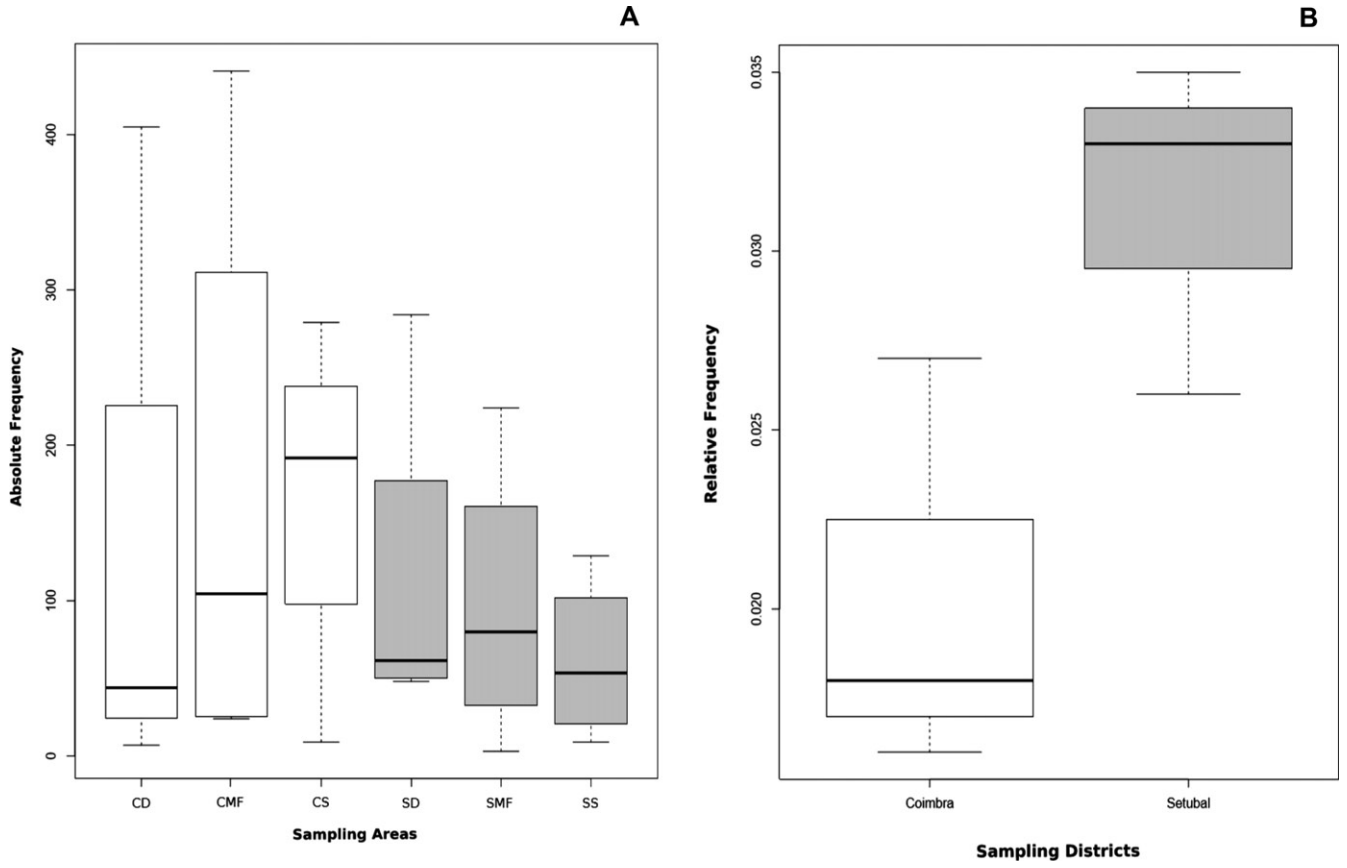

Figure 2. Box plots showing the absolute frequencies of all common taxa obtained in the six sampling areas (A) and the relative frequencies of all taxa diversity in the Coimbra and Setubal sampling districts (B). White box plots refer to the Coimbra District sampling areas; grey box plots refer to the Setubal District sampling areas. CD – Coimbra dunes, CMF – Coimbra mixed forest, CS – Coimbra scrub, SD – Setubal dunes, SMF – Setubal mixed forest, SS – Setubal scrub.

each habitat. As a result, the forensic potential of surface soil palynological profiles in these habitats has been revealed.

Concerning the palynomorph diversity for each sampling area, the identified taxa found in soil surface samples were directly correlated with the habitat vegetation at the location or nearby, as expected (Bruce and Dettmann 1996; Coyle 2005; Wiltshire 2016). It was also verified that each district is unique in terms of the representative plant taxa, as already supported in previous studies (Wiltshire 2009; Wiltshire 2016), showing different plant associations, through the pollen associations, even within the same habitat (dunes, mixed forest or scrub).

Therefore, it is strongly suggested that a given palynomorph diversity can be used to trace a vegetation community profile in a given study region, which consequently supports the use of tracing items containing such a profile to a certain geographical origin (Bruce and Dettmann 1996). Evidence in this study and previous research (Wiltshire 2009) shows marked differences of pollen taxa frequency and composition observed between different soil samples collected only 100–400 m away from each other. It is likely, therefore, that each sample from a crime scene and each association obtained from a locality will be unique and should therefore be evaluated individually (Wiltshire 2006).

Although the main objective was to trace a general palynological profile of each collection area, acquiring and analysing 200 palynomorphs by site, this amount was impossible to collect in some areas. Specifically, limited pollen was found in the dunes sampling sites closer to the seawater (as in CD1 and SD1) because these sites are constantly subject to water erosion and transport, and sandy soils are poor pollen retainers (Horrocks et al. 1999). In future studies, 300 pollen grains would be counted, which is the minimum number considered to be viable for reliable results in palynological analysis of soil samples (Adams-Groom 2017).

Some unexpected observations were recorded in the dataset, such as the presence of *Olea* genus at both dunes habitats (CD and SD), which is rarely seen in the area but which can be justified because this type of pollen is an amphiphilous species (i.e. both insect and wind pollinated). Also, due to the selection through time of varieties with high flower and pollen production, the anemophilous character (wind-borne) is more pronounced (Sofiev and Bergmann 2013). The presence of one pollen grain of *C. avellana* (collected in CD4), another unexpected result, may be justified because it was probably planted near the collection site, since the flora of this region does not supposedly contain this plant species. *Corylus avellana* grows naturally in Europe, many are planted as ornamentals or in nut production fields (Sofiev and Bergmann 2013). Also, *Pteris* genus (Pteridaceae family) identified in SMF3, a fern which was not registered in any other sampling area, and *Rumex* genus (Polygonaceae family) present in SS, which is generally found in the north of the Iberian Peninsula (Santos et al. 2017), were rare events.

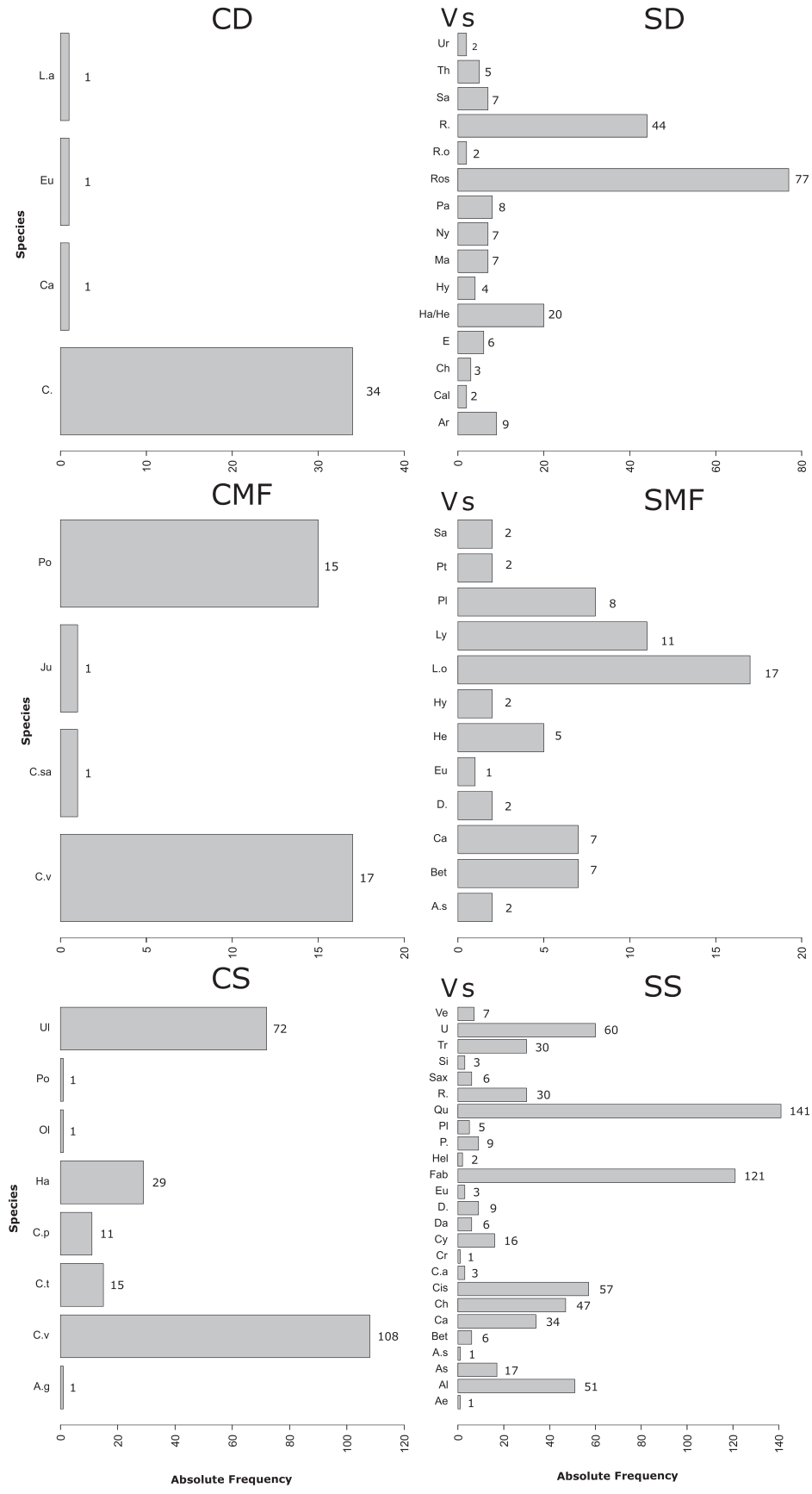

Figure 3. Bar plots showing the relative frequency of difference between palynomorphs by sampling area. CD – Coimbra Dunes, SD – Setubal Dunes, CMF – Coimbra Mixed Forest, SMF – Setubal Mixed Forest, CS – Coimbra Scrub, SS – Setubal Scrub; Ae – *Aegilops*, Al – *Allium*, A.g – *Alnus glutinosa*, Ar – *Artemisia*, As – *Asparagus*, A.s – *Avena sativa*, Bet – *Betula*, C.v – *Calluna vulgaris*, Cal – *Calystegia*, C. – *Carex*, C.sa – *Castanea sativa*, C.t – *Chamaespartium tridentatum*, Ch – *Chenopodiaceae*, C.p – *Cistus psilosepalus*, Cr – *Crytogramma*, Cy – *Cytisus*, Da – *Dactylis*, D. – *Daphne*, E – *Euphorbia*, Fab – *Fabaceae*, Ha/He – *Halimium/Helianthemum*, Hel – *Helicrysum*, Hy – *Hypericum*, Ju – *Juniperus*, L.o – *Lagurus ovatus*, L.a – *Lavandula angustifolia*, Ma – *Malcolmia*, Ny – *Nymphaea*, Ol – *Olea*, Pa – *Pancratium*, P. – *Pimpinella*, Pl – *Plantago*, Po – *Polypodium*, Pt – *Pteris*, Qu – *Quercus*, R.o – *Rosmarinus officinalis*, R. – *Rumex*, Sa – *Salix*, Sax – *Saxifragaceae*, Si – *Silene*, Tr – *Trifolium*, Th – *Thymus*, U – *Umbelliferae*, Ul – *Ulex*, Ur – *Urtica*, Ve – *Verbascum*.

Pinus genus has been included in analyses as a single taxon, despite both *Pinus pinea* and *Pinus pinaster* species being present in CD5 (Santos et al. 2017). However, due to the morphological similarities, it was not possible to identify samples up to the species level. Consequently, this taxon appeared in high frequency in several analyses. However, *Pinus* genus, as well as *Betula* genus and *C. avellana* species, are all wind-pollinated plants which produce large amounts of pollen dispersed for long distances (Wiltshire 2016). This may also be a valid reason for these pollens being commonly acquired in high quantities, although the dispersion capacity can be limited by physical barriers (Wiltshire 2016).

The same issue occurred with *Quercus* taxon, which is known to present seven species in the CMF region (*Quercus pyrenaica*, *Quercus ruber*, *Quercus rotundifolia*, *Quercus faginea*, *Quercus suber*, *Quercus coccifera* and *Quercus ilex*) and two in SMF region (*Q. suber* and *Q. ilex*) (Santos et al. 2017).

Moreover, in this study, the most common palynomorphs found by site and area corresponded to taxa already reported as being distributed in the location. Therefore, to trace a palynological profile it is also essential to undertake a vegetation survey of pollen-producing plants normally represented in the location, when there are plants characteristic of specific habitats. This was, for example, the case for *E. maritimum*, a species commonly found on the Portuguese coast; *Euphorbia* genus, a rhizicola plant taxon frequently found on wet lawns and widely distributed in Portugal; and *Nymphaea* genus, a taxon that was found here due to the proximity of a lagoon (Santo Andre lagoon) at SD5. These three taxa were all found in the dunes habitats.

Regarding statistical analyses, the results show varying palynological profiles occurring even within the same habitats types (CD and SD, CMF and SMF, CS and SS), in terms of diversity and taxa frequency rates, including presence and absence of certain taxa per location. These conclusions were also evidenced in previous works (Bruce and Dettmann 1996) performed in different geographical locations.

Overall, soils may have significant probative value in forensic science, and, additionally, several techniques have been developed to analyse these type of samples, based on physical and chemical properties (Bruce and Dettmann 1996). However, some techniques offer several limitations, such as the prerequisite of a large amount of material available for the analyses. The major advantage of forensic palynology is that in general a small amount of soil (sometimes a teaspoon) is sufficient to perform an accurate analysis (Bruce and Dettmann 1996; Milne et al. 2004; Mildenhall et al. 2006; Wiltshire 2015), but the ideal would be to collect 4 cm^3 of surface soil (Adams-Groom 2017), or even more for sandy soils, in case of having to repeat analyses. Nevertheless, due to taphonomic processes which each palynomorph undergoes in different areas and temporal periods (Wiltshire 2016), there is a need to profile each crime scene as a unique area presenting that palynological assemblage.

Finally, it is important to highlight that even the identification of an individual palynomorph in a forensic sample, especially if present in at least 5%–10% of the sample, might be useful to provide significant information in a forensic case regarding the probable sample original location and/or its similarity to other crime scene and/or samples obtained from a suspect (Faegri and Iversen 1989; Bryant 2013).

5. Conclusion

This study shows the variation in the palynomorph content of different vegetation communities acquired in the Coimbra and Setubal Portuguese districts. The forensic potential of palynological analysis in crime investigations based on soil surface studies was demonstrated, since a precise and unique palynological profile was traceable not only between districts but also between each sampling area (Coimbra and Setubal dunes, mixed forest and scrub habitats) and site.

These results open the way for novel studies, which encompass a search for the preparation and updating of a pollinic map of various regions of Portugal, aiming at the cataloguing of the country, making it easy and efficient to compare samples in a crime scene.

The organisation of this pollinic map does not intend to establish palynological evidence, which is not possible at all, since there are factors that lead to great variability. However, it is intended to be developed to differentiate and mark species of plants and associations of plants characteristic of certain regions or sites (endemic plants, for example), thereby reducing the area of demand within a criminal investigation.

It is important to emphasise that forensic palynology has high potential in the context of criminal investigations, since in many countries, as is the case in Portugal, it is not currently accepted as a forensic support tool and evidence obtained by this analysis method may not yet been recognised in court. Nevertheless, with the increasing number of recent publications related to this forensic area, the validation of palynology as a widespread forensic tool may be about to happen.

As a final remark, it is not only plant analyses that are of interest within forensic science in a habitat context, but also insect analyses (forensic entomology), soil mineral composition evaluation (forensic mineralogy) and many other particles retrieved from crime scenes can be essential to provide evidence proving (or not) contact between a suspect and a scene, to help delimit target search areas and even to establish a time and place of death, and thus none of these tools should be excluded from a forensic analysis.

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Disclosure statement

The authors declare that they have no conflict of interest.

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