Science & Justice 64 (2024) 73–80

Contents lists available at ScienceDirect

Science & Justice

journal homepage: www.elsevier.com/locate/scijus

Research Paper

Use of pollen assemblages as forensic evidence in non-seasonal high-altitude soils

Vanesa Serrudo Gonzáles [a,b], Teresa Ortuño Limarino [c], Pablo Duchen [d,*]

[a] Carrera de Biología, Facultad de Ciencias Puras y Naturales, Universidad Mayor de San Andrés, Cota Cota 27 St, Campus Universitario, Bolivia
[b] Instituto de Investigaciones Técnico Científicas de la Universidad Policial Mcal. Antonio José de Sucre, 7404 Hugo Ernest Ave, La Paz, Bolivia
[c] Carrera de Ingeniería Agronómica, Unidad Académica Campesina Carmen Pampa, Universidad Católica Boliviana San Pablo, La Paz, Bolivia
[d] Faculty of Biology, iomE, Johannes Gutenberg University of Mainz, Hans-Dieter-Hüsch-Weg 15, Mainz, Germany

ARTICLE INFO

Keywords:
Pollen rain
Andean montane ecoregion
Footwear
Indicator species

ABSTRACT

Forensic palynology is a tool in criminalistics that uses spores and pollen grains to link a certain geographical location with a crime scene. The comparison of the pollen assemblage of a crime-scene soil and that of footwear of suspects and victims proved to be very useful as judicial evidence in multiple environments with marked seasonality. However, its usefulness in non-seasonal high-altitude soils has not been experimentally evaluated to the same extent. For this reason, the present study addressed this information gap by undertaking a palynological study in areas with high crime rates in the city of La Paz, Bolivia. To do this, we carried out multiple experimental samplings in three locations with different types of soil and different degrees of urbanization. Specifically, we compared whether the vegetation present at the time of taking the reference samples, was reflected in the pollen rain. Results showed that the vast majority of the species present in the vegetation were found in the pollen rain, with the exception of some plant species with entomophilous pollination syndrome. We also show that the transfer between assemblages from pollen rain to footwear happened effectively, which helped identify their geographical origin, and unveiled a great number of useful indicator species.

1. Introduction

Palynology is the study of pollen, spores, and dinoflagellate cysts, together known as "palynomorphs" [1]. Such palynomorphs are often covered by an exine composed of sporopollenin, leaving a visible mark and allowing them to last for thousands of years [1]. Because of this, palynology has become a useful forensic tool since the late 1950s [2]. In forensic palynology, the small size of pollen (average size range between 10 and 100 μm) is essential, making it invisible to the naked eye, and preventing its concealment by perpetrators. In addition to its long-lasting capabilities, pollen has high production and adheres well to objects, characteristics that adequately coincide with the principles of criminalistics, namely, reconstruction of phenomena or events, probability, exchange, and correspondence of characteristics [3–7]. Therefore, the study of spore and pollen assemblages can provide concrete information about a crime, and later be used as additional evidence or material objects in court [6].

It has been shown that pollen on the soil surface largely reflects the current vegetation of a specific plant community, particularly in wind-dispersed plants [8–10], with only some biases caused by taphonomic factors such as differential pollen production [11,12], mechanisms and means of pollen dispersal [11], and rates of pollen degradation depending on the soil composition [13]. For pollen to be used as evidence in a forensic case we need to evaluate the similarity of pollen diagrams between pollen samples obtained at a crime scene (reference samples) and pollen samples from other objects (such as footwear), in order to demonstrate their resemblance in species composition and relative abundance [6,14–17]. Moreover, the pollen assemblage found at a crime site must agree to a high degree with that of objects and/or people involved [17,18].

There are several examples of the use of pollen assemblages to solve forensic cases [18–21]. In all such cases, the soil was the final substrate of the pollen rain, which represented the plant community around it [17], so it is expected that pollen assemblages are transferred from the soil surface to objects such as footwear. Given that shoes are commonly used as forensic evidence to verify the footprints at a crime scene, it has become common practice to collect palynomorphs together with sediments [22]. For these cases, a recovery of approximately 90 % of the

* Corresponding author at: Faculty of Biology, iomE, Johannes Gutenberg University of Mainz, Hans-Dieter-Hüsch-Weg 15, Mainz, Germany.
E-mail address: pduchnbo@uni-mainz.de (P. Duchen).

https://doi.org/10.1016/j.scijus.2023.11.008
Received 15 August 2023; Received in revised form 25 October 2023; Accepted 26 November 2023
Available online 1 December 2023

palynomorphs has been observed [5,9,19,23], verifying a dilution phenomenon. In other words, the pollen found on clothing and footwear can reflect: 1) the predominance of the pollen from the last site visited by a suspect/victim, 2) a discrimination between localities distant from each other by an average of 300 m [22], 3) plantations separated by 50 m [22], and 4) localities of different plant composition, separated by less than a kilometer [9].

Given the usefulness of pollen assemblages in forensic cases the existence of pre-existing databases of local palynomorphs is extremely helpful for a speedy identification of samples. Although pollen analyses can be delayed when there are no specialists on the subject, the use of pollen databases has proven useful in the early investigative stages [21]. There, the statements of suspects were evaluated, regarding the sites where events occurred, often leading to early confessions, and avoiding lengthy legal proceedings. For this reason, palynological analyses are becoming more frequently accepted by judicial courts in several European [3,21,24] and Latin American countries. In Latin America, this is already the case in Argentina and Brazil [16,17,23,25,26], where they have successfully incorporated forensic palynology in the criminal investigation area.

Overall, resolution of criminal cases using pollen assemblages obtained from footwear were carried out in deciduous forests, pine plantations, oak groves [22], poplar groves, *Acacia-Eucalyptus* forests, dry and humid sclerophyll forests [9] and grasslands [27]. Furthermore, experimental setups have been carried out in England and Spain [9,10,14] in temperate latitude environments, where seasonal characteristics are quite distinguishable. However, to our knowledge, there are no reports of experimental designs nor published investigations in forensic palynology that emulate climatic conditions of tropical, non-seasonal high-altitude environments. Here, we addressed this issue by performing a full experimental forensic palynology study across different sites in a high-altitude region in Bolivia. According to Police data from the city of La Paz, there have been a great number of homicides (49 in the past 12 months) in the southern part of the city [28,29]. For this reason, we carried out an experimental analysis of pollen assemblages in three sites located along this area, including an urban site with introduced flora, an urban site with native flora, and a rural site with native flora. Based on this experimental information, our aim was to evaluate the taxonomic similarity and relative abundance of the pollen assemblage in footwear to determine its geographical origin within the city, and to demonstrate the applicability of forensic palynology in a non-seasonal high-altitude environment.

2. Material and methods

2.1. Study area

The study sites were in the southern area of the city of La Paz, Bolivia (Fig. 1), a city where cases of violence have increased in recent years: 2545 cases so far this year (post-pandemic), compared to a total of 2085 cases in 2018 (pre-pandemic) [28–30]. The data collected here constitutes the beginning of a database of reference species that provide information about the indicators of pollen types in each study site. To select study sites two criteria were chosen: 1) areas with a history of homicides, and 2) areas open to the population.

The first site is called "Cañadón de Wilacota" (CW), located at 3574 m above sea level (m.a.s.l.) (16°31'26,4'' S, 68°2'26,1'' W), a rural site located in the south-eastern area of La Paz, part of the ''Valle de las Ánimas'' canyon formation, and characterized by wild vegetation and some crop species. Among the identified wild species there are predominantly: *Medicago lupulina*, *Clinopodium bolivianum*, *Cortaderia atacamensis*, *Baccharis latifolia*, *Senecio* spp., *Lupinus altimontanus*; and typical crops of the high Andean ecosystem, such as broad beans and potatoes. The second site is the "Parque Gimnasio Achumani" (PGA), located at 3395 m.a.s.l. (16°31'42,4''S, 68°4'27,9'' W), an urban recreational park open to the public. This site has representatives of the native vegetation of the city of La Paz. The following plant species were identified: *Dunalia sp*., *Schinus molle*, *Solanum spp*., and *Viguiera sp*. among others. The third site is the "Espacio Sociocultural Jardín Japonés" (ESJJ), located at 3657 m.a.s.l. (16°32'26,9''S, 68°5' 30,8'' W), an urban park characterized by introduced flora. The following species are present: *Pennisetum clandestinum*, *Hebe speciosa*, *Polylepis besseri*, and *Ruta graveolens*, among others. All the study sites belong to the highlands, with a moderately cold climate (4–10 °C), average annual rainfall of 500 to 600 mm/year, sandy loam soil, with typical growth of small shrubs, trees, and succulent plants [31].

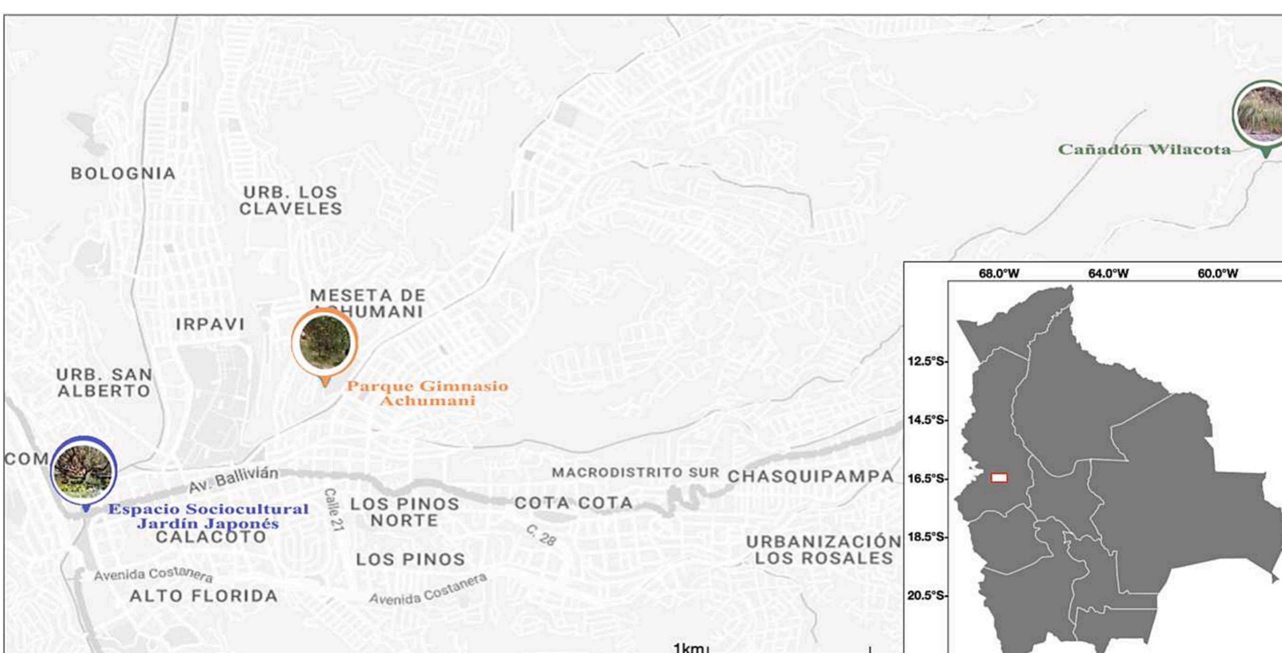

Fig. 1. Map with the geographic location of the three study sites: CW, PGA, and ESJJ within the city of La Paz. The inset in the lower right corner shows Bolivia, with geographic coordinates, and with a small red rectangle indicating the study area.

2.2. Floristic survey

For each study site we generated a complete list of plant species. To elaborate such a list, we collected (per site) three specimens of each plant morphospecies within a 30 m^2 plot, as well as 10 m outside the plot. Plant collection took place several times throughout the year to ensure completeness and presence of reproductive structures. More precisely, we collected in the months of February to April, July to September, and November to December. Collection methodology followed the protocol proposed by [32]. Briefly, a terminal portion of the sample was collected from a branch with leaves and reproductive structures, which were then pressed separately. For small herbs, the complete individual was collected. The vegetation cover was also obtained using the protocol proposed in [32]. For the identification of plant species, we worked with the National Herbarium of Bolivia (LPB), as well as with virtual herbaria and tropical databases of the Missouri Botanical Garden [33], Kew Botanical Garden [34], International Plant Names Index [35], Vascular Plants Australian Plant Census (APC) [36], Catálogo de Plantas Vasculares del Cono Sur [37] and Herbário virtual REFLORA [38].

2.3. Generating pollen reference collections

Given the lack of pollen databases in this area, we started a pollen reference collection (Supplementary Information, Fig. SI-1). For this, we extracted flowers from the specimens collected in the floristic survey and obtained reference pollen slides based on the protocol described in [39]. The photographs of the slides were taken at 400x and 1000x with the Motic BA210 light microscope and included in the World Virtual Catalog of the Pollen Catalog Network (RCPol) [40]. The pollen reference slides were deposited at the Palynotheca of the Universidad Policial Mcal. Antonio José de Sucre (UNIPOL). The pollen types obtained were characterized according to the criteria of the Pollen Catalog Network (RCPol) catalog, namely: pollen dispersal unit, symmetry, polarity, scope, type of opening, characteristic of the colpus, characteristic of the pore, number of openings, ornamentation of the exine, pollen grain size, pollen shape (P:E ratio), size of the polar and equatorial axis, size of the diameter (major and minor), and thickness of the exine.

2.4. Analysis of the pollen rain

To obtain samples of pollen rain within each 30 m^2 plot, the Wiltshire (2016) protocol [41] was used. Briefly, ten random subsamples of 0.1 m of soil surface at each site were taken (Table 1), and subsequently homogenized inside a Ziploc bag. To generate pollen slides, a modified protocol of Erdtman (1960) [42] was used. Briefly, *Lycopodium* pellets were added as positive controls, then centrifuged with 1 ml of 10 % HCl, washed with distilled water, added 3 ml of 10 % KOH 10 % to obtain a sediment, added HF (twice the amount of the sediment obtained), centrifuged with water, acetolyzed with a mixture of anhydrous acetic acid and sulfuric acid 9:1, heated in a water bath for 5 min and centrifuged, and finally washed with distilled water and placed in tubes with 50 % glycerin. This mix was then mounted on slides, where photographs of the palynomorphs were taken at 1000x, to be then identified with the reference pollen slides, the Roubik guide (1991)[43], the NOA Pollen Atlas [44], and the PalDat database [45]. By following this procedure, we obtained a total count of 200 palynomorphs per site. Palynomorphs not identified up to the species or genus level were reported up to the family level.

Table 1
Summary of experiments undertaken to sample palynomorphs from the pollen rain and footwear.

Experiment	Sampling procedure	Number of palynomorphs retrieved
Pollen rain	10 homogenized samples from each site (CW, PGA, and ESJJ).	200 for each site.
Footwear	4x 30-minute walks inside each plot (CW, PGA, and ESJJ).	800 for each site.

2.5. Analysis of samples from footwear

Sampling of palynomorphs. To obtain samples adhered to the soles of footwear, we asked four male volunteers of average weight and height for La Paz (ca. 70 kg and 1.7 m) [46] to walk for half an hour inside each plot, wearing OXDANS sports shoes. Volunteers used a unique pair of shoes each and carried out this scheme once per day and per site, for a total of four consecutive days, under the same meteorological conditions. Each pair of shoes was washed between each day, and the same pair of shoes was used until the end of the experiment (Table 1). At the end of the fourth day, the shoes were stored in a dark place at room temperature for one week (to simulate the time it takes for the local judicial body to request the analysis of the crime scene). For this same reason, the sampling of pollen rain and vegetation cover was also carried out a week after the pollen sampling from footwear.

Sample preparation. The soles of the footwear were rinsed with a 1:1 ethanol:distilled-water solution using a new and sterile toothbrush, until a volume equal to 62,5 ml was obtained. We performed this only on one shoe per pair, because the corresponding pair was considered as a forensic countersample (L. Povilauskas 2022, pers. commun.). Then, to obtain pollen slides in the same way as the surface soil samples, the rinsed substrate was centrifuged until almost dry samples were obtained, which were then processed with the same modified protocol of Erdtman (1960) [42]. We obtained a total of 200 palynomorphs per site per shoe, making a total of 800 palynomorphs per site (recall the experiment was repeated during four consecutive days). Additionally, the soil type was recorded and analyzed, using the classification made by the United States Department of Agriculture [47].

2.6. Data analysis

*Comparison between pollen rain and vegetation***.** We tabulated the list of species present in the pollen rain and the surrounding vegetation per plot as follows: 1) Pollen and/or spores, corresponding to the presence or absence of the species found in the pollen rain, and 2) Vegetation, corresponding to the presence or absence of the plant species present in the plot, collected on the same day the soil samples were taken. With this table it was possible to identify those species that are not represented in the pollen rain but present in the current vegetation and vice versa (Table 2).

Comparison between pollen rain and footwear. To evaluate the transfer of pollen grains from the pollen rain to the soles of the shoes we built a matrix (Table S3) following the protocol of Uitdehaag (2021) [48], and analyzed it using Detrended Correspondence Analysis (DCA). DCA is a type of multivariate analysis, in which communities of species represented in a sparse matrix are evaluated for different factors or gradients. Specifically, columns and rows are ordered simultaneously on complementary axes, the “trend” technique eliminates the arc effect, by dividing the first axis into a number of equal segments, within which the segments fit the order scores to a mean of zero, causing the data plots to stretch and straighten [49]. We applied this analysis to the combined dataset of palynomorphs and study sites, for which we used the *decorana* function of the *Vegan* package (R version 4.2.2).

In addition to DCA, we also used the Morisita-Horn similarity index (*abdiv* package in R: https://search.r-project.org/CRAN/refmans/abdiv/html/morisita.html) to statistically confirm the resemblance of the pollen assemblages between the pollen rain and the footwear. The *abdiv* package actually implements the dissimilarity index d(x,y), so here we report the similarity index as 1-d(x,y), using the counts of palynomorphs by collection site and by footwear and the combined average of footwear by collection site as input. Finally, using the same input data

Table 2
Presence/absence matrix of plant species and palynomorphs in the floristic survey, pollen rain and footwear for each study site. Presence is recorded with a "+", and absence with a "-". Geographic correspondence according to the DCA analysis is also shown.

Palynomorph	Floristic survey			Pollen rain			Footwear			Assemblage
	CW	PGA	ESJJ	CW	PGA	ESJJ	CW	PGA	ESJJ	(DCA)
Adesmia spp.	+	–	–	+	–	–	+	–	–	CW
Clinopodium bolivianum	+	–	–	+	–	–	+	–	–	CW
Cortaderia atacamensis	+	–	–	+	–	–	+	–	–	CW
Lepidium bipinnatifidum	+	–	–	+	–	–	+	–	–	CW
Oenothera sp.	+	–	–	+	–	–	+	–	–	CW
Salvinia sp.	–	–	–	+	–	–	+	–	–	CW
Senecio spp.	+	–	–	+	–	–	+	–	–	CW
Solanum nitidum	+	–	–	+	–	–	+	–	–	CW
Berberis commutata	+	–	–	+	–	–	–	–	–	CW
Gnaphalium sp.	+	–	–	+	–	–	–	–	–	CW
Lupinus altimontanus	+	–	–	+	–	–	–	–	–	CW
Calceolaria buchtieniana	+	–	–	–	–	–	–	–	–	x
Baccharis latifolia	+	–	–	+	+	–	+	+	–	CW
Medicago sp.	+	+	–	+	+	–	+	+	–	CW
Paraserianthes lophantha	+	+	–	+	+	–	+	+	–	CW
Gamochaeta americana	+	+	–	+	+	–	+	–	–	CW
Hieracium sp.	+	–	–	–	+	–	+	–	–	CW
Poaceaewil1ach	–	–	–	+	–	–	+	+	–	CW
Poaceaewil2ach	–	–	–	+	+	–	+	+	–	CW
Lycianthes lycioides	–	–	–	–	+	–	+	+	–	PGA
Deyeuxia sp.	+	+	–	+	+	–	+	+	–	PGA
Dunalia brachyacantha	–	+	–	–	+	–	–	+	–	PGA
Sedum sp.	–	+	–	–	+	–	–	+	–	PGA
Teucrium fruticans	–	+	–	–	+	–	–	+	–	PGA
Verbena litoralis	–	+	–	–	+	–	–	+	–	PGA
Solanaceae 1a	–	–	–	–	+	–	–	+	–	PGA
Opuntia sp.	–	+	–	–	–	–	–	+	–	PGA
Schinus molle	–	+	–	–	–	–	–	–	–	x
Rapistrum rugosum	–	+	–	–	+	+	–	+	+	PGA
Ipomoea pubescens	–	+	+	–	+	+	–	+	–	PGA
Viguiera procumbens	–	+	–	–	+	+	–	+	–	PGA
Antirrhinum majus	–	–	+	–	–	+	–	–	+	ESJJ
Hebe speciosa	–	–	+	–	–	+	–	–	+	ESJJ
Ligustrum sp.	–	–	+	–	–	+	–	–	+	ESJJ
Melaleuca citrinus	–	–	+	–	–	+	–	–	+	ESJJ
Petunia sp.	–	–	+	–	–	+	–	–	+	ESJJ
Pyracantha coccinea	–	–	+	–	–	+	–	–	+	ESJJ
Asteraceae 1j	–	–	–	–	–	+	–	–	+	ESJJ
Poaceae j	–	–	–	–	–	+	–	–	+	ESJJ
Dianthus caryophyllus	–	–	+	–	–	+	–	–	–	x
Inga sp	–	–	–	–	–	+	–	–	–	ESJJ
Iris pseudacorus	–	–	+	–	–	+	–	–	–	ESJJ
Juniperus virginiana	–	–	+	–	–	+	–	–	–	ESJJ
Malvaceae spp.	–	–	–	–	–	+	–	–	–	ESJJ
Argyranthemum sp.	–	–	+	–	–	–	–	–	–	x
Jasminum spp.	–	–	+	–	–	–	–	–	+	ESJJ
Ruta graveolens	–	–	+	–	–	–	–	–	+	ESJJ
Polylepis besseri	–	–	+	+	–	+	+	–	+	x
Sonchus oleraceus	–	+	–	+	+	+	+	–	+	CW
Amaranthaceae	–	–	–	+	+	+	+	+	–	ESJJ
Cyperaceae	+	+	+	+	+	+	+	+	+	CW
Pennisetum clandestinum	+	+	+	+	+	+	+	+	+	ESJJ
Gymnosperm 1	–	+	+	+	+	+	+	+	+	PGA

matrix for the DCA analysis, we generated pollen diagrams using the software TILIA (version 2.6). Here, only the indicator species of each site (including both pollen rain and footwear) were plotted.

3. Results

3.1. Floristic survey and pollen reference collection

A total of 53 plant species were collected: 19 at site CW, 17 at site PGA, and 17 at site ESJJ (Table 2). For all collected specimens identified to species level (17 at CW, 9 at PGA, and 14 at ESJJ) we took photographs of their plant habit and flower or inflorescence and built a database with photographic records. Additionally, with these specimens we generated reference pollen slides (see Methods, section 2.3 for details, and Supplementary Information, Fig. SI-1) and deposited them at the UNIPOL Palynotheca.

3.2. Analysis of pollen rain and samples from footwear

Pollen rain and floristic survey. The number of common species between the pollen rain and the vegetation (floristic survey) within each site was high. For instance, there were 17 such common species at the CW site, 15 at the PGA site, and 14 at the ESJJ site (Table 2). Other species not present in the vegetation but present in the pollen rain corresponded to palynomorphs of the families Amaranthaceae and Poaceae.

Pollen rain and footwear. We found 18 species with good representation and exclusivity at each study site, as well as high percentages in the pollen rain (Fig. 2). These species are considered "indicator" species for each site. Their percentages in the pollen rain (1) and the average

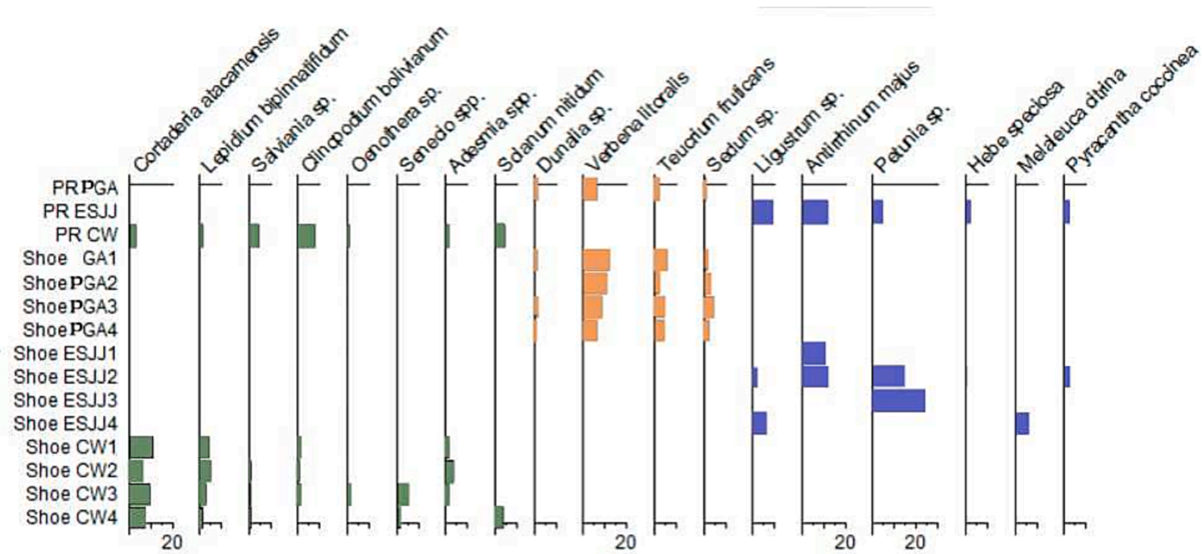

Fig. 2. Pollen diagram that shows the percentages of each indicator species for the pollen rain (PR) and for the footwear (shoe). Each site is represented with different colors: CW (green), PGA (orange), and ESJJ (blue).

among all four shoes (2) are described as follows: For site CW there were 8: *Cortaderia atacamensis* (Poa): (1) 3,4%, (2) 8,1%; *Lepidium bipinnatifidum* (Fab): (1) 1,5%, (2) 3,3%; *Salvinia* sp. (Salv): (1) 4,4%, (2) 0,7%, *Clinopodium bolivianum* (Lam): (1) 7,8%, (2) 0,7%; *Oenothera* sp. (Onag): (1) 1 %, (2) 0,3%; *Senecio* spp. (Aste): (1) 0,5%, (2) 1,6%, *Adesmia* spp. (Fab): (1) 1,5%, (2) 1,6% and *Solanum nitidum* (Solan): (1) 4,9% (2) 0,9%. For site PGA there were 4: *Dunalia brachyacantha* (Solan): (1) 1,2% (2) 0,6%; *Verbena litoralis* (Verb): (1) 6,1% (2) 9,2%; *Teucrium fruticans* (Lam): (1) 2 % (2) 4,2% and *Sedum* sp. (Crass): (1) 1,2% (2) 3 %. Finally, for site ESJJ there were 6: *Ligustrum* sp. (Oleac): (1) 9,1% (2) 1,9%; *Antirrhinum majus* (Plant): (1) 12,1% (2) 10 %; *Petunia* sp. (Solan): (1) 5,1% (2) 13,9%; *Hebe speciosa* (Plant): (1) 2 % (2) 0,4%; *Melaleuca citrinus* (Myr): (1) 0,5% (2) 0,8% and *Pyracantha coccinea* (Rosa): (1) 2 % (2) 1,9%.

3.3. Geographical origin and transfer of palynomorphs to footwear

Pollen assemblages from the surface soil were effectively transferred to the footwear at all sites. The results of the DCA analysis, which includes species from both the pollen rain and the footwear experiment, show a clear separation of the assemblages by geographic origin (Fig. 3). This separation can be seen both at the level of pollen rain and footwear (Fig. 3A) and by palynomorph assemblage (Fig. 3B). In both cases, assemblages aligned nicely with their corresponding sampling sites along the first DCA axis (DCA1).

Site PGA has the pollen assemblage with the largest number of shared species with other sites. Unique to PGA are *Verbena litoralis*, *Teucrium fruticans*, *Dunalia sp.* and *Sedum sp*. This site also shares species with ESJJ, such as *Viguiera procumbens*, *Ipomoea pubescens*, *Rapistrum rugosum*, as well as with site CW: *Polylepis besserii*, and *Sonchus oleraceus*

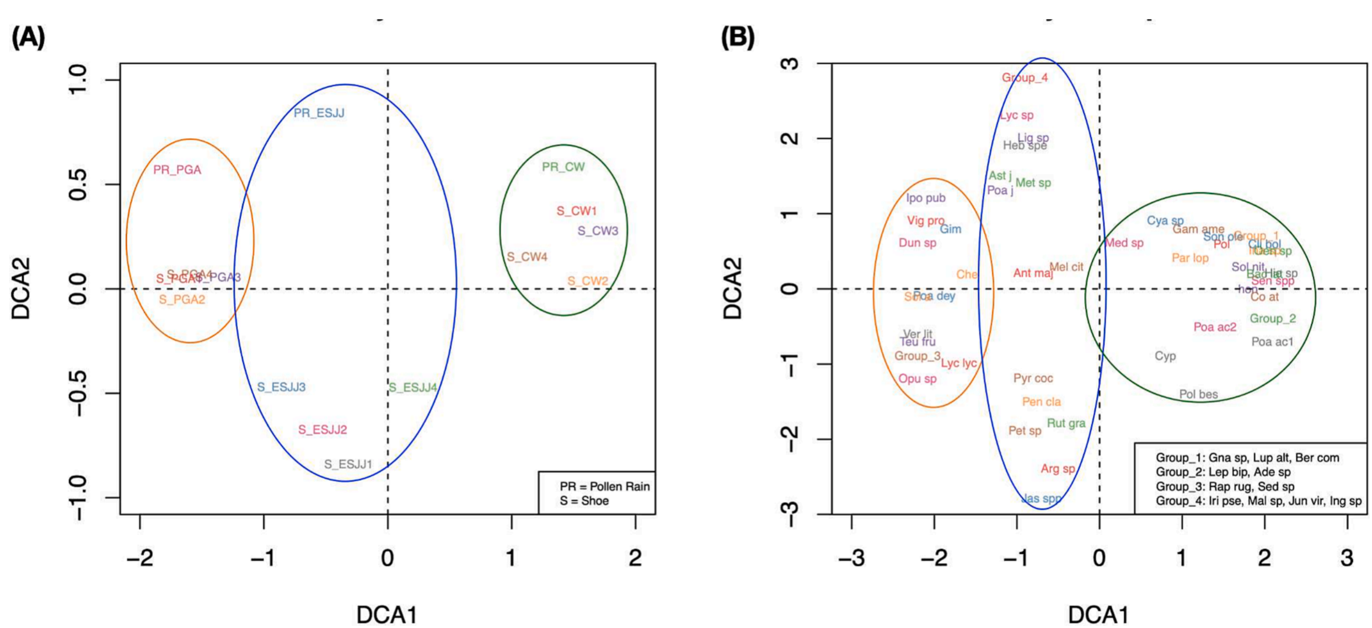

Fig. 3. (A) DCA results I: Pollen assemblages based on pollen rain (PR) and footwear (Shoe). The first DCA axis (DCA1) groups each repetition of the footwear experiment with the corresponding pollen assemblage of the pollen rain. (B) DCA results II: Distribution of palynomorphs. Colors of circles correspond to CW (green), PGA (orange), and ESJJ (blue). Abbreviations can be found on Table S1.

(Fig. 3B). At site ESJJ, several exclusive species could be observed, such as *Iris pseudacorus*, *Juniperus virginiana*, *Inga* sp., *Ligustrum sp.*, *Jasminum spp.*, *Hebe speciosa*, *Antirrhinum majus*, *Melaleuca citrina*, and *Petunia sp.*, all cultivated species. Shared with PGA and CW were *Pennisetum clandestinum* and *Sonchus oleraceus*. The site whose assemblage showed the least similarity with that of other sites was CW (rural natural site), which presents the following indicator species: *Cortaderia atacamensis*, *Lepidium bipinnatifidum*, *Salvinia* sp., *Clinopodium bolivianum*, *Oenothera* sp., *Senecio* spp., *Adesmia* spp. and *Solanum nitidum*.

The results of the DCA analysis and the degree of shared pollen assemblages among sites and footwear was corroborated by the similarity analysis (as depicted by the Morisita-Horn similarity index). Here, the shared presence of palynomorphs in the pollen rain and footwear showed that PGA was the site where the similarity between the indicator species from pollen rain and footwear was the greatest (0.98), followed by ESJJ (0.75) and CW (0.49) (Table 3 for the combined average over all footwear repetitions per site, and Table S2 for each individual repetition).

4. Discussion

4.1. Protocols for evidence processing

For taking palynomorph samples out of footwear, we initially used Wiltshire's protocol [21] which is based on scraping the sediment off the sole. However, a combination of the techniques developed by Povilauskas (2020) [50] and Laurence & Bryant (2019) [51] proved much more efficient for our case. These techniques are based on immersing the footwear for a period of time of at least 24 h in a 1:1 solution of distilled water and 95 % ethanol, which shows the importance of diluting the substrate, instead of scraping it. Like this, the water containing the submerged footwear will provide a representative sample with all the palynomorphs present. It is important to keep in mind that counting 200 palynomorphs in just one shoe (as was our case for the CW sample) represents a significant effort. This should be considered when collecting evidence, for the timely delivery of results without incurring in any delay of justice.

4.2. Study region

Bolivia is a tropical country located close to the equator in the Tropic of Capricorn, with multiple ecoregions distributed throughout the country. Around the area of La Paz, we find several ecoregions including dry inter-Andean forest and pre-Puna. Each of these ecoregions have temperatures and mean annual precipitation that vary throughout the year depending on the geographical location, which is why we collected samples at various time points throughout the year (see Methods). The method used here is optimized in and around La Paz but it could also be used elsewhere in the country. For instance, in [52], a similar sampling strategy as the one used here has been successfully applied across all ecoregions in Bolivia. Thus, for this method to be applied throughout the country, the only aspect needing optimization would be the sampling process of pollen from footwear. The method used here does not only improve the reference database for palynological forensic studies, but it also helps reconstruct past vegetation and past ecosystem types through fossil pollen records.

Table 3
Morisita-Horn similarity index using the average count (across repetitions) of palynomorphs of indicator species for the pollen rain (PR) and footwear for each study site.

	PR CW	PR PGA	PR ESJJ	Shoes CW	Shoes PGA	Shoes ESJJ
PR CW	1	–	–	–	–	–
PR PGA	0	1	–	–	–	–
PR ESJJ	0	0	1	–	–	–
Shoes CW	0.49	0	0	1	–	–
Shoes PGA	0	0.98	0	0	1	–
Shoes ESJJ	0	0	0.75	0	0	1

4.3. Pollen rain and vegetation survey

Our results show that in all three study sites, most of the species listed in the vegetation survey were also recorded in the soil samples from pollen rain, with only a few exceptions. These exceptions constitute species with limited flower numbers (e.g. *Hieracium sp.* in CW), or species pollinated by animals (insects, birds, etc.), which likely consumed most of the pollen resource [53–55], such as bee-pollinated *Calceolaria buchtieniana* (Calceolariaceae) in CW and *Opuntia sp.* in PGA. During pollination, bees profit from the presence of oil-secreting glands in the flowers, which are used as an additive to the food of their larvae and as nest lining material [53], which in turn causes pollen to adhere to their bodies and not fall to the ground. Similarly, at ESJJ the species absent in the pollen rain were *Ruta graveolens* (Rutaceae) and *Jasminum spp.* (Oleaceae), both pollinated by insects [51]. Concerning anemophilous plants (wind pollinated), soil samples of pollen rain showed a high percentage of Poaceae (Fig. 2), a family which generally produces a lot of pollen [56]. Similarly, other families that were found in all the sites with anemophilous pollination are species within Amaranthaceae and Gymnospermae. The long distances traveled by this pollen make them likely to come from places outside our study area [13,56,57], explaining their absence in the vegetation survey. It is worth mentioning that moss cushions can be better pollen catchers than surface soils [58], although in our study sites moss cushions were mostly absent.

Pollen rain is an accumulation of sedimented pollen at different points in time [56], which is why we also found a high abundance of pollen from species that were not in flower at the time of sampling. An example of this was observed at PGA, where *Rapistrum rugosum* (Brassicaceae), a naturalized herb [31] and indicator species of this site, was the species with the highest relative abundance in the pollen rain (17 %) (Table 2) but was not flowering at the time of sampling. This species flowers in summer and spring (September to December in Bolivia) [59].

The greatest diversity of species recorded in the pollen rain (N = 16) and the greatest number of palynomorphs absent in the vegetation survey (N = 8) was obtained at CW (Table 2), which could be because this place does not have cultivated ornamental species. Aside from some bean crops around the Ovejuyo river the vegetation is mostly native. Moreover, the presence of the river makes the dragging of palynomorphs from other sites likely [57,60]. At site ESJJ, the species with the highest abundance, *Pennisetum clandestinum* (Poaceae), was considered an indicator, because it had the highest percentage of coverage on that site (45 %). However, since it was present in the other two sites as well (although with less relative abundance), it cannot be used as the only indicator species, hence the importance of working with assemblages, rather than merely indicator species. At PGA, species of Gymnospermae (28 %) could be considered as indicators, as they are more abundant compared to the other sites (7 % at ESJJ, and 3 % at CW). However, other works such as that of Uitdehaag (2021) [48], propose to exclude overrepresented palynomorphs with high pollen production, as is the case of pine trees.

4.4. Pollen transfer from pollen rain to footwear

The site with the highest number of shared species was PGA (Fig. 3B). This could be because this site presents both native and ruderal species, including *Polylepis besserii* (Rosaceae), *Gamochaeta americana* (Asteraceae), *Lycianthes lycioides* (Solanaceae) and *Sonchus oleraceus* (Asteraceae) which were shared with CW. According to García (1987) [61], ruderal species appear in various places within the city of La Paz, such as *Rapistrum rugosum*, *Viguiera procumbens* and *Sonchus oleraceus* (the latter found in all three sites, Fig. 3B). These species could be used as indicators only after evaluating their relative abundances, since the abundance must be similar between the pollen rain samples and the samples from the footwear. Overall, the pollen assemblage of the natural site showed a greater number of indicator species and pollen biodiversity than the sites located in urban parks. This could be explained by the contribution of "non-indicator" species from urban surroundings, which does not occur in natural sites.

In cases where footwear is processed, it is very important to know the indicator species of the area and to recover the largest amount of pollen retained on the footwear (adherence). Adherence does not only depend on the material of the shoe sole, it also improves when the substrate is humid or wet, and the opposite is true when the substrate is dry [10]. In La Paz, the number of months in the dry season is greater than the number of months in the wet season (seven vs. five). For this reason, the present study was carried out during the wet season, with precipitation events days before the experiments, to which we attribute the good results obtained here. Thus, it is recommended to know the meteorological conditions that occurred in a real forensic case, to interpret the results found.

4.5. Relative abundance of pollen from pollen rain and footwear

At CW and PGA the total count of 200 palynomorphs was reached in every repetition. However, at ESJJ, the total count of 200 palynomorphs was reached only in one of the repetitions, the possible reason being the mechanical effect generated by *Pennisetum clandestinum* creating a layer of leaves, which makes it difficult for pollen to settle on the site. In addition, according to Povilauskas (2022, pers. commun.), the smooth sole of sports shoes hinders the accumulation of sediment, contrary to the findings of [7].

Regarding the indicator species, although taxonomic similarity was achieved (finding the same palynomorphs between the pollen rain and the footwear in the three evaluated sites (Fig. 2), the similarity in terms of relative abundance (Table 3) was not greater than 0.5 in CW, which could be interpreted as a low similarity. Such a value might not be enough evidence before the courts, for which it might be necessary to rely on other indications such as garments or objects where pollen could have been attached, or even rely on geological [19,62] and geographical indicators [51]. In our case, as previously discussed, CW showed conditions that possibly degraded the palynomorphs, generating the lowest similarity value (0.49). Alternatively, the lower similarity index at CW could also be attributed to the intrinsic variation of each type of walk made by each volunteer in terms of their speed and frequency of steps [63]. In any case, the DCA analysis was able to correctly identify the correspondence of each shoe with its geographical origin (Fig. 3A and Fig. 3B), which demonstrates its forensic utility, as confirmed in [16,17,20,21]. For this reason, a combination of similarity indices (such as Morrisita-Horn) and DCA has proven here to be an excellent method to correlate palynological samples and geographic locations that generate quantitative information for the forensic field.

4.6. Conclusions and future directions

A DCA analysis using pollen assemblages, such as the one presented here, made it possible to correctly identify the geographic origin of footwear, showcasing the forensic utility of this method in various non-seasonal high-altitude soils such as the ones studied here. Our results demonstrated the usefulness of the protocol used to obtain substrate samples from footwear [50], meaning that this method can be applied to real cases and similar environments. After successfully applying this method in all the sites here studied, the next steps include: 1) continue with the development of a pollen database of the high-crime zones, and 2) use the information of these databases to develop a pollen atlas, which will accelerate and improve police investigations in criminal cases.

5. Author statement

VSG conceived the project. VSG, TOL, and PD participated in the project design and performed data analyses. VSG and PD wrote the manuscript. TOL and PD supervised the project. All authors approved the current version of this paper.

6. Ethics statement

All the people involved in this study (namely, the four volunteers that wore and walked on the footwear used for this research) have given their consent to participate in this study. Additionally, our institution "Universidad Policial Mariscal Antonio José de Sucre" has given an ethical approval (Approval number: NF-DIC-069/23) for the use of human participants in this study. This research was conducted in accordance with the principles embodied in the Declaration of Helsinki and in accordance with local statutory requirements.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

We are grateful to the "Instituto de Investigaciones Técnico Científicas de la Universidad Policial Mcal. Antonio José de Sucre", the Institute of Geological and Environmental Research (IGEMA), Dr. Gloria Rodrigo and Dr. Isabel Morales (Carrera de Biología, Universidad Mayor de San Andrés) for providing laboratories, equipment and supplies. We are also grateful to the National Herbarium of Bolivia (LPB) for providing the facilities for pressing and identification work. To Emilia García Estigarribia and Rosember Hurtado Ulloa (from LPB) for their support in the identification of plant species. To the "Empresa Municipal de Áreas Verdes, Parques y Forestación" (EMAVERDE) for the permits to work in its facilities. To Daniel Larrea, Daniela Arteaga, Leticia Povilauskas, and the reviewers for their insightful comments, which helped improve the quality of this study. Finally, to Raúl Rodríguez Salvatierra, Yamil Narci Maidana Tuco and Eduardo Aguirre Mazzi for their help with the experiments.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scijus.2023.11.008.

References

[1] M. Hesse, H. Halbritter, R. Zetter, M. Weber, R. Buchner, A. Frosch-Radivo, S. Ulrich, Pollen Terminology. An illustrated handbook, 1st ed., Springer-Verlag, Wien, 2009.
[2] D.C. Mildenhall, P.E.J. Wiltshire, V.M. Bryant, Forensic palynology: Why do it and how it works, Forensic Sci. Int. 163 (2006), https://doi.org/10.1016/j.forsciint.2006.07.012.
[3] D.C. Mildenhall, P.E.J. Wiltshire, V.M. Bryant, Forensic palynology, Forensic Sci. Int. 163 (2006) 161–162.
[4] K.A.J. Walsh, M. Horrocks, Palynology: It's position in the field of forensic science, J. Forensic Sci. 53 (2008) 1053–1060.
[5] P.A. Bull, R.N. Morgan, A. Sagovsky, G.J.A. Hughes, The transfer and persistence of trace particulates: experimental studies using clothing fabrics, Science & Justice. 46 (n.d.) 185–195.
[6] F. Aristizábal, O. Riaño, Indicio, evidencia y prueba, in: J.A. Reyes, C. Sanabria, H. R. Valero, A.A. Fuentes, L. Diaz, A. Diaz, F.J. Aristizabal, J.R. Castro, J.R. Santos, H. J. Agudelo (Eds.), Enciclopedia Criminalística, Criminología e Investigación, 1ra ed., Sigma, Bogotá, 2015, p. 175.
[7] M. Boi, Pollen attachment in different materials, Aerobiología 31 (2015) 261–270.

[8] R.G. Bruce, M.E. Dettmann, Palynological analyses of Australian surface soils and their potential in forensic science, Forensic Sci. Int. 81 (1996) 77–94, https://doi.org/10.1016/S0379-0738(96)01973-1.
[9] M. Martínez-Sánchez, S. Fernández, J. Carrión, Palinología y escenario forense. Un Caso De Estudio Del Sureste De España, Anal. Biol. 30 (2008) 43–54.
[10] J. Ochando, M. Munuera, J.S. Carrión, S. Fernández, G. Amorós, J. Recalde, Forensic palynology revisited: Case studies from semi-arid Spain, Rev. Palaeobot. Palynol. 259 (2018) 29–38, https://doi.org/10.1016/j.revpalbo.2018.09.015.
[11] P. Wiltshire, Consideration of some taphonomic variables of relevance to forensic palynological investigation in the United Kingdom, Forensic Sci. Int. 163 (2006) 173–182.
[12] A. Nawaz, M. Firoze, J. Dubey, P. Morthekai, P. Bisht, P. Pandey, M. Shekhar, R. Ghosh, Surface pollen distribution in alpine zone of the higher Himalaya: a case study from the Kalla glacier valley, India, Bot. Lett. 167 (2020) 340–352.
[13] W. Phuphumirat, D.C. Mildenhall, C. Purintavaragul, Pollen deterioration in a tropical surface soil and its impact on forensic palynology, Open Forensic Sci. J. 2 (2009) 34–40.
[14] J.B. Riding, B.G. Rawlins, K.H. Coley, Changes in soil pollen assemblages on footwear worn at different sites, Palynology 31 (2007) 135–151.
[15] E. Prueger, El práctico en la investigación de homicidios, 1st ed., Córdova, 2009.
[16] L.M. Passarelli, Forensic palynology in Argentina, an alternative treatment for tape method proposed, Forensic Sci. Today 1 (2016) 001–003.
[17] L. Povilauskas, M.A. Gómez, Escena del crimen: Análisis palinológico en zonas de cultivos de cebollas, en la provincia de San Juan, Rev. Ciencias Jurídicas Polít. 6 (2022) 123–131.
[18] P. Wiltshire, Hair as a source of forensic evidence in murder investigations, Forensic Sci. Int. 163 (2006) 241–248.
[19] R.M. Morgan, J. Freudiger-Bonzon, K.H. Nichols, T. Jellis, S. Dunkerley, P. Zelazowski, P.A. Bull, The forensic analysis of sediments recovered from footwear, in: Criminal and Environmental Soil Forensics, Springer Netherlands, 2009: pp. 253–269. 10.1007/978-1-4020-9204-6_16.
[20] L. Povilauskas, Análisis palinológico de un homicidio en la Provincia de Buenos Aires, Argentina, Revista Brasileira de Criminalística. 6 (2017) 30. 10.15260/rbc.v6i3.189.
[21] P. Wiltshire, A rapid and efficient method for evaluation of suspect testimony: Palynological scanning, J. Forensic Sci. 60 (2015) 1441–1450.
[22] P.A. Bull, A. Parker, R.M. Morgan, The forensic analysis of soils and sediment taken from the cast of a footprint, Forensic Sci. Int. 162 (2006) 6–12.
[23] L. Povilauskas, Nuevas metodologías en el protocolo de procesamiento en Palinología Forense, Centro De Estudios Universitarios (2020) 45–54.
[24] Q. Zhang, L. Kaiqing, L. Jinfeng, J. Jinfeng, P. Wang, C. Hu, J. Zhu, Y. Wang, Y. Yao, Application of Judicial Palynology in Judicial Identification. Judicial Identification, Forensic Science Seminar. 8 (2018) 20–24.
[25] V. Manvailer, T. Leandro, E. Scremin-Dias, Botanical Knowledge in the Law Enforcement: a Preliminary Approach Towards its Potential Use in Brazilian Criminal Caseworks, Brazilian Archives of Biology and Technology. 0 (61AD). 10.1590/1678-4324-2018160442.
[26] L. Povilauskas, La aplicación de la palinología forense en casos de femicidios, Rev. Centro Estudios Criminol. Universitarios 14 (2020) 12–23.
[27] V.M. Bryant, G.D. Jones, Forensic palynology: Current status of a rarely used technique in the United States of America, Forensic Sci. Int. 163 (2006) 183–197, https://doi.org/10.1016/j.forsciint.2005.11.021.
[28] J.D.N.E., Remisión de datos estadísticos de la F.E.L.C.C y F.E.LC.V. Departamento Nacional de Estadística., La Paz, 2019.
[29] FELCV, Estadísticas de la base de datos de la Fuerza Especial de Lucha Contra la Violencia, 2023.
[30] G. Reyes, Violencia e inseguridad en las tres principales ciudades de Bolivia:, Wilson Center: Latin American Program. (2017) 1–25. https://www.wilsoncenter.org/sites/default/files/media/documents/article/violencia_e_inseguridad_en_las_ciudades_-_bolivia_final.pdf.
[31] S. Beck, E. García, E.L.N. Thompson, R.I. Meneses, F. Zenteno, R. López, A. Fuentes, Paisajes, eco-regiones y vegetación, in: M. I., M. R., J. Sarmiento (Eds.), Historia Natural de Un Valle En Los Andes: La Paz, 2nd ed., Instituto de Ecología y Museo Nacional de Historia Natural, La Paz, 2015: pp. 113–158.
[32] C. Marín, G.F. Medina, D.I. Jiménez, M.V. Sarmiento, O.A. León, J.E. Díaz, J. E. Paiba, Instituto de Ecología y Museo Nacional de Historia Natural, in: C. E. Sarmiento, O.A. León (Eds.), Transición Bosque-Páramo: Bases Conceptuales y Métodos Para Su Identificación En Los Andes Colombianos, 1st ed., Instituto de Investigación de Recursos Biológicos Alexander Von Humboldt, Bogotá, 2016, pp. 76–79.
[33] Missouri Botanical Garden, Tropicos, Https://Www.Tropicos.Org/Home. (2020). https://www.tropicos.org/home (accessed March 31, 2023).
[34] Royal Botanical Garden Kew, Royal Botanical Garden Kew, (2023). https://www.kew.org/.
[35] H.U.H.& L. and A.N.H. The Royal Botanic Gardens, Kew, International Plant Names Index. Published on the Internet, Https://Www.Ipni.Org/Citeus. (2020). https://www.ipni.org/citeus.
[36] A.P.C. (APC), Australian Plant Census (APC), (2020). https://biodiversity.org.au/nsl/services/search/taxonomy.
[37] Catálogo de plantas vasculares del Cono Sur, (2023). http://www.darwin.edu.ar/proyectos/floraargentina/Familias.asp.
[38] Reflora - Brasil de Plantas: Rescate HIstórico y Herbario Virtual para el Conocimiento y Conservación de la Flora e Funga do Brasil, (2020).
[39] C.I. Silva, V.L. Imperatriz-fonseca, M. Groppo, S.G. Bauermann, A.M. Saraiva, E.P. Queiroz, A.C.P. Evaldt, K.P. Aleixo, J.P. Castro, M.M.N. Castro, L.B. Faria, M.J.F. Caliman, J.L. Wolff, H.F. Paulino Neto, C.A. Garófalo, Catálogo polínico das plantas usadas por abelhas no Campus da USP de Ribeirão Preto, 2014.
[40] A. Veiga, A.M. Saraiva, C. Silva, The Online Pollen Catalogs Network (RCPol), Biodivers. Inform. Sci. Standards 2 (2018).
[41] P.E.J. Wiltshire, Protocols for forensic palynology, Palynology 40 (2016) 4–24, https://doi.org/10.1080/01916122.2015.1091138.
[42] G. Erdtman, The acetolysis method, Svensk Botanisk Tidsskrift 54 (1960) 561–564.
[43] D.W. Roubik, J.E.M. P., Pollen and Spores of Barro Colorado Island, Kew Bulletin. 47 (1992) 791. 10.2307/4110734.
[44] M.E. García, N.J.F. Reyes, H.G. Ríos, Compendio fotográfico de granos de polen de plantas nativas y cultivadas del noroeste argentino, 1st ed., Editorial Académica Española, 2012.
[45] H. Heigl, PalDat - Palynological Database, Https://Www.Paldat.Org/. (2020). https://www.paldat.org/.
[46] ANF, Estatura: los bolivianos están entre los más bajitos de la región, Https://Www.Noticiasfides.Com/Nacional/Sociedad/. (2020). https://www.noticiasfides.com/nacional/sociedad/.
[47] FAO, Sistema Unificado de Clasificación de Suelos, https://www.Fao.Org/Soils-Portal/Soil-Survey/Clasificacion-de-Suelos/Sistema-Unificado-de-Clasificacion-de-Suelos/Es/. (2020).
[48] S. Uitdehaag, Forensic soil comparison: towards objective methods for a more robust evidential value, Utrecht University, 2021.
[49] K. McGarigal, S. Stafford, S. Cushman, Multivariate Statistics for Wildlife and Ecology Research, Springer New York, 2000. 10.1007/978-1-4612-1288-1.
[50] L. Povilauskas, Metodología en la toma de muestras palinológicas, Centro de Estudios Criminológicos Universitarios. (2020) 30–41.
[51] A.R. Laurence, V.M. Bryant, Forensic palynology and the search for geolocation: Factors for analysis and the Baby Doe case, Forensic Sci. Int. 302 (2019), https://doi.org/10.1016/j.forsciint.2019.109903.
[52] T. Ortuño, M.-P. Ledru, R. Cheddadi, A. Kuentz, C. Favier, S. Beck, Modern pollen rain, vegetation and climate in Bolivian ecoregions, Rev. Palaeobot. Palynol. 165 (2011) 61–74.
[53] S.L. Buchmann, The ecology of oil flowers and their bees, Annu. Rev. Ecol. Syst. 18 (1987) 343–369, https://doi.org/10.1146/annurev.ecolsys.18.1.343.
[54] G. Manzanarez-Villasana, M.C. Mandujano, Visitantes y polinizadores en los morfos florales de Opuntia streptacantha lem. (Cactaceae), Sociedad Científica Mexicana de Ecología. (2021). https://scme.mx/visitantes-y-polinizadores-en-los-morfos-florales-de-opuntia-streptacantha-lem-cactaceae/.
[55] J.S.R. Aluri, Floral biology and pollination of Jasminum angustifolium Willd. and J. cuspidatum Rottler (Oleaceae), 58 (2022) 893–897.
[56] K. Faegri, L. Iversen, Textbook of pollen analysis, 4th ed., John Wiley & Sons, Chichester, 1989.
[57] E. Novenko, N. Mazei, M. Kusilman, Tree pollen representation in surface pollen assemblages from different vegetation zones of European Russia, Ecol. Quest. 26 (2017) 61–65.
[58] M.D.F. Quamar, S.K. Bera, Pollen analysis of modern tree bark samples from the Manendragarh Forest Range of the Koriya district, Chhattisgarh, India, Grana 56 (2017) 137–146, https://doi.org/10.1080/00173134.2016.1152293.
[59] A. Simpson, P. Fuller, K. Faccenda, N. Evenhuis, N. Matsunaga, M. Bowser, United States Register of Introduced and Invasive Species, 2022. 10.5066/P95XL09Q.
[60] L. Povilauskas, Análisis de la caída de granos de polen en el cauce de un río, Rev. Mexicana Med. Forense 5 (2020) 17–23.
[61] E. García, Flora y vegetación ruderal de la ciudad de La Paz, Universidad Mayor de San Andrés, 1987.
[62] A.G. Brown, The use of forensic botany and geology in war crimes investigations in NE Bosnia, Forensic Sci. Int. 163 (2006) 204–210, https://doi.org/10.1016/j.forsciint.2006.05.025.
[63] A. Pachi, T. Ji, Frequency and velocity of people walking, Struct. Eng. 83 (2005) 36–40.