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Pollen-monitoring: between analyst proficiency testing

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Abstract This study presents the results of a Europe-wide training and Quality Control (QC) exercise carried out within the framework of the European Aerobiology Society's QC Working Group and European COST Action FA1203 entitled "sustainable management of *Ambrosia artemisiifolia* in Europe (SMARTER)" with the aim of ensuring that pollen counters in Europe are confident in the identification of *Ambrosia* pollen grains. A total of 69 analysts from 20 countries examined a test slide by light microscopy, which contained *Ambrosia* pollen and pollen from other Asteraceae that could be recorded in the atmosphere at the same time of year (i.e. *Artemisia, Iva,* and *Xanthium*). Daily average pollen concentrations produced by individual participants were compared with the assigned value and the bias was measured by *z*-score. Both the assigned value and standard deviation for proficiency testing were calculated following the consensus value principle (ISO13528:2005) from the results reported by all the participants in the test. It took a total of 531 days from when the exercise commenced until all 69 analysts reported their results. The most outliers were reported for *Artemisia* pollen concentrations followed by *Xanthium* and *Iva*. The poor results for *Artemisia* and *Xanthium* were probably caused by low concentrations on the test slide leading to larger bias due to the unequal distribution of pollen over the microscope slide. Participants performed the best in identifying and quantifying *Ambrosia* pollen. Performing inter-laboratory ring tests with the same sample is very time consuming and might not be appropriate for large-scale proficiency testing in aerobiology. Pollen with similar morphology should be included in the education process of aerobiologists.

Keywords Aerobiology · *Ambrosia* · Asteraceae · Quality assurance · Quality Control

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1 Introduction

The use of standard procedures facilitates cooperation between laboratories and assists in the exchange of information allowing the comparison of data between sites. Proficiency testing is part of Quality Control

(QC), and is a prerequisite for the application of aerobiological data in a wide range of disciplines (i.e. agronomy, allergology, climatology, environmental health, forestry, meteorology, and phenology).

A notable effort has been made to assess representativeness and reproducibility of the methodology used in routine aerobiological monitoring (Galán et al. (2014) and references therein). This effort has, so far, culminated in minimum requirements for aerobiological sampling and analysis being published by the European Aerobiology Society's Working Group in Quality Control (QC), which also included a Europe-wide proficiency test focused on identifying and counting commonly found pollen grains in Northern Europe (i.e. *Betula* and Poaceae) and Central and Southern Europe (i.e. *Olea* and Poaceae) (Galán et al. 2014).

Common ragweed (*Ambrosia arthemisiifolia* L.) is a noxious invasive species both known as an important weed in agriculture and a source of highly allergenic pollen. As such, it is considered as an environmental health threat, not only in its native North America but also in many parts of the world where it has been introduced (Smith et al. (2013) and Essl et al. (2015) and references therein). In Aerobiology, airborne pollen plays important roles in evaluating the impact, mapping the sources and monitoring the spread and changes in abundance of this noxious invasive alien plant (Skjøth et al. 2010; Thibaudon et al. 2014; Bonini et al. 2015a, b; Karrer et al. 2015).

This study presents the results of a Europe-wide training and Quality Control (QC) exercise carried out within the framework of the European Aerobiology Society's QC Working Group and European COST Action FA1203 entitled "Sustainable management of *Ambrosia artemisiifolia* in Europe (SMARTER)". The main aim of this exercise is to ensure that pollen counters in Europe are confident in the identification of *Ambrosia* pollen grains, and pollen from other Asteraceae that could be recorded in the atmosphere at the same time of year (i.e. *Artemisia, Iva,* and *Xanthium*), in areas where *Ambrosia* is naturalized as well as areas where the plant and atmospheric pollen are scarce. This was achieved by: (1) performing an inter-laboratory ring test with the same sample slide in order to determine reproducibility of analysis; (2) providing adequate training material to aid identification.

2 Materials and methods

2.1 Material for the analysis

A call for participation in this QC exercise was sent by the European Aerobiology Society's QC Working Group to active aerobiological monitoring stations in Europe. A total of 69 analysts ("Appendix 1") participated in the study from 20 countries, which included regions where *Ambrosia* pollen is abundant in the atmosphere as well as areas where the pollen is rarely recorded (Fig. 1) (Skjøth et al. 2013): Austria, Belarus, Croatia, France, Georgia, Greece, Hungary, Italy, Lithuania, Poland, Portugal, Romania, Serbia, Slovakia, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and Ukraine.

A test slide containing airborne pollen collected by a volumetric trap of the Hirst (1952) design was used in the ring test. The sample for the test was collected at Novi Sad in Serbia, in an area of the Pannonian Plain considered to be one of centres of *Ambrosia* pollen distribution in Europe (Smith et al. (2013) and references therein). The pollen sample was taken on 27 August, 2013 during the main *Ambrosia* flowering season (Šikoparija et al. 2006).

Participants were instructed to count pollen on the test slide using the method commonly applied in their laboratory and to report daily pollen counts (raw data) and daily average pollen concentrations (pollen/m^3) for all pollen types registered in the slide. It was requested that at least 10% of the slide be examined, as described in the European Aerobiology Society's minimum recommendations (Galán et al. 2014). The pollen concentration is a product of the area of the slide sampled. For this reason, participants were also asked to supply the counting method (longitudinal or latitudinal transects), number of lines counted and field of view of the microscope used.

Participants received training material to help distinguish the pollen grains in the test slide: (1) three reference slides containing *Ambrosia*, *Iva* or *Xanthium* pollen; (2) Palynological descriptions of these pollen grains (Wodehouse 1965; Punt and Hoen 2009) ("Appendix 2"). Reference slides were prepared by dusting pollen grains on to the microscope slides directly from flowers of *A. artemisiifolia* L., *Iva xanthifolia* Nutt., and *Xanthium strumarium* L. The slides were mounted with basic fuchsine-stained glycerine jelly and covered by cover slip. Correct

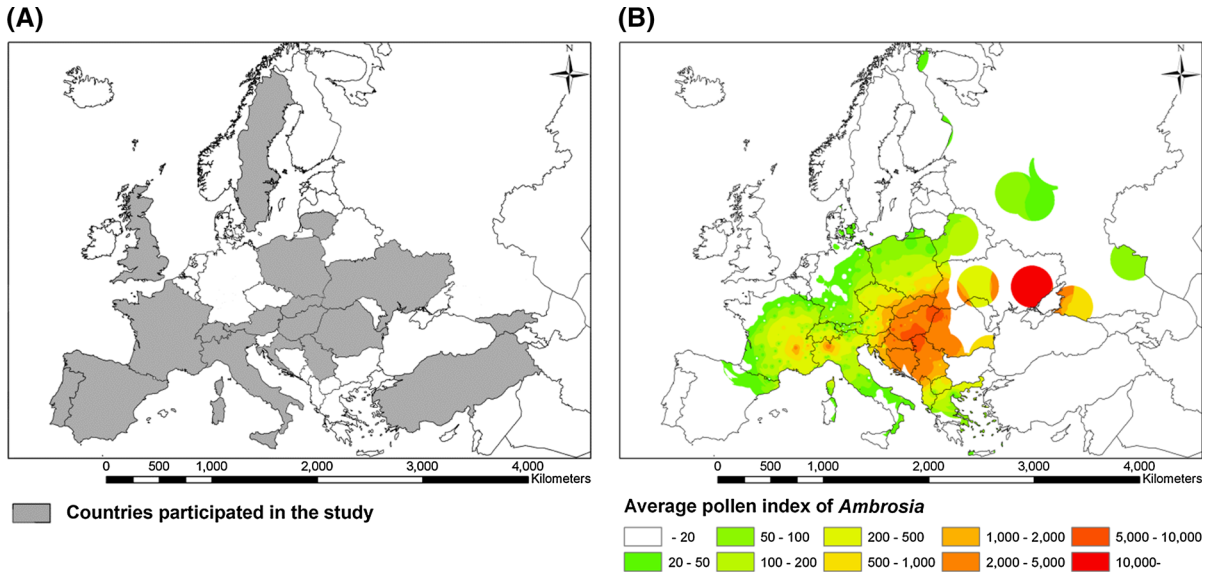

Fig. 1 **a** Countries where the participants in the QC exercise were working; **b** spatial estimate of airborne *Ambrosia* pollen abundance based on the mean annual pollen index of airborne *Ambrosia* pollen from Skjøth et al. (2013)

plant identification was confirmed by Herbarium BUNS (http://www.dbe.uns.ac.rs/o_depart-manu/laboratorije/herbarijum). Reference slides were not supplied for *Artemisia,* because this taxa is widely distributed in Europe and its pollen grains are frequently recorded in pollen traps in late summer.

2.2 Analysis and interpretation

Ambrosia, *Artemisia*, *Iva* and *Xanthium*, as well as the sum of *Ambrosia* and *Iva* daily pollen concentrations were tested using the Shapiro–Wilk test, which is considered the most powerful test for all types of distribution and sample sizes (Razali and Wah 2011). The data were found to be not normally distributed. The following parameters were calculated according to ISO (ISO13528 2005): median value (Me), mean value (m), standard deviation (s), robust standard deviation (s^*), assigned value (X), standard uncertainty of the assigned value (u_X), standard deviation for the proficiency testing (s_{pt}), relative standard deviation of reproducibility, and z-score (z).

Daily average pollen concentrations produced by individual participants were compared with the assigned value. The bias was measured by z-score $[z = (x_i - X)/s_{pt}]$ where x corresponds to the tested measurement, X to the assigned value and s_{pt} to the standard deviation for proficiency testing. By using z-scores for assessing bias, we were able to indicate the degree by which the result deviates from the expected value. It is suggested that the results were considered satisfactory if $|z| \leq 2$. Results with $2 < |z| < 3$ give a "warning" signal (ISO13528 2005) and is designated as questionable according to the IUPAC International Harmonized Protocol (Thompson et al. 2006), while those $|z| \geq 3$ require an "action" to improve the analysis procedure (ISO13528 2005) and is designated as unacceptable or unsatisfactory according to the IUPAC International Harmonized Protocol (Thompson et al. 2006).

The methods used for determining the assigned value and standard deviation for proficiency testing is sensitive to values that notably deviate from the average, and so outliers were identified and removed prior to calculation (ISO5725 1994). Hampel's test was used for identifying outliers. Unlike other tests used in previous QC studies in aerobiology (Oteros et al. 2013; Galán et al. 2014), Hampel's test is not sensitive to outliers since it uses outlier-resistant alternatives to mean and standard deviation (i.e. median and median absolute deviation from the median) (Pearson 2002). In performing Hampel's test, we calculated the median (Me) of all data (x_i), the absolute residuals (r_i) of each single data point from the median $[r_i = (x_i - \text{Me})]$, and the median of the absolute residuals (Me$|r_i|$). Any data point with

$r_i > 4.5$ Me$|r_i|$ is considered as an outlier (Konieczka and Namiesnik 2009). The removal of outliers normalized the distribution of the analysed datasets.

Both the assigned value and standard deviation for proficiency testing from the results reported by all the participants in the testing round were determined following the consensus value principle (ISO13528 2005). Following Algorithm A in ISO13528 (2005), the assigned value for each pollen type (the "robust average") and the standard deviation for proficiency testing (the "robust standard deviation") were calculated. The algorithm is an iterative calculation of average and standard deviation until the process converges. The initial value for calculation assigned value is a median of daily average pollen concentrations produced by individual participants after outliers were excluded (x^*). The initial value for calculation s_{pt} is s^* calculated as 1.483 Me $|x_i - x^*|$. These values were updated as follows:In the first step calculate $\delta = 1.5$ s* and for each x_i calculate (Eq. 1)

$$x_i^* = \begin{cases} x^* - \delta, & \text{if} \quad x_i < x^* - \delta \\ x^* + \delta, & \text{if} \quad x_i > x^* + \delta \\ x_i, & \text{otherwise} \end{cases} \tag{1}$$

In the second step calculate the new values of x^* and s^* from (Eq. 2)

$$\begin{aligned} x^* &= \sum x_i^*/p \\ s^* &= 1,134\sqrt{\sum (x_i^* - x^*)^2/(p-1)} \end{aligned} \tag{2}$$

The second step should be repeated until both x^* and s^* remain unchanged from the previous iteration. The resultant x^* is taken as the assigned value while the resultant s^* is taken as the standard deviation for proficiency testing (p denotes to items of data, sorted into increasing order).

3 Results and discussion

Notable variations in pollen levels can be detected in atmospheric concentrations of pollen recorded in Hirst type traps situated close to one another [e.g. Buters et al. (2010), Tormo Molina et al. (2013) and Velasco-Jiménez et al. (2013)]. For this reason, the QC exercise to determine reproducibility of analysis was performed on the same sample. This made the whole process extremely time consuming and demanding from a logistical point of view. The exercise commenced in May 2014 and it took 531 days until all 69 participants reported their results (~8 days per participant to analyse the slide and send it on to the next laboratory). However, the time spent with participants ranged from <1 day to as much as 68 days. In addition, we faced a number of delays due to holidays and customs procedures when the shipment was sent to countries outside of the EU. For future QC tests, we recommend organizing rounds with fewer participants if the results are going to be used for annual inter-laboratory testing as required by ISO17025 (2005).

For all assigned values, uncertainty was lower than 30% of the standard deviation for the proficiency test (Table 1). As a result, these were deemed negligible and not included in the interpretation of the results of the proficiency test (ISO13528 2005).

The most outliers were detected for reported airborne pollen concentrations of *Artemisia* ($n = 5$), followed by *Iva* ($n = 3$), *Xanthium* ($n = 3$), *Ambrosia* ($n = 1$), and the sum of *Ambrosia* and *Xanthium* ($n = 1$) (Table 1).

Most participants performed well in identifying and quantifying airborne *Ambrosia* pollen, and only one unsatisfactory result was recorded ($|z| \geq 3$). This was to be expected, since *Ambrosia* pollen is included in the training that many aerobiologists receive, e.g. in the European Basic courses and International Advanced courses in aerobiology run by the European Aerobiology Society (EAS) and the International Association for Aerobiology (IAA) as well as local training and participation in internal QC exercises. Training and internal validation are two of the minimum requirements in the EAS Working Group on Quality Control (Galán et al. 2014), as it helps to ensure operators are able to identify a range of different pollen types even in regions where they are not commonly recorded in the atmosphere.

With this in mind, it is surprising to see that the most unsatisfactory results ($|z| \geq 3$) were found for airborne *Artemisia* pollen concentrations (Fig. 2). This pollen type is widely distributed throughout Europe and as such it is frequently recorded by pollen-monitoring stations and also routinely included in training programmes. *Artemisia* is also considered to be easily identifiable among anemophilous Asteraceae pollen types. Despite its wide distribution, however, *Artemisia* pollen grains are not always recorded in large numbers. It is therefore possible that not all

Table 1 Descriptive statistics

	Ambrosia	*Artemisia*	*Iva*	*Xanthium*	*Ambrosia* + *Iva*
Submitted data	69	66	68	68	68
Median (Me)	160	8	16	31	177
Mean (x)	156	10	18	31	174
Standard deviation (s)	29.37	4.19	11.75	8.25	29.79
Number of removed outliers	1	5	3	3	1
Assigned value (X)	158	9	16	32	176
Standard deviation for the proficiency testing (s_{pt})	27.60	2.37	7.51	5.88	27.06
Standard uncertainty of the assigned value (u_X)	4.18	0.38	1.16	0.91	4.13
Number of questionable results ($2 < \|z\| < 3$)	1	3	3	6	1
Number of unsatisfactory results ($\|z\| \geq 3$)	1	5	3	3	1

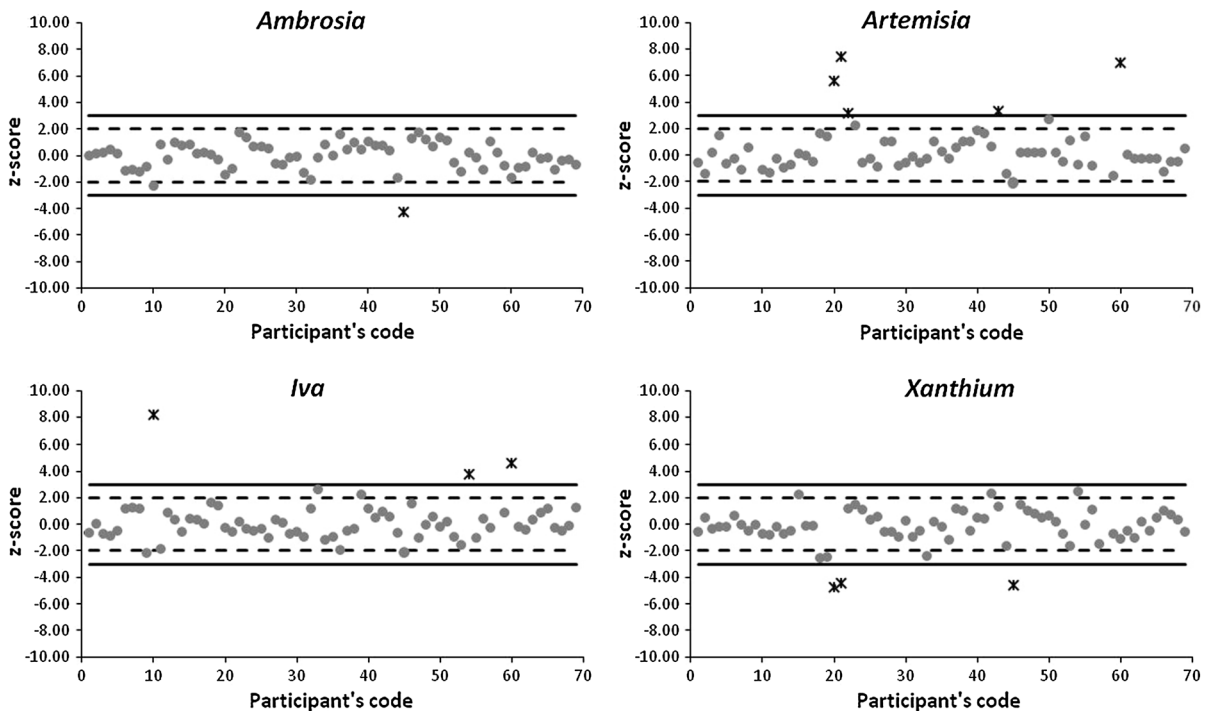

Fig. 2 z-scores for reported *Ambrosia*, *Artemisia*, *Iva*, and *Xanthium* pollen concentrations. Outliers detected using Hampel's test are indicated with asterisk. Solid black lines indicate limits for unsatisfactory results and *dashed black lines* indicate limit for questionable results (Thompson et al. 2006)

counters involved in the study had the same level of proficiency when it came to identifying *Artemisia* pollen, and so the inclusion of reference slides could have helped improve the results. It should be noted that with three unsatisfactory ($|z| \geq 3$) and six questionable ($2 < |z| < 3$) results *Xanthium* seemed to cause the most trouble for a number of participants although reference slides from this pollen type were available to participants. However, the amounts of *Artemisia* pollen (10 pollen/m^3 on average) and *Xanthium* pollen (31 pollen/m^3 on average) on the test slide were low. Such low mean concentrations are expected to influence the amount of variation (Galán et al. 2014). In addition, when a fraction of a slide is analysed, as is commonly done in aerobiology, unequal distribution of pollen over the microscope slide would add to the bias for low pollen concentrations. Discrepancies in the results for *Artemisia* and

Table 2 Reported pollen concentrations and corresponding *z*-scores for questionable and unsatisfactory *Ambrosia*, *Iva*, and sum of these two morphologically similar pollen types (Outliers identified by using Hampel's test are indicated by bold numbers)

Pollen type	*Ambrosia*		*Iva*		*Ambrosia* + *Iva*	
Participant's code	PG/m^3	*z*-score	PG/m^3	*z*-score	PG/m^3	*z*-score
QC_09	134	−0.88	0	−2.18[a]	134	−1.57
QC_10	95	−2.29[a]	**78**	8.22[b]	173	−0.13
QC_33	154	−0.14	36	2.65[a]	191	0.52
QC_45	**41**	−4.25[b]	0	−2.12[a]	**41**	−4.99[b]
QC_54	163	0.17	**44**	3.74[b]	208	1.15
QC_60	111	−1.73	**51**	4.64[a]	162	−0.54

[a] Questionable result

[b] Unsatisfactory result

Xanthium are therefore likely to be caused by relatively low average concentrations of these pollen types on the sample slide.

Three unsatisfactory ($|z| \geq 3$) and three questionable ($2 < |z| < 3$) results (Fig. 2) makes *Iva* pollen the third most problematic. Here the problem could come from large morphological similarity between *Iva* and *Ambrosia* pollen (Wodehouse 1965). It is interesting to note that when summing *Ambrosia* and *Iva* pollen concentrations for participants with questionable and unsatisfactory results, *z*-score values notably improve (Table 2). This indicates that morphological similarity resulted in misidentification of these two pollen types.

There is an increased interest for using aerobiological data to either analyse distribution and abundance of ragweed pollen sources (Skjøth et al. 2010; Thibaudon et al. 2014; Bonini et al. 2015a, b; Karrer et al. 2015) or validate the performance of tools developed for modelling the introduction and spread of non-native species (Chapman et al. 2016). This clearly emphasizes the importance of accurate identification of *Ambrosia* pollen from aerobiological samples and avoiding grouping Asteraceae pollen grains with similar morphology as *Ambrosia* "type" pollen grains (Kalinovych et al. 2007).

Ambrosia pollen is usually expected to be much more abundant in air samples compared to the other pollen types examined in this study. It is, however, important to record *Iva* and *Xanthium* pollen grains separately as this provides valuable data for monitoring potential distribution changes of these weed species. This is particularly important for *Iva xanthifolia*, which has not fully realized its invasion potential in Europe (Follak et al. 2013). In some regions, *Iva xanthifolia* pollen, as well as *Ambrosia*, can make a notable contribution to the airborne pollen spectrum (Weryszko-Chmielewska et al. 2003). In such cases, the correct quantification of *Iva xantifolia* is especially critical because, from clinical point of view, *Iva* and *Ambrosia* pollen should be treated separately when formulating allergen immunotherapy (Weber 2008).

4 Conclusions

Performing the between laboratory and analysts reproducibility on the same sample is very time consuming and might not be appropriate for large-scale proficiency testing in aerobiology. It is important to suggest the least time consuming approach for future proficiency testing in order to enable organization such QC exercises more frequently. It is also important to include pollen that can potentially cause misidentification in the education of aerobiologists. Particular attention should be given to invasive species, since correct identification in airborne pollen samples is prerequisite for assessment of changes in their distribution range. It is important for pollen-monitoring networks to actively search for airborne *Ambrosia* pollen, especially in areas where the plant and the pollen is not commonly recorded as this will provide an early warning of its expansion to new areas. It is therefore necessary for workers to become confident in identifying both the plant and its pollen. For this reason, various training schools and proficiency tests such as this have been implemented by International Association for Aerobiology (International Advanced Course in Aerobiology), the European Aerobiology Society (European Basic Course in Aerobiology) and COST SMARTER. Frequent

proficiency testing and continuous education of both novice and experienced agrobiologists is required to maximise the quality of the datasets they provide.

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Appendix 1: The following counters participated in this QC exercise

Abramidze, T; Adams-Groom, B; Albertini, R; Anelli, P; Bastl, K; Bigagli, V; Bonini, M; Bócsi, E; Bucher, E; Caeiro, E; Celenk, S; Cerovac, Ž; Chłopek K; Cislaghi, G; Clot, B; Cortonesi, B; Cristofori, A; Dahl, A; Della Bella, V; Dupuy, N; Dušička, J; Ferro, R; Flori, C; Graber, M-J; Hrastovćak, S; Ianovici, N; Józsa, E; Kasprzyk, I; Kmenta, M; Kofler, V; Magyar, D; Mányoki, G; Maya Manzano, J.M.; Myszkowska, D; Nowak, M; Oliver, G; Paganoni, B; Palamarchuk, O; Parati, S; Pashley, C; Pini, A; Piotrowska, K; Prentović, M; Rachoud, A-M; Radišić, P; Rapp Benito, A; Rodinkova., V; Russo, M; Sallin, C; Satchwell, J; Sindt, C; Smith, M; Szymanska, A; Šaulienė, I; Ščevková, J; Šikoparija, B; Tassan Mazzocco, F; Testoni, C; Tomičić Žabčić, V; Udvardy, O; Ugolotti, M; Vadassy, R; Vannini, J; Vecenaj, A; Velasco-Jiménez, M.J.; Verardo, P; Viola, MC; Vuillemin, F; Zemmer, F.

Appendix 2

See Table 3.

Table 3 Palynological descriptions of *Ambrosia*, *Iva*, and *Xanthium* pollen grains taken from literature were given to participants as supporting material

Ambrosia artemisiiaefolia (Punt and Hoen 2009)	*Xanthium strumarium* (Punt and Hoen 2009)	*Iva xanthifolia* under *Cyclachaena xanthifolia* (in Wodehouse 1965)
Pollen class: 3-zonocolporate, rarely 4-zonocolporate	*Pollen class*: 3-zonocolporate, rarely 4-zonocolporate	*Pollen class*: 3-zonocolporate
Apertures: Ectoaperture-colpus, short to very short and narrow, irregular in outline, slightly or not sunken; margins distinct, irregular, without a margo; ends distinct acute; colpus membrane indistinct, probably smooth; apocolpium index large. Mesoaperture—absent. Endoaperture—indistinct, probably a lolongate porus; margins indistinct; ends diffuse; no costae	*Apertures*: Ectoaperture-colpus, very short and narrow, irregular in outline, slightly or not sunken; margins distinct, irregular, without a margo; ends acute; colpus membrane smooth; apocolpium index large. Mesoaperture—often absent if present an indistinct lolongate porus. Endo aperture—usually indistinct, if present a lolongate porus; margins diffuse and indistinct; no costae	*Apertures*: Ectoaperture-colpus, long
Exine: Thick. Nexine thin. Cavea distinct usually broad. Sexine 1 missing. Sexine 2 a thin tectum. Sexine 3 rather thick, consisting of slender, not digitate columellae, densely set, not or only slightly longer under the echinae. Sexine 4 an udulating tectum without puncta with low echinae which are as broad as high, without a cavity in the top; sides of the echinae straight	*Exine*: Fairly thin to thick. Nexine thin. Cavea distinct or indistinct, if distinct broad in the mesocolpium and thinner at the apocolpium. Sexine 2 a thin internal tectum. Sexine 3 consisting of short, slender, not digitate, densely set columellae, not longer under the microechinae. Sexine 4 an undulating tectum without puncta and with microechinae, which are acute at the top; basis of microechinae very broad (verrucae)	*Exine*

Table 3 continued

Ambrosia artemisiiaefolia (Punt and Hoen 2009)	*Xanthium strumarium* (Punt and Hoen 2009)	*Iva xanthifolia* under *Cyclachaena xanthifolia* (in Wodehouse 1965)
Ornamentation: Echinate or microechinate. In polar view about 30 to 35 echinae in the equatorial plane. Columellae small, circular, crowded	*Ornamentation*: Microechinate. In polar view about 30 microechinae visible in the equatorial plane. Columellae small, circular, crowded	*Ornamentation* Echinate or microechinate (subechinate)
Outlines: Equatorial view—elliptical to circular. Polar view—circular to triangular, angles obtuse, sides convex, apertures in the middle of the sides; colpi intruding	*Outlines*: Equatorial view—circular or elliptic. Polar view—triangular; angles obtuse, colpi situated in the middle of the sides only slightly intruding; sides straight to slightly convex	
Measurements:	*Measurements*	*Measurements*
Glycerine jelly—P 18.5–26.0 µm; E 19.0–29.0 µm; colpi 4–8 µm long and 1–2 µm broad; exine varying from about 2 µm (without cavea) up to 5 µm with cavea; echinae up to 2 µm high and 2–3 µm broad.	Glycerine jelly—P 26.0–30.5 µm; E 27.5–33.0 µm; exine about 2 µm; microechinae less than 1 :m high, basis 1–1.5 µm broad.	19.1–21.0 µm
Silicone oil—P 21.5–25.5 µm; E 24.5–28.0 µm	Silicone oil—P 23.5–31.5 µm; E 27.5–33.0 µm;	colpi about 10 µm long (almost from pole to pole)

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