A morphometric approach to track opium poppy domestication

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Opium poppy (Papaver somniferum L. subsp. somniferum) was likely domesticated in the Western Mediterranean, where its putative wild ancestor is indigenous, and then spread to central and northern Europe. While opium poppy seeds are regularly identified in archaeobotanical studies, the absence of morphological criteria to distinguish the seeds of wild and domestic forms prevents the documentation of their respective historical and geographical occurrences and of the process of opium domestication as a whole. To fill this gap and better understand the status of this crop in the Neolithic, we combined seed outline analyses, namely elliptic Fourier transforms, with other morphometric descriptors to describe and identify Papaver setigerum, Papaver somniferum and other Papaver taxa. The combination of all measured parameters gives the most precise predictions for the identification of all seven taxa. We finally provide a case study on a Neolithic assemblage from a pile-dwelling site in Switzerland (Zurich-Parkhaus Opéra, ca. 3170 BC). Our results indicate the presence of mixed populations of domestic and wild seeds belonging to the P. somniferum group, suggesting that the plant was already in the process of domestication at the end of 4th millennium BC. Altogether, these results pave the way to understand the geography and history of the poppy domestication and its spread into Europe.

Opium poppy (Papaver somniferum L.), as the principal source of opium and opiate drugs, today, as in the past, is a most controversial species. This plant has multiple uses including medicine (e.g. morphine), decoration (as an ornamental plant) and food. Poppy seeds can be used for making porridge, eaten raw or pressed for edible oil1. Unlike the founder crops (different cereals, pulses and flax) that are known in Europe, arriving from the Near-East during the Neolithic period (ca. 6500–3500 BC), opium poppy is currently supposed to have been domesticated outside of the Fertile Crescent. Its domestication probably took place in the Western Mediterranean area from where the putative progenitor is native and still growing wild today, Papaver somniferum subsp. setigerum (DC.) Arcang.2,3 (from now on P. setigerum). Papaver somniferum/setigerum seeds are reported in the archaeological record starting from the Neolithic period (6th-millennium cal. BC)4,5,6,7. Regrettably, these are not identified to subspecies/status level (i.e. at the wild/domesticated level) because no clear criterion exists for these seeds to be distinguished. This paper aims to fill this methodological gap to further gain knowledge for the archaeological and the botanical sides of Papaver domestication history. The goals of the paper are to distinguish the wild from domestic species in modern Papaver through the application of traditional and geometric morphometrics on seeds. Then we use this methodology to establish the status of this plant during the Neolithic period using archaeological seeds from a case study in central Europe, Zurich-Parkhaus Opéra (ca. 3170 BC). This is the first time this approach is used to study the domestication process of opium poppy.

The genus Papaver encompasses more than 80 different species8 of annual, biennial and perennial plants distributed in central and south-western Asia, central and southern Europe and northern Africa9. All species of Papaver grow in open and unevenly disturbed habitats. Perennials and biennials are mountain taxa growing above 1000 m while annuals are mostly lowland taxa10. Papaver species encountered in western and southern Europe and identified in the archaeological record are: P. album; P. hybridum; P. rhoeas; P. argemone; as well as different subspecies and one variety of the P. somniferum group (here referred at the species level for the sake of simplicity): P. somniferum, P. setigerum and P. nigrum.

Papaver taxonomy is still debated, Kadereit11, Zohary et al.12 and Carolan et al.13 all argued that P. somniferum has two subspecies: setigerum and somniferum, the latter being the domesticated descendant. Whether P. setigerum and P. somniferum represent two distinct species or whether they should be considered as two subspecies is still debated. Using sequences of the plastid gene rpl16 and the rpl16-rpl14 Hosokawa et al.13 argued that both

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species were identical. Likewise, a phylogenetic study of *Papaver* based on DNA sequences was unable to distinguish these two taxa13. The opium poppy (*P. somniferum*) is an annual herb, 30–150 cm high, self-pollinated and most of the actual cultivars are diploid. *Papaver setigerum* is an annual plant, 60 cm high, a field weed occurring in disturbed grounds14 and native to the western Mediterranean14 in Algeria, France, Italy, Morocco, Portugal, Spain, Tunisia15. *P. setigerum* is both diploid and tetraploid and inter-fertile with the *P. somniferum* cultivars16.

The history and mechanisms of opium poppy domestication remain unclear despite the abundance of archaeological seeds in sites dated to the Neolithic period, particularly in the Alpine Foreland1. Archaeobotanical remains are usually broadly identified as *P. somniferum*, yet their domesticated status is unclear17. The domestication syndrome of opium poppy encompasses the increase in the size of the capsule and seeds, as well as capsule indehiscence18. Previous studies attempted to distinguish wild from domestic opium poppy seeds based on the size, comparing archaeological seeds to modern species19–22. However, the size of the seed alone has not proven to be a good discriminating criterium20 since it overlaps between the two species.

This paper addresses two questions: (i) can we distinguish between modern seeds of the wild (*Papaver setigerum*), domestic (*Papaver somniferum*) and other *Papaver* species? If so, (ii) can we distinguish *Papaver* species in archaeological assemblages previously identified as *P. setigerum/somniferum*?

The modern plant material consisted of 270 seeds belonging to seven *Papaver* taxa (30 seeds per taxon) obtained from the seed collection of the Integrative Prähistorische und Naturwissenschaftliche Archäologie (IPNA/IPAS) at the University of Basel, Switzerland (Supplementary Material Table 1). Two additional sets of 30 seeds of *P. somniferum* and *P. setigerum* were obtained from the Grainerie (seed collection) of the National Museum of Natural History (MNHN) in Paris, France. We first established new identification criteria between *Papaver* species, and chiefly between *P. setigerum* and *P. somniferum*. We applied traditional and geometric morphometrics on seeds, considering the number of cells, size measurements and shape using outline analysis. Outline analysis has been successfully used to identify archaeobotanical remains of an array of species such as grape pips23, olive stones24,25 cereals26–28, dates29 and cherry stones30. The technical challenge for *Papaver* seed lies in the millimetric size of the seeds and their globoid shape. Prior to any morphometric analysis, repeatability tests were performed to establish the effects of taking the photos, cleaning and landmarking in the observed seed morphometric variation.

This protocol was then applied to 33 uncharred poppy seeds preserved in waterlogging conditions from a Neolithic pile-dwelling site in the Alpine Foreland (Zurich-Parkhaus Opéra, dendro-dated to ca. 3170 BC31). This site is an ideal starting point since the Swiss Plateau is outside of the natural area of spread of *P. setigerum*, thus suggesting a human introduction. *P. somniferum* seeds are known in Switzerland since ca. 5000–4800 BC in the Valais region32, and seed and capsule fragments were recovered in large quantities in pile-dwelling sites starting from 4300 BC, indicating widespread cultivation33. Zurich-Parkhaus Opéra is, therefore, a perfect case study to test our methodology, since opium poppy had been cultivated in the area for ca. 1000 years and might therefore show morphometrical signs of domestication. Furthermore, the waterlogging conditions maintained the original seed shape and size, unlike what is known to occur to charred remains34.

### Results

**Measurement error.** The error measurements was quantified by acquiring data 3 times on 5 seeds independently for the three species (Table 1), which allow to test for the different steps of the protocol: positioning, image cleaning and landmarking. These three steps yield contrasting results. Positioning error is high (between 68 and 85%) (Table 1). This originates from the difficulty to orientate the seed consistently under the stereomicroscope, due to the small size and globoid shape of poppy seeds. On the other hand, cleaning and landmarking errors are much lower. Despite the existence of a certain positioning error, this does not prevent taxonomic identification (see below) and the protocol can therefore be used for the purpose of this study.

| Taxa            | ANOVA F | P-value | %ME |
|-----------------|---------|---------|-----|
| Position test   |         |         |     |
| *P. somniferum* | 1.3208  | 0.093   | 85.339 |
| *P. setigerum*  | 2.3065  | 0.039   | 79.283 |
| *P. nigrum*     | 3.2988  | 0.013   | 68.504 |
| Cleaning test   |         |         |     |
| *P. somniferum* | 4.4525  | 0.004   | 46.493 |
| *P. setigerum*  | 17.502  | 0.001   | 15.382 |
| *P. nigrum*     | 7.079   | 0.001   | 33.043 |
| Landmark test   |         |         |     |
| *P. somniferum* | 37.649  | 0.001   | 7.566  |
| *P. setigerum*  | 13.648  | 0.001   | 19.171 |
| *P. nigrum*     | 27.335  | 0.001   | 10.226 |

Table 1: Results of the reproducibility tests (seed positioning, photography cleaning and landmarking) performed through Anova: F: Fisher statistics, P-value and measuring error (%) when comparing the three taxa of *P. somniferum*. 

Phenotypic variation among species. Length and width show considerable variation with significant differences between the various Papaver species (Fig. 1), as reported by the results of the Kruskal–Wallis tests (length: \( \chi^2 = 231.78, \text{df} = 7, P < 10^{-16} \); width: \( \chi^2 = 243.76, \text{df} = 7, P < 10^{-16} \)). The two domestic species (P. somniferum and P. nigrum) have bigger seeds than the wild species, especially in width (Wilcoxon rank tests, all \( P \) values < 10\(^{-11} \)). The seeds of P. somniferum and P. setigerum are different in size (Wilcoxon rank tests, all \( P \) values < 10\(^{-8} \)). For both species, the two investigated samples are close, yet some dimensions appear different (Wilcoxon rank tests, \( P \) values = 0.003; P. somniferum and P. setigerum, width \( P \) = 0.005). The number of cells also present differences between species (Fig. 1, Kruskal–Wallis tests number cells: \( \chi^2 = 233.21, \text{df} = 7, P < 10^{-16} \)) and between samples of P. setigerum. P. argemone is clearly the species with more cells. P. nigrum is also different from the other species of the P. somniferum group. P. somniferum and P. setigerum and consequently the archaeological seeds are very close regarding this criterion.

The first two PCs (Fig. 2) gathered 84\% of the total shape variation. Shape changes along PC1 (46\%) are related to roundness while changes along PC2 (38\%) correspond to an asymmetry component between the two parts of the seed. Asymmetry mostly represents intraspecific variability. It is higher for the species with the most rounded seeds (P. setigerum and P. somniferum).

The permutational MANOVA (df = 6, F = 32.126, adj. \( r^2 = 0.42, P = 0.001 \)) showed differences in shape between taxa. The species with the most elongated seeds (P. argemone and P. hybridum) are clearly distinguished from the other species with proportionally rounder seeds. Shape overlapping is particularly important between P. setigerum, P. somniferum, P. nigrum both on PC1 and PC2.

The hierarchical clustering performed on the euclidean distance matrix computed on the coefficients averaged per taxa confirmed the shape proximity between P. setigerum and P. somniferum, as well as between P. rhoeas and P. dubium and, in the other branch, P. hybridum and P. argemone. (Fig. 3). The slight differences in shape between the species of the P. somniferum group occur in part surrounding the hilum (Fig. 4 and Fig. 1 in Supplementary material).

Identification of modern seeds. The linear discriminant analyses (LDA) on modern material allowed a good identification at the species level. The percentage of accuracy identification using the cells and size ranged between 67 and 73\% for two taxa (P. dubium and P. rhoeas) but for the other five taxa it was above 80\% (Fig. 5).
Figure 2. Principal component analysis performed on shape coefficients. The first two components are shown here gather 84% of the total shape variability. Archaeological seeds (red dots) are added as supplementary individuals, i.e. reprojected, on this biplot.

Figure 3. The unrooted tree obtained with hierarchical clustering on the Euclidean distance matrix between Fourier coefficients averaged per taxa.
**Figure 4.** Mean shapes pairwise comparisons for all *Papaver* taxa studied here. Orange colour corresponds to the taxon of the rows and the blue colour to the taxon of the columns.

**Figure 5.** Benchmarking of linear discriminant analyses on all species and using different proxies. Accuracy per classes and their variability were obtained using 100 permutations on classes-balanced dataset with the error bars.
Although the performance of individual variables (length, width, shape and cell number) provided relatively good discrimination, the best percentages were obtained when all traditional and shape parameters were combined (Fig. 5: 73–100%).

The results were similar if we considered only the species from the *P. somniferum* group with two taxa and three taxa (Figs. 2 and 3 in supplementary material). Combining all variables, more than 87% of the seeds were correctly identified to their specific taxon (Supplementary data Fig. 2).

**Assignation of archaeological seeds.** Length and width showed considerable variation between modern seeds of *Papaver* and archaeological seeds (Fig. 1). The width of the archaeological seeds of Zurich-Parkhaus Opéra is closer to modern *P. setigerum* seeds while the length is intermediate between *P. setigerum* and *P. somniferum* modern seeds (Fig. 1). The LDAs using various sets of descriptors were used to infer species on the archaeological material. The archaeological seeds from Zurich-Parkhaus Opéra were identified as *P. setigerum* and *P. somniferum* (see Fig. 6A). Some seeds were identified as *P. nigrum* only when using one of the descriptors: number of cells (3%) and shape (21%). When all criteria are combined, no seed is allocated to *P. nigrum*. In every case, and more especially when all criteria are combined, about half of the seeds are attributed to *P. setigerum* and a half to *P. somniferum*.

**Discussion**

Here we show that the combined application of morphometric descriptors, number of cells and shape analysis outline elliptic Fourier transforms (EFT) allows the discrimination of seven modern species of *Papaver* genus.

In spite of a high positioning error, due to the small size of this material, morphometrics can be done. The various species and sub-species are well discriminated, which validates the methodology used (Table 1). Indeed, the most interesting result was that by using this method, with all descriptors, the LDA gave optimal results when distinguishing between *P. setigerum* and *P. somniferum* as well as when compared to other *Papaver* related modern seed species. The second finding of this study was that allow for the first time the application of this method to archaeological seeds.

**Phenotypic variation among modern seeds.** The first question that this study sought to answer was if it is possible to discriminate between the different taxa. The LDA results (Fig. 5) show that it is possible to distinguish the taxa with high accuracy results by using all the descriptors or the combination of the number of cells and size.

According to our results, adding the cell number to the size descriptors gives a better prediction for *P. dubium, P. rhoesas* and *P. somniferum* (Fig. 5). These are also the taxa where the shape yielded the lowest additional
Papaver species can be used for this type of analysis.

In our results, 18 seeds were attributed to P. setigerum and 15 seeds to P. somniferum. This may be interpreted in two ways: the population at Zurich-Parkhaus Opéra is a mixture of wild and domestic forms belonging to a population in an intermediate stage of domestication, or we have a fully domestic form with some wild individuals still surviving as weeds in the fields. This opens several scenarios to explain the process of domestication of poppy. There is evidence of the use and potential cultivation of opium poppy in the Western Mediterranean since ca. 5600 BC, according to the finds at the lake village of La Marmotta, Italy (ca.5620–5480 Cal BCE)\(^{35}\) and in several other sites, for instance, at the pile-dwelling site of La Draga, Spain, ca. 5200 BC\(^{3,8}\). The authors of both archaeobotanical studies actually suggest that opium poppy was cultivated, based on the number of finds and their ubiquity\(^{3,35}\). Nevertheless, it is possible that the plant was still morphologically wild since these sites fall within the native area of P. setigerum and isolation of the cultivated population would have been more difficult. Thus, it is unclear if opium poppy spread northwards as morphologically wild, not fully domesticated, or as a domesticated plant. It is actually possible that opium poppy arrived at the Alpine foreland as a domesticated form along with some wild P. setigerum forms as weeds.

The domestication process could have been accelerated with the beginning of cultivation of opium poppy outside of the area of the natural distribution of the wild subspecies such as in the Alpine foreland around 4300 BC\(^{3,8}\). After ca. 1100–1200 years of cultivation of opium poppy in this region, the Zurich-Parkhaus Opéra seeds seem to indicate that the plant is still in the process of being domesticated. This may be interpreted as an indication of a protracted domestication process\(^{3,8}\), as observed with other plants domesticated in the Neolithic period. In order to test this hypothesis, similar analyses should be performed on opium poppy seeds from archaeological sites located in the Western Mediterranean region.

Another critical factor is that some of the seemingly early finds of opium poppy seeds outside of P. setigerum natural zone are not dated. Early deposits found in Israel\(^{37}\) but also in central Europe\(^{38,39}\) lack radiocarbon dates on the seeds or on direct contexts where the seeds were found. New efforts on dating these seeds and their contexts should be made before interpreting the route of cultivation/domestication\(^{3,4}\). Future studies on poppy seeds should integrate the morphometric as well as the direct dating approaches\(^{36}\). Likewise, it is foreseen to attempt to obtain aDNA from the archaeological seeds and so confirm, if possible, their status as domestic or cultivated.

**Conclusions**

The present paper provides the first results of geometric morphometrics for Papaver taxa. The combination of descriptors such as the number of cells, size and shape of different modern species of Papaver allows to classify the seeds with good accuracy despite the methodological challenge due to the small size and globoid shape of poppy seeds. The classification model from the modern species used to assign archaeological seeds recovered at the late Neolithic site of Zurich-Parkhaus Opéra was also successful as it did attribute them to either P. setigerum and P. somniferum. The seeds were actually distributed within these two subspecies in equal parts, which might suggest that the plant has not yet acquired the morphometric characteristics of modern domestic seed. Further studies should be done in order to test the classification model. Future research should consider the study of
opium poppy seeds from historical periods to confirm their assignation to the domesticated subspecies, as well as the study of earlier Neolithic finds in the Western Mediterranean in order to trace the pace of the domestication process.

Methods

Archeological material. One archaeological case included in the AgriChange Project40 was used in this paper: Zurich-Parkhaus Opéra (ZHOPE) located in Zurich, Switzerland is a Neolithic lake-dwelling site. A total of 33 whole and well-preserved uncharred waterlogged seeds (with visible cells) identified as *P. somniferum*41 were used. All seeds were obtained from the sample 12015.1B in layer 13 dated by dendrochronology between 3176 and 3153 BC (middle Horgen Culture42). Zurich-Parkhaus Opéra was excavated during the construction of subterranean parking in 2010 and 2011. Located in the northern shore of the Lake Zurich, eight settlement phases were identified and dendro-dated to 3234–2727 BC. In this late Neolithic site, archaeological deposits related to pile-dwelling houses are preserved in a waterlogged state where thousands of plant remains are present in charred and uncharred states, especially in layer 13, large quantities of opium poppy seeds were found concentrated mostly within building limits41.

These analyses were non-destructive and therefore no special permissions were required. Permission for the use of the archaeological seeds of Parkhaus Opéra for this study was granted by the scientific director of the project, Dr. Niels Bleicher (Office for Urbanism Zurich). Permission for the use of modern seed reference material was granted by the Graineterie of the National Museum of Natural History (MNHN) and no permission was necessary for the use of our own seed collection of the Integrative Prähistorische und Naturwissenschaftliche Archäologie (IPNA/IPAS).

Data collection. All *Papaver* seeds were photographed from a lateral view, with the hilum to the right. In this angle, it shows the cells, including those close to the hilum (Fig. 7A). The broader part of the seed at the bottom. The background of the seeds was a white surface to ease further background removal. The photos were made using Leica Z16 APO Binocular Stereo Microscope with a digital camera Leica DFC 420 and Leica Application Suite software (LAS 4.0, Leica), creating one mounted photo from several single photos that are stacked together to give depth of field to the seed and enable the counting of the number of seed cells. The background of the photo was removed manually using Photoshop 6 (Adobe) as well as the yellow soft tissue on the hilum part, in both archaeological and modern individuals (Fig. 7B). Then a mask (a black shape over a white background) was created using Photoshop (Fig. 7C). In order to normalise the outlines before elliptic Fourier transforms (EFT), coordinates of five landmarks were recorded using ImageJ43. The position of the landmarks was chosen in order to be the most reproducible as possible: two landmarks were positioned at the top and bottom extremes of the seeds and three around the hilum part (Fig. 7D). The landmark points covered most critical biological traits, from seed length (ldk: 4–5) to the hilum arch (ldk:1–3).

Outline analysis. Seed shape was analysed using outline analysis based on elliptic Fourier transforms (EFT) using Momocs 1.3.044 in a R 4.0.0 environment45. The elliptic Fourier transforms is a progressive decomposition of the outline (x; y) coordinates into a series of trigonometric functions called harmonics, associated with coefficients, used as quantitative shape variables. Here, outlines were normalised for their position, size and orientation using full generalised Procrustes alignment46. Landmark n°2 was used as the initial point for each outline. Then EFT was calculated from 360 points equally spaced along the curvilinear abscissa, and two landmarks (4 and 5) were extracted on each image. Based on harmonic power, five harmonics were retained and gathered 95% of the total harmonic power; more details on EFT can be found in Bonhomme et al.44.

Measurement error. The poppy seeds are small and round and thus difficult to position in a specific orientation under the stereomicroscope. To minimise the error and aid with its reproducibility, some precautions were taken: the use of the same protocol, the same equipment, a single operator (RS) took the photos, a single operator (AJ) did all the cleaning and landmarking. As a preliminary step, all measurements were tested for the overall reproducibility. We used analyses of variance (ANOVA) following Claude34. The percentage of measurement error (%ME) is defined as “the ratio of the within measurement component of variance on the sum of
the within- and among-measurement component. A set of five photos from three different taxa from the \( P. \) \textit{somniferum} group (\( P. \) \textit{setigerum}, \( P. \) \textit{somniferum} and \( P. \) \textit{nigrum}) were used in three different tests. The position test compares five photos of the same 15 seeds of the three different taxa by one single operator (RS). The cleaning and landmarking tests compared the repetition of the same action of digitalising cleaning and landmarks on the same photos (same 15 photos, same three species, three times).

**Phenotypic variation among species.** The size (length and width of the bounding box) of the seed was recorded using the rectangular tool in ImageJ. The number of cells was counted for every seed using the multi-point tool in ImageJ. Length and width of the seeds were log-transformed. Distributions of seed lengths, widths and cell number values were illustrated using boxplots. For each univariate variable (length measurements, cells number), overall differences were tested using Kruskal–Wallis non-parametric rank tests for multi-group comparison and Wilcoxon’s tests between each pairs of species.

To explore the overall shape variability, we used a principal component analysis (PCA) on the full matrix of Fourier coefficients and added the archaeological seeds as supplementary individuals. The first two principal components (see Results) were used as synthetic shape variables.

Then we used the coefficients on the first five harmonics in a permutational MANOVA using the package vegan, to test for differences between taxa. A hierarchical clustering using UPGMA on the euclidean distance matrix between coefficients averaged per taxa is presented as an unrooted tree obtained with the package ape.

To benchmark the performance of the different descriptors (width, length, number of cells, shape) at identifying species, we used linear discriminant analyses (LDA) provided by the package MASS. Different combinations were used: first to all modern species, then only to \( P. \) \textit{somniferum} group (\( P. \) \textit{setigerum}, \( P. \) \textit{somniferum} and \( P. \) \textit{nigrum}) and finally only to \( P. \) \textit{setigerum} and \( P. \) \textit{somniferum}. To cope with unbalanced group sizes between sets due to the repeated \( P. \) \textit{setigerum} and \( P. \) \textit{somniferum}), we used random sampling of the over-represented classes so that they all sum up to 30. The process was repeated for 1000 permutations. To compare the model performances, we obtained the percentages of specimens correctly classified by using leave-one-out cross-validation. To visualise mean species shapes, we averaged Fourier coefficients and reconstructed seed outlines for each taxon.

**Archaeological identification.** Each archaeological seed was classified using the “predictive” linear discriminant analyses trained on the modern material. For each seed, the dominant classification obtained along the 100 permutations was considered as the predicted class. The archaeological seeds were classified within three taxa of \( P. \) \textit{somniferum}: \( P. \) \textit{nigrum}, \( P. \) \textit{setigerum} and \( P. \) \textit{somniferum} first, and after only classified within \( P. \) \textit{setigerum} or \( P. \) \textit{somniferum}. All descriptors (length, width, shape and number of cells) were used.

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Author contributions
F.A. and L.B designed the research and share senior authorship of the paper; R.S. took all photographs; A.J. generated the data; A.J., VB and R.S. prepared the figures; A.J., R.S., S.I., A.E. performed error measurement tests and analysis; V.B. wrote the R script for the analysis and AE did the R script for the Error analysis; A.J., VB, A.E., L.B., S.I. validated and analysed the data, F.A. provided the funding; F.A and A.S. supplied the samples. All authors contributed to the final manuscript.

Competing interests
The authors declare no competing interests.

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