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BRIEF REPORT

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Three-dimensional X-ray-computed tomography of 3300- to 6000-year-old *Citrullus* seeds from Libya and Egypt compared to extant seeds throws doubts on species assignments

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Societal Impact Statement

The watermelon (*Citrullus lanatus* subsp. *vulgaris*) is among the world's most important fruit crops. We here use C-14 dating and morphometric analysis to test whether ancient seeds can be identified to species level, which would help document food expansion, innovation, and diversity in Northeastern Africa. We dated a Libyan seed to 6182–6001 calibrated years BP, making it the oldest *Citrullus* seed known. Morphometric analysis could not reliably assign ancient seeds to particular species, but several seeds showed breakage patterns characteristic of modern watermelon seeds cracked by human teeth. Our study contributes to the understanding of the early history of watermelon use by humans, who may have mostly snacked on the seeds, and cautions against the use of morphology alone to identify *Citrullus* archaeological samples.

KEYWORDS
Citrullus, colocynth, Egyptian tombs, micro-CT scanning, seed shape, watermelons

1 | INTRODUCTION

With a production of 161 million tons in 2019, watermelon (*Citrullus lanatus* subsp. *vulgaris*) is among the world's most important fruit crops (FAO, accessed 4 May, 2021). Based on the geographic occurrence of all its wild relatives (Chomicki & Renner, 2015), watermelon originated in the African continent. Molecular phylogenies show plants from Sudan as the earliest branches within *C. lanatus*, pointing to northeast Africa as the most likely region of its domestication (Guo et al., 2019; Renner et al., 2021; Wu et al., 2019). Molecular phylogenies alone, however, do not reveal the full history of when and how humans began to use a certain plant or animal. Instead, phylogenies are starting points for further exploration through archaeological, linguistic, and population-genetic research (Paris, 2015; Prendergast et al., 2019). Archaeological research on the domestication history of watermelons has focused on the seeds, the thick, hard coats of which preserve well and which are found at settlements where people either ate the fruit pulp or the seeds themselves or may have used the bitter oil extracted from *C. colocynthis* seeds (Akbar, 2020; Cox & van der Veen, 2008; van der Veen, 1995, 1996, 2011; Wasylikowa & van der Veen, 2004).

Besides *C. lanatus* and *C. colocynthis*, the genus *Citrullus* comprises five other species, of which at least two are also used by humans. One is *C. mucosospermus* (or *C. lanatus* subsp. *mucosospermus*), which is a crop in West Africa where its soft-coated seeds are used in so-called egusi soups or stews (Achigan-Dako et al., 2015; Oyolu, 1977; Prothro et al., 2012). The other is *C. amarus*, the pulp and thick fruit rind of which are boiled with sugar to make jam (Laghetti & Hammer, 2007) and which is also a source of animal fodder (McGregor, 2016). The other three species in the genus—*C. ecirrhosus*, *C. naudinianus*, and *C. rehmii*—are not used by humans (authors of all taxonomic names are in Table 1).

The earliest seeds assigned to *Citrullus* come from the Libyan archaeological site Uan Muhuggiag in the Tadrart Acacus range, occupied by pastoralists during the early to mid-Holocene (van der Veen, 1995). The layer in which the seeds were found was radiocarbon dated to 7784–7545 and 8162–7520 calBP (calibrated years before the present), but stratigraphic evidence suggested that it had been disturbed and contained younger intrusive material (Wasylikowa & van der Veen, 2004; the paper mentions several seeds of *C. colocynthis* and a single seed of *C. lanatus*, but more seeds of both species were found later). Other seeds, dated to 1500–1480 BC, came from the area of the Aswan Dam Reservoir (Lake Nubia; Van Zeist, 1983) but appear to have been lost (S. Blomsma, Groningen Institute of Archaeology, pers. comm. to SSR on 30 March 2017). *Citrullus* seeds have also been found at Amara West (desert site 2-R-65) in Northern Sudan, dated to 3455–3373 calBP (P. Ryan, Royal Botanic Gardens, Kew, pers. comm. to SSR of 12 Nov. 2019) and by H. Carter and P.E. Newberry in 11 baskets in Tutankhamun's tomb at Thebes in Egypt, mixed with *Ziziphus* seeds and clearly intended as a snack (http://www.griffith.ox.ac.uk/gri/carter/369-p1626.html). The tomb has been dated to about 3300 BP (Ramsey et al., 2010). Some of the seeds from basket number 527a are kept at Kew (accession H. Carter 4.1965), others in the Dokki Agricultural Museum in Cairo (accession 4198; reproduced in Warid, 1995). When found, "they were still in remarkably good condition as flat seeds about 8 mm (5/16 in) long. When fresh, they would have been lightly roasted and eaten by cracking the brittle outer cover with the front teeth" (Hepper, 2009, p. 56).

The identification of the seeds found at all these archaeological sites naturally has been based on drawings, photographs, and comparison with modern seeds. These identification efforts, however, were limited by (i) incomplete sampling of the seeds of the extant species, some of which, such as *C. rehmii*, were only discovered or recognized as species in the 1990s or later; (ii) the difficulty of describing and comparing three-dimensional (3D) shapes; and (iii) preservation artifacts or damage caused by humans that have resulted in broken seeds (Cox & van der Veen, 2008). In contrast to linear measurements on real objects or photos, 3D morphometrics can unveil morphological variation that otherwise remains hidden, and such analyses have proven particularly useful for archaeological samples because complete shapes can be reconstructed even with fragmented material (e.g., Bonhomme et al., 2017; Caracuta et al., 2016). Analyses via micro-X-ray-computed tomography (micro-CT) of modern and ancient seeds therefore may allow more reliable distinction of the seeds of colocynths, watermelon, and other wild or semidomesticated species of *Citrullus* (above) and thereby shed light on the early uses and domestication of watermelons. In this study, we apply this technique to seeds from Uan Muhuggiag and Tutankhamun's tomb, as well as modern *Citrullus* seeds representing all species except the soft-seeded West African *C. mucosospermus*.

The specific question we wanted to answer was if morphometric analysis based on micro-CT would reliably distinguish *C. lanatus* and *C. colocynthis*, which both have been reported from Uan Muhuggiag and other sites in North Africa (Kahlheber, 2004; Nixon et al., 2011; van der Veen, 1995; Wasylikowa, 1993; Wasylikowa & van der Veen, 2004). Distinguishing between these species has implications for the uses of plants in North Africa during the Neolithic period because of their highly divergent properties (above). The difficulty of distinguishing these species' seeds led at least one scholar to the idea that the sweet watermelon originated in India and was brought to Africa during the Islamic period, based on the view that all pre-Islamic African *Citrullus* seeds might represent *C. colocynthis* (Watson, 1983).

2 | MATERIALS AND METHODS

2.1 | Plant material and herbarium vouchers

Fossil seeds collected at Uan Muhuggiag were provided by K. Wasylikowa from the Palaeobotanical Museum of the W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków. The presumed *colocynthis* seeds came from Samples 14 and 25, and the presumed *lanatus* seeds from Sample 6 as listed in Wasylikowa (1993). Seeds collected by H. Carter and P.E. Newberry in Tutankhamun's tomb at Thebes, Egypt, and identified as *C. lanatus*, were lent to the Royal Botanic Gardens, Kew in 1932 for study by Leonard Boodle, and remain on deposit in Kew's Economic Botany Collection (de Vartavan, 1999). Modern seeds were purchased commercially or obtained from germplasm collections or colleagues working in Oman and Sudan. Herbarium vouchers and numbers of seeds used for micro-CT scanning are shown in Table 1. The taxonomy follows Renner et al. (2017).

2.2 | Radiocarbon (C-14) dating

One of the Uan Muhuggiag presumed *C. lanatus* seeds from Sample 6, identified by K. Wasylikowa, was sent to the Laboratory of Ion Beam Physics, ETH Zurich (lab code ETH-102414), where its radiocarbon age was calculated using the measured ^{14}C content after correction for

TABLE 1 Species, sources of material, herbarium vouchers, and letters corresponding to Figure 1 where they are shown

Species	Age	Source	Determined by whom and when	Herbarium voucher	No. seeds; letter used in Figure 1
Citrullus amarus Schrad.	Modern	Bought commercially	S.S. Renner and G. Chomicki, 2017	S.S. Renner and M. Silber 2866 (M)	20 seeds, n
Citrullus colocynthis (L.) Schrad.	Modern	Oman, Sharqiyah Sand Desert, 22°15′492″N, 58°46′522″E	A. Patzelt, 2017	A. Patzelt 4872 (M)	20 seeds, l
	Ancient	Libya, Uan Muhuggiag from Sample 14 (UMA14)	Wasylikowa and van der Veen, 2004[a]	Not applicable	2 seeds, e
	Ancient	Libya, Uan Muhuggiag from Sample 25 (UMA25)	Wasylikowa and van der Veen, 2004[a]	Not applicable	1 seed, 2 fragments, h–j
Citrullus ecirrhosus Cogn.	Modern	Namibia, USDA Griffin, GRIF 16056	S.S. Renner, 20 I 6	S.S. Renner 2855 (M)	9 seeds, o
Citrullus lanatus (Thunb.) Matsum. & Nakai	Modern	Bought commercially from Rareplants.eu		M. Silber 19 (M)	40 seeds, k
	Ancient	Egypt, desiccated seed from Tutankhamun tomb (accession H. Carter 4. 1965)	P. Newberry, sometime after 1891	Not applicable	5 seeds, a–c
	Ancient	Libya, Tadrart Acacus Mts., Uan Muhuggiag Sample 6 (UMB6)	Wasylikowa and van der Veen, 2004[a]	Not applicable	3 seeds, 2 fragments, d, f, g
Citrullus lanatus subsp. *vulgaris* var. *cordophanus* (Ter-Avan.) Fursa	Modern	Sudan, North Darfur, Al Fashir (Al Fasher), 13°37′N, 25°21′E	S.S. Renner, 2017	M. Sir El Khatim 1 (M)	19 seeds
Citrullus naudinianus Hook.f.	Modern	Bought commercially from Rareplants.eu	S.S. Renner, 2018	J.-L. Gatard s.n. (M)	8 seeds, m
Citrullus rehmii De Winter	Modern	Bought commercially from Rareplants.eu	S.S. Renner, 2017	S.S. Renner and M. Silber 2864 (M)	8 seeds, p

[a] Wasylikowa and van der Veen, 2004. An archaeological contribution to the history of watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai (syn. *C. vulgaris* Schrad.). Vegetation History and Archaeobotany 13: 213–217.

standards, blank values, and fractionation (δ^{13}C values were measured semi-simultaneously on graphite). There were too few presumed *C. colocynthis* seeds for C-14 dating, because several were needed for CT scanning. The reported conventional age in years BP (before 1950 AD or CE) was calibrated to a calendar age using OxCal Version 4.3.2 (Bronk Ramsey, 2017) and the IntCal13 atmospheric curve (Reimer et al., 2013).

2.3 | Micro-CT

Image analysis of modern and fossil watermelon seeds was performed using micro-CT. Seeds were mounted untreated in batches inside pipette tubes filled with acryl foam (Staedler et al., 2013), an approach that reduces artifacts and facilitate downstream analysis (Staedler et al., 2018). Micro-CT scans were performed using an XCT-200 system (Zeiss Microscopy) at the Department of Botany and Biodiversity Research of the University of Vienna. X-ray detection was performed via scintillator crystals (Zeiss Microscopy) that convert X-rays into visible light. The light was then focused (Nikon optical lens) and detected via a charge-coupled device (CCD) camera. We used 25 to 35 kV from an L9421-02 microfocus X-ray source from Hamamatsu electronics (Dataset S1 shows all scanning conditions). XMReconstructor (XRadia Inc.) was used to perform 3D reconstruction from raw scan data.

2.4 | Landmark placement on digital 3D surface models

Resulting DICOM datasets were imported into Amira Software Version 5.4.5 (FEI Visualization Sciences Group, Burlington MA, USA). Datasets were converted to Amira files (*.am) and visualized using the Volren module in Project View. Datasets were then cropped to contain a maximum of four seeds using the Crop Editor. To view specimens in XYZ planes that are comparable to standard biological longitudinal (XZ, YZ) or cross (XY) sections, datasets were transformed to biologically relevant orientations (along planes of seed symmetry) for the largest seed using the Transform Editor for the largest seed (Mode: extended, Preserve: voxel size, Padding Value: 0). Excess blank space resulting from transformations was removed, along with standardization of voxel sizes (4.3234 × 4.3234 × 4.3234 μm) and XYZ coordinates (0, 0, 0) using the Crop Editor.

The Segmentation Editor was used to create "outer" (exotesta) and "inner" (endotesta) label fields for the largest seed from each dataset. For segmentation, the Magic Wand tool along with Segmentation → Remove Islands, Fill Holes, was used when sufficient contrast was present. For lower contrast regions and/or datasets, the Blow Tool and Paintbrush were used. Labels were smoothed using Smooth Labels (Size: 6, 3 dimensions). Surfaces were generated from smoothed label fields using the SurfaceGen module in the Project Editor. All micro-CT datasets and seed 3D surface models shown in Figure 1 have been deposited in MorphoSource under project id 0000C1170 (https://www.morphosource.org/projects/0000C1170?id=0000C1170&locale=en&page=2#media).

Landmarks ($N = 32$) were chosen to accurately represent seed shape in both planes of symmetry. They were placed on specimen surfaces using a Landmarks Object (one set). The OrthoSlice module was used to find seed midsection slice numbers in XY and YZ planes, and then a Surface Cut module was used to cut surfaces at chosen midsections and place landmarks. For fossil datasets with incomplete/partial specimens, missing landmarks were recorded for manual NA entries during post-processing. Landmark sets for each dataset were saved as *.landmarkAscii files from Amira and converted to non-proprietary text files for analysis (File S1).

2.5 | Geometric morphometric analysis

Landmark sets were combined to a single file containing all fossil and modern datasets formatted like a 3D TPS file (*.tps) with a row for landmark number (ex: LM = 3) and identifier (ex: ID=C_colo_foss). TPS-formatted Landmark files were then imported into R Version 4.0.2 (R Core Team, 2014) and analysis was carried out using the packages geomorph Version 4.0.0 (Adams & Collyer, 2018) and Morpho Version 2.8 (Schlager, 2020). For landmark pairs across planes of symmetry, missing landmarks for fossil specimens were estimated using geomorph fixLMmirror in cases where one landmark was present and could be mirrored to provide the missing landmark coordinates (see Section 4 for limitations of assuming symmetry for botanical specimens). For the remaining missing fossil landmarks, we used thin-plate-spline interpolation with Morpho fixLMtps, with generalized procrustes alignments using geomorph gpagen to retain shape variation but eliminate statistical differences resulting from scale, translation, and rotation (Bookstein, 1992). Principal component analysis (PCA) was then performed on aligned datasets using geomorph gm.prcomp to quantify shape variation among seeds. Figures were created using geomorph plotting functions in R (geomorph.prcomp.plot, plotRefToTarget, plotOutliers; accompanying code available at github.com/aubricot/Citrullus_3D_GMM), with point and cluster labels manually added in Adobe Photoshop CS6.

3 | RESULTS

3.1 | C-14 dating

A presumed *C. lanatus* seed from Uan Muhuggiag (identified as this species by K. Wasylikowa, but not included in our morphometric analysis because it was instead used for C-14 dating) was dated to 5313 uncalibrated years BP or 6182–6001 calBP.

3.2 | Geometric morphometrics

The visualized ancient and modern *Citrullus* seeds with their landmarks are shown in Figure 1. The inclusion of five highly fragmented seeds (Figure 1d,f,i,g,h) substantially increased variation in the PCA

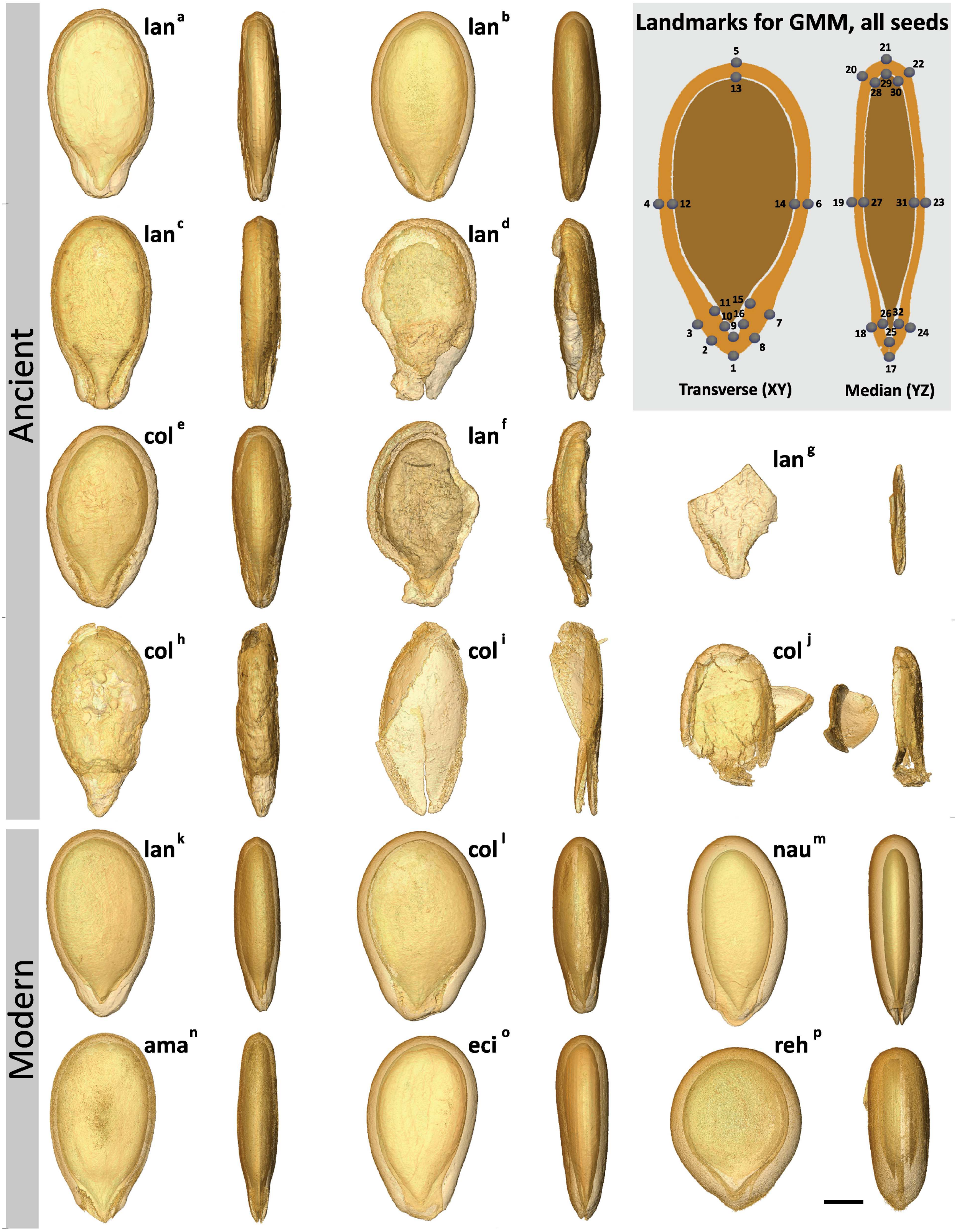

FIGURE 1 Visualized ancient and modern *Citrullus* seeds and the landmarks used for geometric morphometric analysis (GMM). For ancient seeds (top), all datasets are shown. For modern seeds (bottom), one seed per species is shown. Fragmented seeds d, f, and g of *C. lanatus* show possible evidence of human seed consumption. Seeds d, f, i, g, and j were excluded from GMM analysis because they were too fragmented. Key to species abbreviations: ama (*amarus*), col (*colocynthis*), eci (*ecirrhosus*), lan (*lanatus*), nau (*naudinianus*), reh (*rehmii*). Scale bar represents 1 mm. Visualized using SurfaceView (Draw Style: Fancy Alpha, Base Trans: 0.62) in Amira version 5.4.5 (FEI Visualization Sciences Group, Burlington MA, USA)

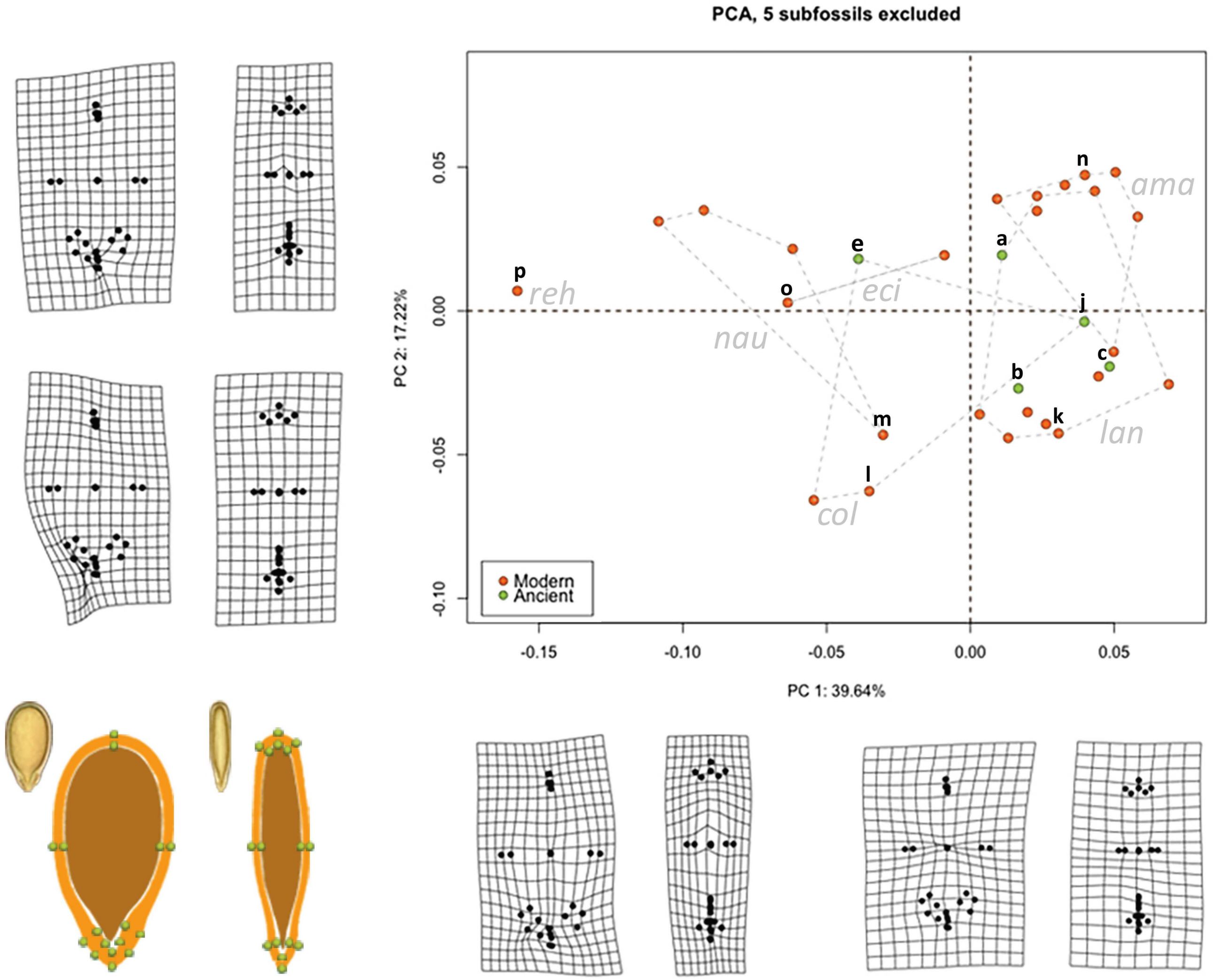

FIGURE 2 Principal component analysis (PCA) of *Citrullus* species. Ancient and modern seeds. Thin-plate-spline (TPS) deformation grids show shape changes along principal components 1 and 2 on transverse and median planes. Shape changes represent the difference between mean and min(−)/max(+) shape for each axis. Polygons are minimum convex hulls for each species. Principal components 1 and 2 represent 39.64% and 17.22% of the cumulative variance between watermelon seed shapes. Two clusters are present. The left cluster contains *C. colocynthis* ($n = 3$), *naudinianus* (4), and *ecirrhosus* (2). The right cluster contains *C. lanatus* ($n = 12$), *amarus* (7), and *colocynthis* (1). Lettered labels correspond to seeds in Figure 1. Key to species abbreviations: ama (*amarus*), col (*colocynthis*), eci (*ecirrhosus*), lan (*lanatus*), nau (*naudinianus*), reh (*rehmii*). Five ancient seeds were excluded as outliers because they were too fragmented to precisely estimate missing geometry. For a plot indicating outliers that were removed, see Figure S2

morphospace (Figure S2 and File S1), and they were therefore excluded in our final PCA (Figure 2). With these seeds excluded, principal components 1 and 2 represent 39.64% and 17.22% of the cumulative variance between watermelon seed shapes (Figure 2). Two overlapping clusters are apparent, one comprising three of four included *C. colocynthis* seeds (including one ancient seed from Uan Muhuggiag, e in Figure 1) along with all *C. naudinianus* and *C. ecirrhosus* seeds and the other comprising all *C. lanatus* seeds (including those from Tutankhamun's tomb), all *C. amarus* seeds, and one ancient *C. colocynthis* from Uan Muhuggiag (j in Figure S1). *C. lanatus* and *C. amarus* overlap almost completely. *C. rehmii* (p in Figure 2) is clearly distinct from all other species in our PCA and by simple visual inspection (Figures 1 and S2). The unusual ancient *C. colocynthis* seed (j) was not a statistical outlier (Figure S2) but is highly fragmented, and its placement within *C. lanatus* in our PCA plot may be due to landmark estimation error (compare Figure 1a,b,c,e,j and Figure 2). Notably, due to its fragmented condition, it also may not initially have been properly identified using morphology.

Several *C. lanatus* seeds showed breakage patterns that may point to their consumption as a masticatory item (compare figure 7 in Cox & van der Veen, 2008 with the seeds in Figure 1d,f,g).

4 | DISCUSSION

Our paper provides C-14 dating for the oldest *Citrullus* seeds yet identified. These seeds were collected at Uan Muhuggiag in Libya in layers that were previously radiocarbon dated to 7784–7545 and

8162–7520 calBP (Wasylikowa, 1993; Wasylikowa & van der Veen, 2004). Based on the results of this study, these ancient seeds, some originally identified as *C. lanatus* and others as *C. colocynthis* (Wasylikowa, 1993; Wasylikowa & van der Veen, 2004), cannot reliably be identified to species. Nevertheless, that they were found within a human settlement and sometimes show traces of cracking from human teeth (our Figure 1d,f,g) suggests that they were actively collected and that *Citrullus* may have been cultivated by the early to mid-Holocene. The cracked seeds, suggesting their consumption as a snack, match the baskets with mixed *Ziziphus* and *Citrullus* seeds found in Tutankhamun's tomb at Thebes.

With the exception of the soft- and large-seeded West African taxon lacking from our sampling (*C. mucosospermus*), the sizes of *Citrullus* seeds per se do not allow unambiguous species assignments. For instance, *C. colocynthis* seeds from Uan Muhuggiag measure 6.3 (5.6–7.1) × 3.8 (3.1–4.4) mm in size (10 measurements; Wasylikowa & van der Veen, 2004), but as these authors point out, these sizes fall within the range of variation of both *C. colocynthis* and *C. lanatus*, so are not diagnostic. More recent seeds of *C. lanatus* also exhibit a considerable size range (Van der Veen, 2011: figures 3.18 and 3.19: maximum seed length range of Late Roman seeds: 8–13.3 mm; maximum seed width range of Late Roman seeds: 5–7.3 mm).

Our morphological analysis was hampered by small sample sizes (Table 1), which was due to *C. ecirrhosus*, *C. lanatus* var. *cordophanus*, *C. naudinianus*, and *C. rehmii* not being in cultivation. We tried to grow these species, self-fertilize them, and thus achieve fruit set, but without success. *Citrullus* seeds are not available from herbarium specimens because of obvious issues with drying and pressing mature watermelon fruits. Nevertheless, we were able to include seeds of more species than any previous archaeological study (none of which included taxa other than *C. lanatus* and *C. colocynthis*; Cox & van der Veen, 2008; Nixon et al., 2011; van der Veen, 1995, 1996, 2011; Wasylikowa & van der Veen, 2004; Ziyada & Elhussien, 2008). Our images also provide the first 3D "cyber specimens" of *Citrullus* and are available in an open-source repository for future study.

The use of micro-CT scanning on archaeological samples is debated with regard to the damage it may cause to the DNA that can remain in sub-fossilized tissues. Certainly, if within-species variation is large enough, relying on photographs for an initial analysis is one option (e.g., following the approach of Bonhomme et al., 2017). However, if, as in our case, there is little to differentiate species, micro-CT scanning is a good alternative as it allows landmark placements that capture the full 3D morphology of the sample. Also, physical specimens only need to be handled once, and the resulting cyber specimens can be infinitely reused for downstream measurements without damaging archival material. A test with mammal bones showed minimal DNA damage from standard micro-CT imaging with low dosage and brief exposure as used here (Immel et al., 2016), although we know of no tests with plant material yet.

Geometric morphometrics with paleontological specimens often face the dilemma of either retaining partial (broken) material and possibly introducing bias or instead excluding such material and losing valuable information. We estimated missing seed fragments by mirroring across axes of symmetry. Such estimates have limitations because botanical specimens are rarely perfectly symmetrical. Even so, geometric morphometrics has leveraged symmetry for the analysis of flowers (Savriama, 2018; Savriama & Klingenberg, 2011) and fossils (Dumoncel et al., 2016). Although we generated surface models of all seeds studied, we used landmarks placed on these surfaces as an approximation of overall 3D morphology. Using landmarks alone is less computationally intensive than comparing entire 3D surfaces and is standard procedure for geometric morphometric studies to date. With more computing power available in coming years, new automated approaches to compare entire meshes present an exciting opportunity to find nuanced differences with less introduced bias from landmark selection (Boyer et al., 2015, 2018; Gao et al., 2018; Gonzalez et al., 2016; Renaud et al., 2018). Methods for shape estimation also will continue to improve with more widespread use of micro-CT imaging, collection digitization efforts, and advances in machine learning technology (Dumoncel et al., 2016; Schlager et al., 2018; Senck et al., 2013). For the time being, however, an unambiguous assignment by image analysis of fossil seeds to *C. amarus*, *C. colocynthis*, or *C. lanatus* is not yet possible, and this contributes to the difficulty of fully understanding watermelon use and domestication. Solutions will require integrating collections-based phylogenomics with archaeological records, ancient DNA, and even the genomics of relevant traits (Chomicki et al., 2020; Paris, 2015; Renner et al., 2021).

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AUTHOR CONTRIBUTIONS

KAW performed all 3D analyses, GC and SSR conceived of the study, YMS contributed expertise in micro-CT analysis, KW contributed material and archaeobotanical expertise, MN contributed material and archaeobotanical expertise, JS provided access to CT scanner, and SSR contributed material and expertise; all authors worked on the final version of the MS.

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