sulphides can have different Os isotope compositions. Care must therefore be exercised in interpreting whole-rock PGE and Re/Os data without detailed information on these sulphides. ☐

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Starch grains reveal early root crop horticulture in the Panamanian tropical forest

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Native American populations are known to have cultivated a large number of plants and domesticated them for their starch-rich underground organs[1]. Suggestions[2,3] that the likely source of many of these crops, the tropical forest, was an early and influential centre of plant husbandry have long been controversial[4-6] because the organic remains of roots and tubers are poorly preserved in archaeological sediments from the humid tropics. Here we report the occurrence of starch grains identifiable as manioc (*Manihot esculenta* Crantz), yams (*Dioscorea* sp.) and arrowroot (*Maranta arundinacea* L.) on assemblages of plant milling stones from preceramic horizons at the Aguadulce Shelter, Panama, dated between 7,000 and 5,000 years before present (BP). The artefacts also contain maize starch (*Zea mays* L.), indicating that early horticultural systems in this region were mixtures of root and seed crops. The data provide the earliest direct evidence for root crop cultivation in the Americas, and support an ancient and independent emergence of plant domestication in the lowland Neotropical forest.

The site (Ag-13) is located on the Pacific coastal plain of Central Panama approximately 17 km from the sea[7]. Today the region receives 1,600 mm of precipitation annually, distributed on a highly seasonal basis, and has a mean annual temperature of 26 °C. The potential vegetation is a deciduous tropical forest. Excavations carried out in 1973–75 and 1997 uncovered the remains of three distinct human occupations. Zone D, the oldest, is a yellow (inside the dripline) or red (beyond the dripline) silty clay on top of weathered bedrock. It has uncalibrated ^{14}C dates of 10,725 ± 80 BP (NZA-10930), 10,529 ± 184 BP (NZA-10930), 8,423 ± 79 BP

Table 1 Number and types of starch grains on milling stones from the Aguadulce Shelter

Catalogue number	Manioc	Maize	*Dioscorea* spp.	Arrowroot	Other	Total *n*
Zone C, bottom						
Block 3 (42)	8	1	3	0	2	14
Block 2 (26b)	1	2	0	0	0	3
Block 3 (38)	0	1	0	0	0	1
1S3E (439)	0	7	0	0	0	0
Zone C, middle						
Block 2 (26a)	5	15	2	2	5	29
Zone C, top						
1S4E (350)	0	25	16	0	13	54
Zone B, bottom						
Block 2 (23)	0	4	0	0	0	4
Zone B, middle						
1S6E (519)	0	3	0	0	9	12
Zone B, top						
3W1N (243)	0	7	0	0	0	7
ON0E (402)	0	7	0	0	0	7
1S5E (316-1)	0	3	0	0	0	3
3W7N (318)	0	2	0	0	1	3

Block/geographic orientation indicates the excavation unit. Field catalogue number for each artefact is given in parentheses. All artefacts except tool 26a, a boulder milling stone base, are edge-ground cobbles. Artefacts with a block number are from the 1973–1975 excavations, and those with a geographic orientation are from the 1997 excavations. The single bell-shaped grain from tool 26b is very likely to be from manioc.

(NZA-9625), 7,061 ± 81 BP (NZA-9624) and 5,560 ± 80 BP (UCR-3462), all direct accelerator mass spectrometry (AMS) dates on sediment phytoliths[8,9]. The 5,560 ± 80 BP date was run on a very small sample that was recovered in a soil column directly underneath a larger sample dating to 6,910 ± 60 BP. The 5,560 ± 80 BP date is therefore rejected and Zone D is considered to date from about 11,000 to 7,000 BP because it held a chipped stone industry characterized by bifacial reduction that is present in Panama only during the Paleoindian (about 11,000 to 10,000 BP) and Early Preceramic (about 10,000 to 7,000 BP) periods[10]. No milling stones were present.

Zone C, a dark brown clayey silt with angular rock, has two AMS ^{14}C phytolith dates of 6,910 ± 60 BP (UCR-3417) (cited above), at its base, and 6,207 ± 60 BP (NZA-10929) stratigraphically higher. A terminal date is estimated to be 5,000 BP. The initiation of this zone marks a great increase in the intensity of human occupation and the rate of sediment accumulation. As in other sites from the region dated to this period, bifacial reduction disappears from the chipped-stone industry, which is now characterized by expedient core reduction and bipolar reduction[10]. Milling stones, called edge-ground cobbles because the grinding facet is located on the narrow edge of the stone, first occur near the base of this zone, together with boulder milling stone bases. On the basis of use wear and replicative studies, this distinctive tropical forest tool complex may have been used to process roots and tubers[11,12]. Faunal remains, including animal bone and shell, are encountered for the first time in Zone C and are abundant and well preserved in sediments underneath the overhang. Ceramics are absent.

Zone B, a tan silt with much less angular rock than Zone C has a single AMS phytolith date of 4,250 ± 60 BP (UCR-3418), and is marked by the introduction of 'Monagrillo' pottery, the earliest found in Central America[13]. The zone is dated from about 5,000 to 3,000 BP, on the basis of ^{14}C dates from the type-site, the Monagrillo shell midden, and others containing this ceramic tradition in Panama[13]. Milling stones from this zone are similar to those recovered from Zone C, but tend to be more heavily worn.

Preliminary analysis of milling stones from the first set of excavations in 1973–75 indicated that they contained starch grains from a variety of plants[14]. However, the sample size was small, we lacked sediments that had encased the tools in the ground to confirm that the tools were a primary source of the grains, and we had a limited selection of modern comparative starches to identify domesticated plants. The 1997 excavations were designed to increase the sample of milling stones and starch and to study the context and chronology of archaeological starch grains in more detail by recovering tools in a pristine condition, together with sediments that were in immediate proximity to the artefacts. We developed a protocol for the collection and analysis of archaeological starch grains, and a key for starch grain identification based on a considerably enlarged modern reference collection (see Methods). Our descriptions and measurements of grains from domesticated and wild taxa correspond to those reported in other large studies[15–19] and indicate that manioc, yams, arrowroot and maize can be distinguished.

A total of 18 edge-ground cobbles and one boulder milling stone base were analysed. Of these, 12 contained starch residue (Table 1). We recovered 13 bell-shaped starch grains with combinations of attributes that only occur in manioc roots from an edge ground cobble (tool 42) and the boulder milling stone base (tool 26a). The starch grains had rounded and unbanded pressure facet edges, centric and open hila, and no demonstrable lamellae. They often had crossed, winged to y-shaped, or stellated fissures. One of these grains, with pressure facets located on the distal end in the form of a corona with five to eight indentations and a stellated fissure, is a highly idiosyncratic form unique to manioc (Fig. 1a). It occurred on tool 42, which was found near the base of Zone C and should date to nearly 7,000 BP. Eight of the other bell-shaped grains on tool 42 and 26a had crossed, winged to y-shaped, or stellated fissures. Such combinations of fissures on bell-shaped grains with centric hila and without lamellae are unknown in species other than manioc[15]. The remaining four bell-shaped grains had no or simple fissures. These also occur in lower frequencies than do the other bell-shaped grains in modern manioc, demonstrating that grain type distributions in the archaeological samples correspond to modern distributions. The identification of a domesticated *Manihot* is further supported by the fact that starch grains in its closest living wild relative *Manihot esculenta* spp. *flabellifolia* (Pohl) Ciferri, which is native to Brazil[20], exhibit substantial morphological differences (much narrower pressure facets, simple fissures and mostly eccentric hila), and smaller sizes (Fig. 1b) (Table 2).

Ovoid starch grains with a cuneiform-shaped depression,

Table 2 Size of starch grains in domesticated plants and close wild relatives compared with archaeological grains

Modern plants	Taxonomic status	Range and s.d. of mean length (μm)	Range (μm)	n
Manihot esculenta ssp. *flabellifolia*	Wild ancestor of *M. esculenta*	8 ± 2.1	4–12	50
Manihot esculenta	Domesticated	13 ± 2.6 to 16 ± 3.4	6–28	600
Panamanian Poaceae (small-grained, 30 species)	Wild	3 ± 0.9 to 11 ± 2.7	2–18	800
Panamanian Poaceae (large-grained, 5 species)	Wild	14 ± 2.9 to 20 ± 4.8	8–30	250
Zea mays spp. *parviglumis**	Wild ancestor of *Z. mays*	8 ± 2.1 to 9 ± 2.1	4–16	200
Zea mays	Domesticated	8 ± 1.5 to 16 ± 4.9	4–24	750
Dioscorea spp.	Wild	17 ± 4.9 to 63 ± 20	8–100	550
Dioscorea trifida	Domesticated	37 ± 8.3 to 48 ± 12.8	24–84	100

Archaeological grains	Tool number	Mean length	Range	n
Manihot esculenta (all Layer C)	42	14	10–24	8
	26a	18	16–20	5
	26b	18	–	1
Manihot esculenta	Layer C combined	17 ± 3.7	10–24	14
Zea mays, Layer C	42	18	–	1
	26a	15	10–24	15
	26b	13	12–14	2
	439	17	10–22	7
	350	18	10–24	25
	38	14	–	1
Zea mays	Layer C combined	16 ± 4.1	10–24	51
Zea mays	Layer B combined	15 ± 4.9	10–22	26
Dioscorea spp. (all Layer C)	42	15	14–16	3
	26	42	24–60	2
	350	36 ± 7.3	30–52	16

Range of mean length of starch grains in modern plants is the range of means in the populations/races of each species studied. s.d. is the standard deviation for grains in the populations/species having the smallest and largest grains. Range is the range for all starch grains sized in these species. The five wild Panamanian Poaceae (large-grained) with starch grains that demonstrate size overlap with maize (*Cenchrus*, *Anthephora*, *Orthoclada*, *Panicum* spp.) have morphologies distinct from maize.
* Four different populations of *Zea mays* spp. *parviglumis* were studied.

eccentric hila, and lamellae diagnostic of tubers from *Dioscorea* spp. were recovered from these two preceramic tools (42 and 26a), and from another (tool 350) retrieved from the top of Zone C that is probably younger in age than the other two (Fig. 1c). Grains from tools 42 and 26a are morphologically like those of modern wild *Dioscorea* species from Panama, whereas the straight distal edges, morphological uniformity and size of the grains from tool 350 make them identical to those of *D. trifida*, the American domesticated yam. An unidentified, free-growing species of yam from Panama possesses very similar grains, precluding a positive identification of *D. trifida*. Differences in the *Dioscorea* starch residues indicate, however, that at least two species of yams were exploited during the preceramic period. The final tuber starch identified on preceramic artefacts is that of arrowroot (*Maranta arudinaceae*), today a minor root crop of the tropical forest, which possesses diagnostic ovoid grains with eccentric hila and proximal fissures.

Starch grains from grass kernels identified as maize, and which in modern maize are characteristic of races with harder endosperms (such as pop/flint corns), were also found on tools from deep levels in Zone C. As with the manioc grains, the starch assemblage morphologic profiles, presence of individual grains diagnostic of maize (Fig. 1d), and grain size all indicate maize, not wild Poaceae. The same types of maize starches present in Zone C occur in Zone B, which may indicate the persistence of varieties with non-floury endosperms in the ceramic-phase subsistence economy at Ag-13.

The sequence of steps used to analyse the tools and sediments produced the following outcomes (see Methods). Step 1 resulted in starch recovery only from the grinding and/or pounding facets of the tools. In the case of the tools from the 1997 excavations where closely associated sediment was analysed, every starch grain except one was recovered in Steps 1–3, showing that sediments sampled from the immediate proximity of the tools but that were not firmly stuck to artefacts, were nearly devoid of starch. Step 5 yielded only a single starch grain, further indicating that starch preservation in the sediments is very poor, and no starch was recovered from the analysis of four non-artefactual stones and associated sediments.

It appears that starch grains at Ag-13 had a higher probability of survival when they were lodged inside small crevices and cracks on the surfaces of artefacts and, thus, derived protection from decay. Starch research in these contexts opens up a new avenue of palaeoethnobotanical investigation in the Neotropical forest. Formerly, root crop cultivation was indirectly inferred by the presence of edge-ground cobbles and other artefacts that were

thought, on the basis of replicative studies of tool use or an ethnographic analogy, to be used for tuber processing[11,12]. We cannot rule out the possibility that some of the starch grains on the artefacts represent secondary deposition from the site's sediments after the tools were discarded. Nevertheless, the patterns demonstrated by the starch frequencies on tools and in sediments suggest that the tools were used to process the plants identified. Comparing starch in soils and tools has been used in the Old World as a measure of artefact use[21].

The starch remains from tuber crops and maize support phytolith and pollen evidence from the Aguadulce Shelter and lake cores in Panama for the presence of well-developed agricultural systems using a variety of crop plants by 7,000—6,000 BP[22,23]. The Aguadulce starch grains represent the earliest recorded occurrence of manioc in the Americas. Manioc, a staple food in the tropics, was previously thought to have been domesticated in both Mesoamerica and South America, but recent botanical and molecular studies indicate a South American origin[23], particularly a region of southwestern Brazil that supports a vegetation of seasonally moist tropical forest[20]. Manioc pollen grains retrieved from two sites in Belize dated to 4,700 BP may represent the spread of this cultivar into Mesoamerica[24]. Arrowroot's area of origin is unclear, but the seasonally moist forest of northern South America is a likely candidate[23]. Our data indicate that prehistoric societies in tropical America domesticated and dispersed manioc and arrowroot by 7,000–6,000 BP, supporting an early transition from foraging to food production using root crops in the lowland Neotropical forest[2,3,23,25]. □

## Methods

Archaeological field research

Laboratory research

Step 1: Unwashed grinding stones were examined under a stereoscopic microscope at a power of ×100. The point of a fine needle was inserted into cracks and crevices on the grinding facets, pounding edges and non-utilized surfaces of the artefacts to loosen and remove any residue. The residue was mounted in water on a slide and examined with polarized and unpolarized light at a power of ×400. 10–13 locations covering the utilized and non-utilized facets of tools were examined in this manner. Step 2: Tools were washed with a brush underneath running water, and starch was removed from the sediment using techniques described in detail elsewhere[26] (such as addition of a heavy metal liquid at a density of 1.8 made from a solution of caesium chloride, CsCl). Step 3: Washed tools were shaken in an ultrasound for five to ten minutes to further dislodge adhering sediment and starch, and starch was then isolated using CsCl, as in Step 2. Step 4: 10–20 cm^3 of sediment sampled from beneath and around the peripheries of tools while the excavations were in progress was analysed for starch content, as in Step 2. Step 5: 24 sediment samples from two stratigraphic columns (from excavation squares 1N6E and 1S5E) were analysed for starch content as in Step 2. Four non-artefactual angular rocks that were a natural component of the site and closely associated sediment were sampled from the bottom to the top of the excavations and were analysed using steps 1–4. All archaeological starch grains possessed the starch-specific extinction cross when examined under cross-polarized light.

Starch grain identification

We used a modern reference collection of 170 species from 36 families of plants. It comprises: (1) most domesticated plants known or thought to have been used in pre-Columbian Panama and close wild relatives: (2) non-cultivated species used as foodstuffs (for example, Fabaceae, Marantaceae, various tree fruits); and (3) plants commonly used for medicinal, craft and ritualistic purposes in local indigenous economies today. Included were 12 different populations of manioc and two populations of *Dioscorea trifida* (domesticated yam) grown by local farmers, 15 races of Latin American maize, the putative wild ancestors of maize (*Zea mays* spp. *parviglumis*) and manioc (*Manihot esculenta* ssp. *flabellifolia*), and 48 wild species of Poaceae together with 12 wild species of *Dioscorea* from Panama. The numerous studies available on starch grain morphology were also consulted[15–19,27].

Our starch keys and classifications emphasize attributes demonstrated by these studies to be useful in identification: overall grain shape (spherical, bell-shaped, ovoid); contour
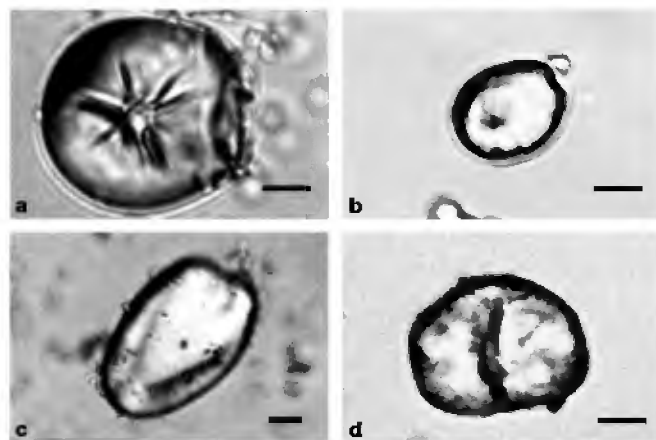
Figure 1 Various starch grains. **a**, A starch grain from manioc recovered from milling stone 42. **b**, A starch grain from the putative wild ancestor of manioc, *Manihot esculenta* spp. *flabellifolia*. **c**, A starch grain from a species of *Dioscorea* recovered from milling stone 350. **d**, A starch grain from maize from milling stone 42. This type is unique to maize and is found in Race Jala from Mexico. Scale bar, 5 μm (**a**, **b**, **d**); 10 μm (**c**).

and surface features; position and form of the hila (the botanical centre of the grain) and fissure; number and characteristics of pressure facets; and presence or absence of demonstrable lamellae. Manioc, arrowroot, *Dioscorea* spp. and maize contribute unique, individual grains (Fig. 1a, c, d). To confirm identifications of manioc and maize we used attribute combinations in a multiple grain analysis, which is the most conservative means to distinguish species because it provides population signatures for specific species and takes into account intra- and inter-species variation in grain attributes[15,16,19,27].

Starch grains in roots of modern domesticated *Manihot esculenta* are compound and predominantly bell-shaped with smooth and highly transparent surfaces, one to five unbanded pressure facets with rounded edges, hila that are centric and open, and no demonstrable lamellae. Many grains have fissures that are often crossed, winged to y-shaped, or stellated (Fig. 1a). This combination of characteristics distinguishes manioc from all other taxa, including the few others with bell-shaped starches such as *Pachyrrhizus* spp.[18], in which grains have banded and angled pressure facets, closed or semi-open hila, and no or simple fissures. Our results indicate that bell-shaped grains are primarily confined to subterranean organs of plants. Starch grains from maize kernels are predominantly spherical and smooth (flour corns) or mostly angular and four- to five-sided with a rough, grooved surface (popcorns and flint corns). In this and other studies[16,17,27], endosperm type is thus shown to exert considerable influence on starch morphology in maize. Our morphological and size determinations for different species incorporated sampling of multiple reference points within a single specimen, to account for within-sample variability.

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Bacteria have been found associated with a variety of ancient samples[1], however few studies are generally accepted due to questions about sample quality and contamination. When Cano and Borucki[2] isolated a strain of *Bacillus sphaericus* from an extinct bee trapped in 25–30 million-year-old amber, careful sample selection and stringent sterilization techniques were the keys to acceptance. Here we report the isolation and growth of a previously unrecognized spore-forming bacterium (*Bacillus* species, designated 2-9-3) from a brine inclusion within a 250 million-year-old salt crystal from the Permian Salado Formation. Complete gene sequences of the 16S ribosomal DNA show that the organism is part of the lineage of *Bacillus marismortui* and *Virgibacillus pantothenticus*. Delicate crystal structures and sedi-
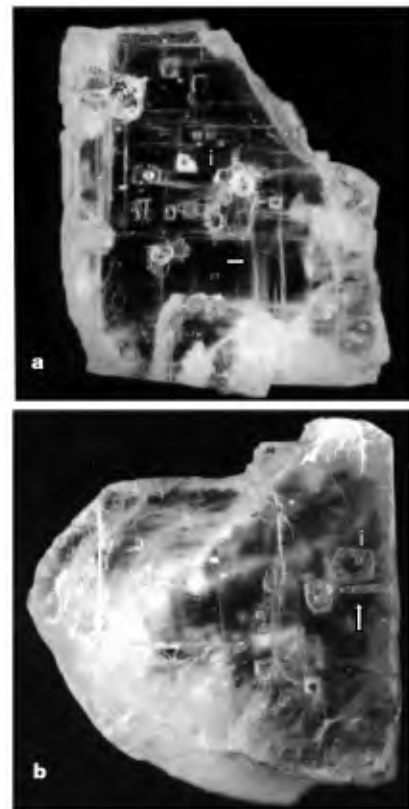
Figure 1 Halite crystals taken from the dissolution pipe at the 569 m (1850 ft) level of the Salado Formation (air intake shaft[18] for the Waste Isolation Pilot Plant, Carlsbad, New Mexico) in October 1998. **a**, This crystal was rejected for sampling. The crystal has numerous fracture lines (arrow) and misshapen inclusions with gas bubbles (i). This crystal measured 3.2 × 2.8 × 0.9 cm. **b**, The sample that yielded *Bacillus* strain 2-9-3. This crystal measured 3.5 × 3.5 × 2.5 cm. The inclusion that contained the bacterium (below i) measured approximately 3 × 3 × 1 mm (9 cubic mm). The drill hole made during sampling of inclusion B (Table 1) is visible above the arrow. The thickness of this crystal obscures some of the internal details (the drill hole for inclusion A) in the photograph.