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Journal of Archaeological Science 32 (2005) 1471–1480

Journal of Archaeological SCIENCE

http://www.elsevier.com/locate/jas

Morphological and genetic studies of waterlogged *Prunus* species from the Roman *vicus Tasgetium* (Eschenz, Switzerland)

Britta Pollmann, Stefanie Jacomet, Angela Schlumbaum *

Institute for Prehistory and Archaeological Science IPAS, University of Basel, Spalenring 145, CH 4055 Basel, Switzerland

Received 24 September 2004; received in revised form 23 March 2005

Abstract

Morphological and genetic studies were performed on waterlogged *Prunus* fruit stones from the Roman *vicus Tasgetium* (Eschenz, Switzerland). Some fruit stones could be identified to species level based on morphological and metric criteria. Other fruit stones found were not identifiable to species level. Of the latter, the morphological group *Prunus insititia/spinosa* represents either native sloe, cultivated primitive plum (damson) or a hybrid of both. In one out of 10 individual fruit stones of this group *Prunus* specific chloroplast *trn*L-*trn*F and nuclear ITS1 markers were verifiably amplified. Sequences of *trn*L-*trn*F led to the identification of *Prunus spinosa*. The presence of authentic DNA was confirmed by phylogenetically meaningful sequences from the archaeobotanical group *Prunus avium/cerasus* of the same sample using chloroplast *rbc*L and nuclear ITS1 DNA markers. The results demonstrate the utility of waterlogged plant remains for genetic analysis: as seeds of fruit trees are often preserved waterlogged in the archaeological record. Ancient DNA (aDNA) studies are a promising tool to answer archaeobotanical issues concerning early horticulture, which probably started in the Northern alpine region with the onset of Romanisation.

Keywords: Ancient DNA; *Prunus*; Archaeobotany; Waterlogged; Chloroplast DNA; Ribosomal DNA; Roman Period

1. Introduction

One of the most interesting subjects in archaeology is the history of agriculture and changes in human diet through time. The emergence and evolution of horticulture in the Northern alpine regions started with Romanisation. Major changes in social, economical, and agricultural structures are observed in the Roman provinces. Fruit-growing of walnut, wine, cultivated apple and pear, cherry, peach and plum is commonly assumed to have started North of the Alps during this time: their seeds and fruits are found abundantly and more frequently in all types of Roman settlements than in pre-Roman times [4,14,46].

However, based on traditional archaeobotanical research, it is in most cases not possible to establish if the fruits found in the archaeological record were grown locally or were imported as dried fruits. Written sources that mention horticulture in the Northern provinces of the Roman Empire are too rare to be conclusive. For example Pliny notes special cultivars of cherries grown in some regions of the Province Belgica and on the bank of the river Rhine (*Nat. Hist.* **15**, 103, 1st century AD). The cultivation of Prunoideae today still plays an economically prominent role in these areas.

The precise reconstruction of the agricultural history of these plants is difficult for several reasons. Firstly,

* Corresponding author.
E-mail addresses: britta.pollmann@unibas.ch (B. Pollmann), stefanie.jacomet@unibas.ch (S. Jacomet), angela.schlumbaum@unibas.ch (A. Schlumbaum).

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doi:10.1016/j.jas.2005.04.002

there is the ambiguity in the identification to species level, e.g. sloe (*Prunus spinosa*) versus damson (*Prunus insititia*) or sweet cherry (*Prunus avium*) versus sour cherry (*Prunus cerasus*). Secondly, the assignment to cultivars is morphologically impossible. Thirdly, in the archaeological record complete fruit stones necessary for morphological identification are found nearly exclusively under waterlogged conditions. This condition is rarely found but several Roman sites such as in Eschenz, CH, or Biesheim-Kunheim, F, with structures below the groundwater table, provide a rich source of fruit species [16,38,39].

One possibility to overcome the identification problem and to address further questions about the history of horticulture such as propagation and cultivar diversity in antiquity is the analysis of ancient DNA. The potential of ancient DNA studies for species identification, inference of origins, or domestication process has been shown [11,17,23,35,42]. In the case of investigating fruit-growing it is essential that DNA survives in waterlogged plant remains. Evidence for the survival of aDNA in waterlogged plant remains has been reported [35]. As an initial step to clarify the presence of DNA in waterlogged fruit remains, *Prunus* fruit stones are best suited: individual stones are large with a hard endocarp and are well represented at sites with waterlogged preservation.

Prunus species include wild *P. spinosa* and many cultivated agronomical valued species such as *P. avium*, *P. cerasus*, *Prunus persica*, *P. insititia* and *Prunus domestica*, represented by different cultivars.

Sloe, *P. spinosa*, is a wild tetraploid shrub widely distributed from West and Middle Europe to Asia Minor, the Caucasus region and North Africa [18]. In Middle Europe fruit stones of sloe are found abundantly in the archaeological record beginning in the Neolithic. A high size variability is seen [21,30,41]. Although *P. spinosa* grows naturally in deciduous forests and open or disturbed areas, its intentional use or cultivation by early societies cannot be excluded [18].

The primitive plum (*P. insititia*, damson, German: Haferpflaume) is not native to Middle Europe and was reported first from the Neolithic. The presence of primitive plums similar to *P. insititia* var. *juliana* is published from as early as Bronze Age in Switzerland [22]. However, the identifications of these early finds are highly controversial [30]. Single plum stones occur at excavations of the late pre-Roman Iron Age in Germany [31] and probably in France [47]. Large numbers of this type of primitive plum and furthermore of European plum (*P. domestica*) are identified at Roman and mediaeval sites and for Early Modern Times [1–3,5,28–30,45].

Another well-known phenomenon is the frequent interspecific hybridisation between wild *P. spinosa* and cultivated *P. insititia* and *P. domestica*, leading to a high number of intermediate forms of plants and fruits [30,34,48].

Archaeobotanical investigations suggest the presence of morphologically diverse fruits in antiquity, but the number of cultivars corresponding to the different morphological groups or the underlying genetic diversity is completely unknown. Only recently, with the availability of microsatellites, molecular genetic diversity in modern cultivars is studied, e.g. [12,44,49].

At the Roman *vicus Tasgetium*, excavated in Eschenz, Kanton Thurgau, Switzerland [8,9,24] thousands of morphologically well-preserved waterlogged *Prunus* fruit stones were found, providing an excellent opportunity to study. We identified *Prunus* fruit stones from *Tasgetium* using morphological criteria. Amplification of genetic markers of chloroplast *rbc*L and non-coding *trn*L-*trn*F intergenetic spacer and nuclear ribosomal ITS1 region, commonly used in molecular plant phylogeny and systematics were tested e.g. [7,15,40]. As an initial example fruit stones of morphologically ambiguous sloe/damson were assessed and fruit stones of sweet/sour cherry were used for authentication. We report here the successful amplification of chloroplast *rbc*L and *trn*L-*trn*F regions and nuclear ribosomal ITS1 region from waterlogged individual fruit stones of *Prunus* species from *Tasgetium*. The results provide a basis for answering the question of when fruit-growing started and how it was practised in the Northern alpine region by combining archaeobotanical and genetic analyses.

2. Material and methods

2.1. Archaeological information

The Roman *vicus Tasgetium* (Eschenz, Kanton Thurgau, CH) is located on the South bank of the river Rhine near the Lake of Constance. The settlement was close to the border of the Roman provinces Raetia and Germania Superior and to important trade routes running along and across the Rhine. The *vicus* has been dendrochronologically dated to the beginning of the 1st century until the end of the 3rd century AD. Rich plant assemblages, possibly representing rubbish and/or faeces, are preserved waterlogged in drains, sewers, cesspits and basins constructed of wood [8,9,24] (Fig. 1). Among other plant remains different *Prunus* species were found abundantly, indicating the importance of these fruits at the *vicus* [16,38]. The region around Lake of Constance is an important fruit-growing area until the present day.

2.2. Archaeological plant material

Our morphometric study included well-preserved waterlogged fruit stones of *Prunus* species excavated in

Fig. 1. Wooden basin (basin 1) and sewer in the Roman *vicus Tasgetium* (excavation Eschenz 1999.010), waterlogged preservation.

1999/2000 in what is thought to be the centre of the *vicus*. They are from several sewers/drains and from one wooden basin, containing faeces and rubbish (excavation 1999.010, samples 1, 2, 13, 32, 33, 38 [25–27,38]).

Ten waterlogged fruit stones of *P. insititia*/*spinosa* from one soil sample (sample 33, Eschenz 1999.010) of the wooden basin were selected for molecular analysis after their morphological analysis. In addition, 10 fruit stones of cherry (*P. avium*/*cerasus*) were selected from the same soil sample for authentication. The contents of the basin date to the second half of the 2nd century AD or later (numismatic dating). Soil samples containing plant remains were stored in airproof boxes at temperatures between 10 and 15 °C up to two years. After sieving of the archaeobotanical material, the fruit stones were analysed and then stored without exchange of oxygen in distilled water at 4 °C for almost one year.

2.3. Modern plants

Twigs and buds of *P. spinosa* and *P. insititia* var. *juliana* were obtained from the Botanical Garden Brüglingen (Basel, CH). The nomenclature used in this paper follows the Synonymie-Index ZDSF (http://www.cjb.unige.ch/rsf/deu/index/index.htm) and [45,48], in particular *P. insititia* = primitive plum = damson and *P. domestica* = European plum.

2.4. Morphological identification, measurements and analysis

Prunus fruit stones were identified according to established criteria [1–3,5,30,32,43] and with use of the reference collection at IPAS. The terms used for the morphological traits follow van Zeist & Woldring and Behre [5,45]. From measurements of thickness, length and breadth, we calculated index values which are commonly used for morphological discrimination between *Prunus* species of subfossil provenance [5,30] and also between modern cultivars [43]. In addition, the index value length^2/(thickness × breadth) was calculated which is well established to differentiate between fruit stones of modern cultivars [43]. Here we used the index values to characterise types of *P. insititia*, *P. spinosa* and the intermediate forms of *P. insititia*/*spinosa*. Cherries (*P. avium*/*cerasus*) were assigned to a taxon using morphological features. The data were analysed using the statistic program "jmp" (version 5, The Statistical Discovery Software TM).

2.5. DNA extraction

Ten fruit stones each of the groups *P. insititia*/*spinosa* and *P. avium*/*cerasus* from sample 33 of the excavation 1999.010 were treated as follows. Fruit stones were dried for two days in a desiccator. Through drying some stones cracked, so that the inside of the seed was clearly visible, separated from the wooden endocarp (Fig. 2). Each *Prunus* stone was ground in one Eppendorf tube using a Retsch mill (Schieritz & Hauenstein, CH). Two different extraction protocols were used for each taxon to support the presence of authentic DNA independent of the extraction protocol.

In the case of *P. insititia*/*spinosa*, the powder was incubated for 5 h with 1 ml prewarmed CTAB extraction buffer (2% (w/v) CTAB; 100 mM Tris/HCl pH 8.0; 20 mM EDTA pH 8.0; 1.4 M NaCl) at 60 °C with shaking in a hybridisation oven (GFL). After 3 min

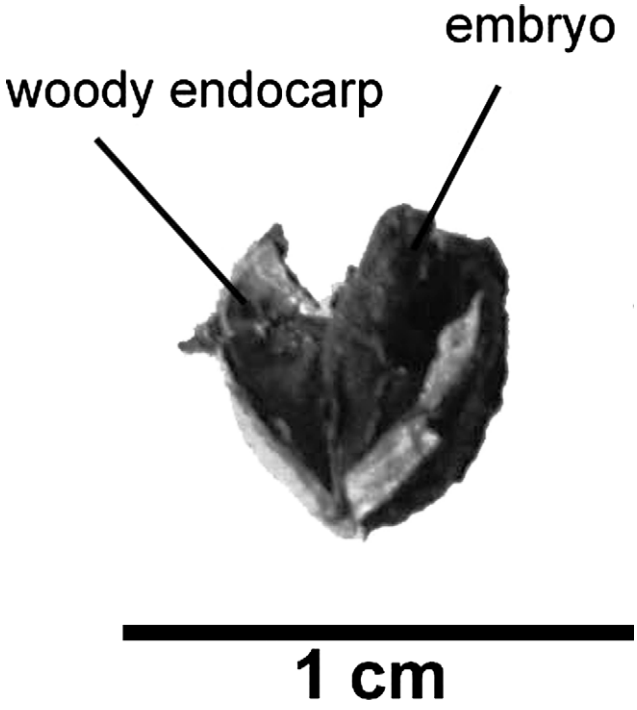

Fig. 2. *Prunus insititia/spinosa* of group ES V, waterlogged fruit stone (B 2) after drying in a desiccator, dehisced fruit stone, where fruit endocarp and seed can be distinguished.

centrifugation the supernatant was collected and an equal volume of 24:1 (v/v) chloroform:isoamyl alcohol was added. Supernatant and chloroform:isoamyl alcohol were mixed by vortexing and centrifuged 2 min at 10,000 rpm. Chloroform:isoamyl alcohol extraction of the aqueous layer was repeated three times. Two volumes of precipitation buffer (1% (w/v) CTAB; 50 mM Tris/HCl pH 8.0; 10 mM EDTA pH 8.0) was added to 1 volume of aqueous layer and incubated overnight at 4 °C. After 8 min centrifugation at 10,000 rpm, the supernatant was discarded and the DNA pellet was washed with 100% EtOH. The DNA was resuspended in 100 µl water.

In the case of *P. avium/cerasus*, a silica-based extraction protocol modified after Höss and Pääbo [19] was used. The silica suspension was prepared after [50]: to 6 g silica (Sigma), 50 ml Eppendorf water was added. This was shaken well and left standing overnight at room temperature. The supernatant (43 ml) was removed and 43 ml fresh Eppendorf water was added, suspension was shaken well and left standing for over 5 h. After taking off 44 ml, 60 µl 37% HCl was added and suspension was stored in the dark at 4 °C until further use. The ground fruit stone was incubated in 800 µl silica extraction buffer (10 M guanidine thiocyanate (GuSCn) (see comment in Ref. [20]), 100 mM Tris/HCl pH 6.4, 40 mM EDTA pH 8.0; 1.3% Triton X 100) for 3 h at 60 °C with shaking. After 5 min centrifugation the supernatant was collected and an equal volume of silica extraction buffer and 40 µl silica suspension was added.

The mixture was incubated for 10 min at room temperature with occasional shaking followed by 2 min centrifugation. The supernatant was discarded and the pellet washed twice with 500 µl washing buffer (10 M GuSCn, 100 mM Tris/HCl pH 6.4) with an incubation time of 10 min. Subsequently the pellet was washed twice with 500 µl 70% EtOH and once with 500 µl acetone without incubating. After 1 min centrifugation at 3000 rpm the supernatant was discarded and the pellet dried at least 20 min at 56 °C. DNA was eluted in 65 µl water.

2.6. PCR amplification and detection

Hotstart-PCR was carried out in 30 µl total volume including 3 µl aDNA extract (1:100 dilution), 1.25 U Amplitaq Gold Polymerase (Applied Biosystems) with commercial buffer containing 15 mM $MgCl_2$, 200 µM each dNTP and 0.3 µM of each primer (its 1 Fi/Ri, *trn*L-F F/R; Table 1).

Table 1
PCR primers used for amplification of chloroplast and nuclear DNA in Roman waterlogged *Prunus* stones

Primer code	Target size	Genetic region	Primer sequence	Specificity	Reference
rbcL F *rbcL* R	131 bp	*rbcL* (1)	ATG TCA CCA CAA ACA GAR AC AGG AST TAC TCG GAA YGC	Plant	Blatter et al. [6]
rbcL Fi *rbcL* Ri	120 bp	*rbcL* (1)	CCA CAA ACA GAR ACT AAA GC TTA CTC GGA AYG CTG CC	Plant	Blatter et al. [6]
trnL-F F *trnL*-F R	181 bp	*trnL-trnF* (2)	CGA ACA TCT TTG AGC AAG GA GAC TTG GGT CTA TGT CAA TTA	*Prunus*	this study
its1 F *its1* R	179 bp	*ITS1* (3)	GGA AGG ATC ATT GTC GAA ACC TG CAA GTT CCT TGG CGC AAT TCG C	*Prunus*	this study
its1 Fi *its1* Ri	156 bp	*ITS1* (3)	CGA AAC CTG CCT AGC AGA ACG AC CAA TTC GCG CCG GTG TTC GTT TG	*Prunus*	this study

(1) Large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (chloroplast DNA), (2) *trn*L-*trn*F intergenic spacer region (chloroplast DNA), (3) internal transcribed spacer 1 (ribosomal nuclear DNA).

Cycle parameters of the PCR were: 10 min at 94 °C, 71 cycles of 1 min at 94 °C, 1 min at 53 °C, 1 min at 72 °C; 5 min final extension at 72 °C.

In the case of the cherry stones nested PCR was carried out in 30 μl total volume with the following conditions: 5 μl aDNA extract (1:50 dilution) or 1 μl of the first PCR, 1.25 U Taq DNA polymerase (Roche) with commercial buffer containing 15 mM $MgCl_2$, 200 μM each dNTP and 0.3 μM of each primer (*rbc*L F/R and *rbc*L Fi/Ri, its 1 F/R and its 1 Fi/Ri; Table 1). Cycle parameters of the first and the nested PCR were: 1.5 min at 94 °C, 36 cycles of 1 min at 94 °C, 1 min at 53 °C, 1 min at 72 °C; 9 min final extension at 72 °C.

PCR products were electrophoresed using 1.5% Nusieve agarose (FMC BioConcept Allschwil, CH) and visualised under UV light.

2.7. Cloning, sequencing and sequence analysis

PCR products were either cloned (pGEM-T Vector Kit, Promega) and sequenced or directly sequenced (Microsynth, Balgach, CH). One to five clones of each PCR product were sequenced. PCR products of modern *Prunus* were directly sequenced. The sequences were submitted to Genbank with accession numbers: AJ809343, AJ810436, AJ810438–AJ810445. All sequences were aligned by eye and compared to entries in EMBL/Genbank database and to own sequences obtained from modern *Prunus* DNA that was amplified in a laboratory for modern DNA analysis in a different building and after finishing analysis of the aDNA (AJ810444, AJ810445). Numbering of sequences starts with the first base of the forward primer as the position 1.

2.8. Authenticity

Criteria have been established to verify the ancient origin of DNA in particular in relation to human remains [13,20]. Criteria for the authenticity of plant-derived aDNA are argued in Ref. [37] and, with particular emphasis on small plant samples where repeated extraction of the same sample is not possible in Ref. [6].

We used physically separated rooms for pre-PCR (one room for DNA extraction and one for PCR setup) and post-PCR steps (one room for PCR cycler and one main lab for analysis of amplified sequences and modern DNA) in two separate buildings. Pre-PCR precautions include dedicated equipment, disposable plastic ware, the use of commercial DNA-free water (Eppendorf) and the treatment of surfaces and equipment with bleach (Migros, CH). Plastic ware and solutions including the commercial water were exposed to at least 20 min UV irradiation. Unidirectional movement of experimentors was strictly followed.

Results obtained in this study were considered authentic if at least two different loci (chloroplast and/or nuclear DNA) were successfully amplified independently in one fruit stone. Sequencing results of each PCR product had to be phylogenetically meaningful and agree with the morphological group assignment. For independent reproduction of results, associated finds of *P. avium/cerasus* from the same sample were analysed with the same criteria.

3. Results

3.1. The morphological analysis of Prunus fruit stones

The six soil samples of the excavation Eschenz 1999.010 contained over 3500 *Prunus* stones and fragments. Almost 90% were fruit stones of *P. avium/cerasus*. The remainder includes 65% *P. spinosa*, 22% *P. insititia*, 5% *P. insititia/spinosa*, 4% *P. domestica*, 3% *P. persica* and 1% of what could be either *P. domestica* or *P. insititia*.

Fruit stones of *P. domestica*, *P. insititia*, *P. spinosa* and their intermediates from the excavation Eschenz 1999.010 were assigned to seven groups (ES I–VII) based on morphological criteria leading to the following identification: group ES I (*P. domestica*), groups ES II and III (*P. insititia*), groups ES IV and V (*P. insititia/spinosa*) and groups ES VI and VII (*P. spinosa*). Drawings of typical members of each group are shown in Fig. 3.

Three of these groups are detailed below: group ES II (*P. insititia*), group ES V (*P. insititia/spinosa*) and group ES VI (*P. spinosa*). These groups share some features and are described as follows:

Group ES II (*P. insititia*): stones are nearly oval with domed sides, a blunt base and a pointed apex. The ventral ridge is well developed. The surface is moderately pitted and weak, short creases radiate from the base. The stones of group ES II are similar to modern stones of *P. insititia* var. *juliana*.

Group ES V (*P. insititia/spinosa*): stones are obovate with a blunt base and a distinct pointed apex. Longitudinal creases start at the base and can reach to half of the length of the stone. The ventral ridge is weakly developed. The surface is moderately pitted.

Group ES VI (*P. spinosa*): stones are fairly round, the apex and the base are domed and the ridge at the ventral side is weakly developed. Stones show few short longitudinal creases at the base and the surface is deeply pitted.

Group ES V shares morphological characters with both group ES II of *Prunus insititia* and group ES VI of

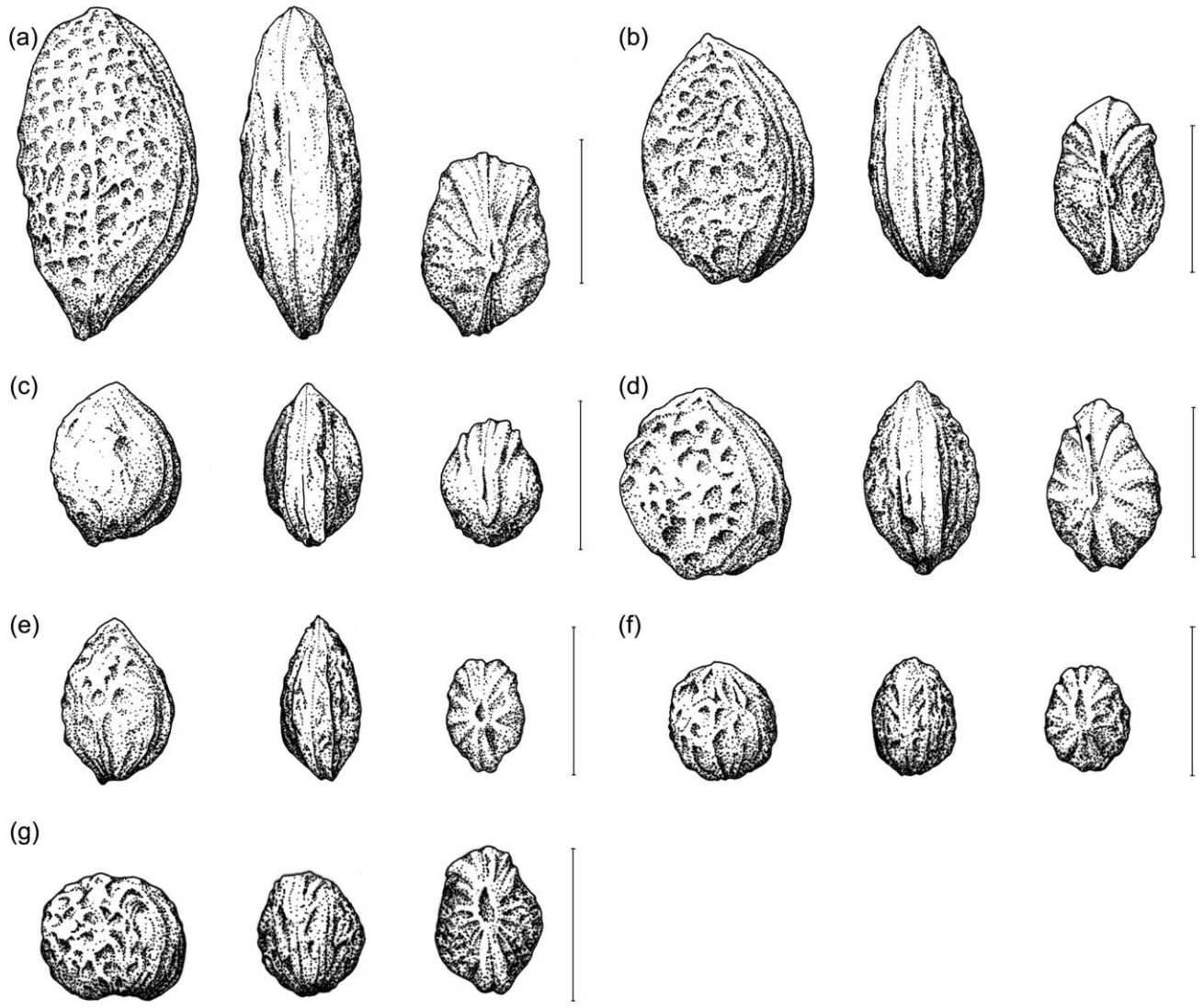

Fig. 3. Typical individual fruit stones from plum and sloe (types of fruit stones: groups ES I–ES VII) identified at the Roman *vicus Tasgetium* (Eschenz, CH), excavation Eschenz 1999.010; group ES I (a): *Prunus domestica*, group ES II (b) and ES III (c): *Prunus insititia*, group ES IV (d) and ES V (e): *Prunus insititia/spinosa*, group ES VI (f) and ES VII (g): *Prunus spinosa*, scale 1 cm.

P. spinosa. The similarity and overlapping of morphological traits between ES II, V and VI are clearly seen in the drawings (Fig. 3), in the measurements and in the index values (Table 2). The measurements of each group are normally distributed. The measurements of *P. insititia/spinosa* (ES V) are significantly different to both other groups, but more similar to those of *P. spinosa* (ES VI). The one-way ANOVA of the three groups ES II, ES V and ES VI with the index values show significant differences except for two combinations: ES V–ES VI with index T/B and ES II–ES VI with index T/L. This supports the intermediate position of group ES V.

Consequently group ES V could not be identified to species level. It was named *P. insititia/spinosa*. The identification relates to the ambiguity in morphological discrimination between the archaeological finds of sloe and plum, i.e. between wild and cultivated. The stones were subsequently analysed genetically.

The fruit stone A2 of *P. avium/cerasus* (before drying) was round with a weak developed ventral ridge. The hilum was fairly flat and large; at the dorsal site a small ridge could be observed. The measured dimensions of the cherry stone were: height = 9.1 mm, thickness = 8.9 mm, breadth = 7.4 mm. The other stones assigned to this group are similar but not identical. Although some authors describe typical morphological features of *P. avium* and *P. cerasus* stones (e.g. [32]) a clear identification of fruit stones from sweet and sour cherry by the morphological criteria is often difficult or impossible [1]. An identification of the fruit stone A2 and the others to species level was not possible.

Table 2
Mean and standard deviation of measurements and index values of plum and sloe stones found at the Roman settlement *Tasgetium* (today: Eschenz, CH), excavation Eschenz 1999.010; group ES II: *Prunus insititia*, group ES V: *Prunus insititia/spinosa*, group ES VI: *Prunus spinosa*

Group	ES II	ES V	ES VI
N	30	18	26
L (mm)	15.4 ± 1.5	9.3 ± 0.7	8.5 ± 0.9
T (mm)	10.6 ± 0.8	6.6 ± 0.3	7.3 ± 0.8
B (mm)	6.9 ± 0.4	4.7 ± 0.3	5.3 ± 0.4
$T/L \times 100$	69.0 ± 4.0	71.1 ± 4.3	86.3 ± 6.5
$B/L \times 100$	45.1 ± 3.6	51.0 ± 5.1	62.3 ± 5.7
$T/B \times 100$	151.9 ± 8.7	140.7 ± 8.6	140.5 ± 11.0
$L^2/(T \times B)$	3.2 ± 0.4	2.8 ± 0.5	1.9 ± 0.3

3.2. aDNA analysis of *Prunus insititia/spinosa* and *P. avium/cerasus* fruit stones

Amplifications of nuclear ribosomal internal transcribed spacer region (ITS1) and chloroplast intergenic spacer *trn*L-*trn*F from extracts of *P. insititia/spinosa* stones were attempted. Sequence polymorphisms of the ITS1 target region used here are specific for *Prunus*/Prunoideae, but are not species-specific. It distinguishes the species *P. spinosa* and *P. domestica* from *P. avium* or *P. cerasus*. The chloroplast *trn*L-*trn*F primers were designed spanning a transversion of "A" to "C" at position "317" which discriminates between *P. spinosa* with "A" (AF318683) and other *Prunus* species such as *P. domestica* (AF318666) with "C".

From nine out of 10 fruit stones of *P. insititia/spinosa* (ES V, stones A1–A6, B1–B4, soil sample 33) from the Roman *vicus* of *Tasgetium* a PCR product of the expected size of 156 bp of ITS1 was amplified. Dilution was necessary to overcome inhibition. The ITS1 sequences obtained from extracts of stone A1 and stone A6 are 100% identical to published *P. domestica* and *P. spinosa*, thus correctly affiliating with expected group. In agreement with the morphological identification, the stones are either sloe or plum (Table 3c).

In two of the extracts the *trn*L-*trn*F target (181 bp) was amplified (stones A4 and A6), confirming the presence of aDNA in stone A6, according to the criteria defined above. The sequences of *trn*L-*trn*F from both stones carry the "A" at position "317" diagnostic for *P. spinosa* (AF318683). It is thus clear that the female parent is *P. spinosa* and not any other *Prunus* including *P. insititia* (Table 3b).

Other mutations compared to the reference were detected. Out of three *trn*L-*trn*F clones of the Roman

Table 3
Details of the DNA sequence alignments from PCR products of different *Prunus* species

a: *rbc* L

	pos 63	
AF227904	g	*P. spinosa*
AF227903	.	*P. insititia*
AF227900	.	*P. cerasifera*
l01947	c	*P. domestica*
AJ809343	**c**	***P. avium/cerasus* fruit stone A2**
AF206813	c	*P. persica*

b: *trn* L-*trn* F

	pos 205	227	233	236	237	241+	252	253	291	317	
AF318683	t	t	c	g	t	-	t	t	t	a	*P. spinosa*
AJ810444	.	.	.	.	g	.	.	.	.	.	*P. spinosa*
AJ810440	.	.	.	**a**	.	.	.	.	.	.	***P. insititia/spinosa* fruit stone A6**
AJ810442	.	.	.	.	.	.	**-**	.	.	.	***P. insititia/spinosa* fruit stone A6**
AJ810441	.	.	.	.	.	.	**-**	.	.	.	***P. insititia/spinosa* fruit stone A4**
AJ810445	.	.	.	.	g	a	.	.	g	c	*P. insititia*
AF318666	.	.	.	.	.	.	.	.	.	c	*P. domestica*
AF318690	a	.	t	.	.	.	-	.	.	c	*P. avium*
AF318694	.	c	.	.	.	.	-	-	.	c	*P. persica*

c: ITS1

	pos 57+	63	64	71	73	75	76	81	82	83	86	87	89	90	91	100	101	106	114	115	116	117	120	127	128	132	133	142	
AF318730	-	a	c	c	g	c	t	g	t	g	c	c	c	g	t	c	t	g	g	t	t	g	c	a	g	g	c	a	*P. spinosa*
AJ810438	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	***P. insititia/spinosa* fruit stone A1**
AJ810439	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	***P. insititia/spinosa* fruit stone A6**
AF318713	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*P. domestica*
AF318729	g	.	k	t	t	-	g	t	c	-	t	g	-	-	-	.	.	a	r	.	.	.	t	.	a	a	-	t	*P. cerasus*
AF318737	g	.	t	t	t	-	g	t	c	-	t	g	-	-	-	.	.	a	a	.	.	.	t	.	a	a	-	t	*P. avium*
AJ810436	**g**	**.**	**t**	**t**	**t**	**-**	**g**	**t**	**c**	**-**	**t**	**g**	**-**	**-**	**-**	**.**	**.**	**a**	**a**	**.**	**.**	**.**	**t**	**.**	**a**	**a**	**-**	**t**	***P. avium/cerasus* fruit stone A2**
AF318741	g	g	.	y	c	-	g	t	c	-	t	g	.	-	-	t	c	.	.	c	g	c	n	g	.	a	-	g	*P. persica*

a: *rbcL* region (reference sequence: *P. spinosa* AF227904): comparison of ancient DNA of *P. avium/cerasus* (fruit stone A2, AJ809343) and modern *P. spinosa* (AF227904), *P. insititia* (AF227903), *P. cerasifera* (AF227900), *P. domestica* (l01947), *P. persica* (AF206813); b: *trn*L-*trn*F intergenic spacer region (reference sequence *P. spinosa* AF318683): comparison of ancient DNA of *P. insititia/spinosa* (fruit stone A4, AJ810441; fruit stone A6, AJ810440 and AJ810442) and modern *P. spinosa* (AF318683 and AJ810444), *P. insititia* var. *juliana* (AJ810445), *P. domestica* (AF318666), *P. avium* (AF318690), *P. persica* (AF318694); c: ITS1 region (reference sequence *P. spinosa* AF318730): comparison of ancient DNA of *P. avium/cerasus* (fruit stone A2, AJ810436) and *P. insititia/spinosa* (fruit stone A1, AJ810438; fruit stone A6, AJ810439) and modern *P. spinosa* (AF318730), *P. domestica* (AF318713), *P. cerasus* (AF318729), *P. avium* (AF318737), *P. persica* (AF318741).

P. insititia/*spinosa*, two clones from independent PCR's carry a poly T sequence of 10 bases, compared to the one clone and the entries of *P. spinosa*, *P. insititia* and *P. domestica*, which all have 11 T's (Table 3b, pos. 252). Chloroplast diversity in *P. spinosa* is high [36], and so far length variations at the poly T positions have been reported in the more distant *Prunus* species *P. avium* (10 T) and *P. persica* (9 T) (Table 3b, pos. 252, 253). Whether this finding has significance in relation to population diversity or is the result of polymerase slipping has to be further explored. Equally we did not further investigate sequence variations in clones of *trn*L-*trn*F as we are interested in species identification which depends on position 317 only.

The site "317" specific for *P. spinosa* was further confirmed by sequences of modern *P. spinosa* obtained by us (AJ810444, Table 3b) which is identical to *P. spinosa* AF318683, except at position 237 where it shares a "G" with *P. insititia* var. *juliana* (AJ810445). *P. insititia* var. *juliana* differs by three mutations from but shares the "C" at position "317" with *P. domestica* (AF318666). This confirms that the maternal parent of the Roman fruit stone is not *P. insititia* but *P. spinosa*.

The presence of authentic DNA in fruit stones from *Tasgetium* is further supported by sequences of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) and ITS1 regions obtained in one out of 10 extracts of fruit stones identified as *P. avium*/*cerasus*. Within the *rbc*L target region, sequences from Prunoideae so far published fall into two groups: e.g. *P. domestica* and *P. persica* differ at position "63" from *P. spinosa*, *P. cerasifera* and *P. insititia*. The Roman cherry *rbc*L sequence (AJ809343) is 100% identical to published sequences of e.g. *P. domestica* (L01947). This supports the survival of *Prunus* DNA, and corresponds to the morphological identification by excluding *P. spinosa*. Sequences of ITS1 are identical to the modern reference accessions of *P. avium* (AF318737) and *P. cerasus* (AF318729), and differ from *P. spinosa* (AF318730) and *P. domestica* (AF318713) (Table 3a and c). This is again the expected affiliation according to the morphology and confirms the authenticity of the sequences. From fruit stones of both morphological groups phylogenetically meaningful sequences were obtained.

4. Discussion

Little is known about the beginning of horticulture in the Northern alpine region and about the appearance of different cultivars. The first evident increase in diversity of cultivated fruits obviously took place with Romanisation. Famous Roman authors like Pliny and Columella reported on different cultivars and on contemporary propagation and grafting techniques (e.g. Columella, *Liber de arboribus* **8**;**9**;**25**;**26**; Pliny, *Nat. Hist.* **12**;**14**;**15**). Based on archaeobotanical results and historical sources the existence of many cultivars can also be assumed for mediaeval and Early Modern Times. The numbers and features of these cultivars are just as unknown as their importance in different periods and geographic regions. Archaeobotanists have attempted to methodically assess cultivar diversity in antiquity. Morphologically similar *Prunus* stones were grouped together (German: "Formenkreis") and compared with modern fruit stones of known cultivars. Limitations of this approach are mainly the imprecise classification of these groups, in some cases only small numbers of fruit stones are found, and the awareness that modern races and cultivars may not be identical to past fruits, e.g. [45]. Therefore it is often difficult or even impossible to identify different groups of archeological *Prunus* stones to species or cultivar level. Our morphological analyses reflected this problem as no assignment to a species level was possible, although the fruit stones were best preserved morphologically. In cases where the archaeobotanical approach is limited, DNA analyses are the obvious choice to address these problems.

We found authentic chloroplast and nuclear DNA in two waterlogged *Prunus* stones (morphologically identified as *P. avium*/*cerasus* and *P. insititia*/*spinosa*) from Roman *Tasgetium* even after two years of storage in distilled water without exchange of oxygen. These results corroborate the earlier report of ancient DNA from waterlogged Roman *Vitis vinifera* seeds [35]. They contrast with the perception that waterlogged preservation is less suitable for aDNA analysis, because hydrolysis is one major mechanism of DNA degradation [33]. One explanation for the preservation of ancient DNA in the *Prunus* fruit stones at *Tasgetium* is the strong fruit endocarp, consisting of hydrophobic lignified tissue that prevents microbial attack. Additionally, the slightly alkaline water conditions at the excavation site (measurements between pH 6.9 and 7.5, mostly pH 7.3) may explain the inhibition of DNA hydrolysis.

Based on chloroplast *trn*L-*trn*F, one fruit of *P. insititia*/*spinosa* was genetically identified as *P. spinosa*. Assuming maternal inheritance of chloroplast DNA, as reported for sour cherry (*P. cerasus*) [10], the fruit stone could then stem from a shrub of *P. spinosa* pollinated either by its own species, *P. spinosa*, or equally by other *Prunus* species.

Several studies on present day sloe and plum show the existence of hybrids, e.g. [30,34]. Damson, European plum, and sloe are all insect-pollinated plants and therefore have a narrow area of pollen distribution. If the stones of *P. insititia*/*spinosa* are hybrids of female *P. spinosa* and male *P. insititia*, the cultivated plum trees must have been grown close to the shrubs. Any male contribution from *P. insititia*/*domestica* detected in the Roman *P. insititia*/*spinosa* fruits would support local growing of primitive, cultivated plums. To investigate

this possibility, the female woody endocarp can easily be separated from the seed and each typed genetically.

Our studies show that ancient DNA can be well preserved at waterlogged excavation sites and that one single *Prunus* fruit stone is enough for successful DNA extraction. Genetic typing of ambiguous plant remains improves the level of identification. The amplification of different phylogenetically meaningful regions can help to solve questions in archaeobotany which cannot be answered with traditional methods. Future analyses of ancient DNA from waterlogged fruit stones will provide a new way for species and cultivar identification of ancient fruit species and will contribute to our understanding of early horticulture in Middle Europe.

Acknowledgement

We thank H. Brem (Amt für Archäologie Kanton Thurgau, CH) for providing the plant material from the excavation Eschenz 1999.010. We are pleased for the help of Marrie Tensen to improve our English. We gratefully acknowledge the funding of this project by the Kanton Thurgau and the Lotteriefonds des Kantons Thurgau, Switzerland.

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