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ORIGINAL ARTICLE

The oldest macroremains of *Vitis* from Slovenia

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Abstract The oldest *Vitis vinifera* ssp. L. (grape) pips (seeds) from Slovenian archaeological sites were found at the late Neolithic (Copper Age) pile-dwelling settlement of Hočevarica on the Ljubljansko barje and date to the 37th/36th century B.C. Various biometric studies were carried out to establish whether the grapes were wild or cultivated. A comparison of several morphological characteristics of grape pip samples was performed. Neolithic pips from Hočevarica, Roman pips from Vrhnika (1st century A.D.) and recent Slovenian cultivated grape pips were included in the study. According to the standard indices, the Neolithic *Vitis* pips were wild. In the case of the Roman pips, it was impossible to determine whether they were wild or cultivated, although they had most probably been cultivated. Since the morphology of grape pips varies considerably (as it did in the Neolithic, as well), the attempted identification of the subspecies *Vitis vinifera* ssp. *sylvestris*/*vinifera*, based on morphological characteristics alone, was unsatisfactory. Therefore, an attempt was made to analyse the DNA, which could help to resolve this question. Initial experiments on extraction of archaeological DNA and amplification of the chloroplast region were performed, but further optimization steps are needed to achieve successful amplification.

Keywords Grape pips · Neolithic · Roman · Morphological features · Biometric studies · DNA extraction

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Introduction

The area of present-day Slovenia is highly suitable for grape growing because of its geological and climatic conditions. Slovenia is considered to be a traditional viticulture land, where many European prehistoric events took place, and there is abundant cultural evidence to prove that. The temperate climate with sunny sand hills on the margins of the Pannonian lowland, the littoral or karst region with its sub-Mediterranean climate and marl terraces—both are very suitable for viticulture.

Slovenia lies within the natural distribution area of *Vitis vinifera* ssp. *sylvestris* (wild grapevine) (for example, Zohary and Hopf 2000); however, because of extensive viticulture in Slovenia, no more natural wild populations of *Vitis* are to be found there. Pejkić (1980) denotes the sandy river banks along the southern stream of the river Sava in Bizeljsko (a region in Slovenia) as the natural habitat for wild *Vitis* plants.

Grapevine, a plant with excellent physiological potential as a sugar collector, was an ideal plant for extensive cultivation by the Neolithic people, who were farmers as well as gatherers of native wild fruits. The genetic centres of the *Vitis* genus (west European group *Proles occidentalis* and

Mare Pontico group *Proles pontica*) (Branas 1974; Pejkić 1980; Rivera Nunez and Walker 1989) provide an eloquent evidence of how grapevines spread to suitable lands. Moreover, the main Slovenian rivers, the Drava and Sava flowing from the north and the Ljubljanica from the southwest, with their sandy river banks or floodplain woods were the best trading routes, through which the grapes or the knowledge of cultivation of the grapevines reached our prehistoric land.

All major lacustrine and marshland prehistoric settlements in Slovenia are to be found within Ljubljansko barje (the Ljubljana Moor) (Velušček 2004a). Since the first discovery of ancient settlements in this location in the second half of the 19th century, Ljubljansko barje has undergone extensive research study, which has produced extremely well preserved archaeological as well as archaeobiological evidence.

Ljubljansko barje is situated at the edge of the south-eastern Alps in central Slovenia (Fig. 1) and covers an area of 163 km^2 (Lah and Adamič 1992). The bog was formed by subsidence about 2 million years ago. Water, sediments and various other deposits subsequently filled the depression that was formed by subsidence (Pavšič 1989).

The sequence of sediments in Ljubljansko barje, which was determined with the help of corings, aided the reconstruction of the dynamically changing circumstances: first the area of the bog was a large gravel plain with individual small ponds, later it was a shallow lake with a rich flora and fauna, then an impassable swamp, and finally it became a peat bog (Pavšič 1989). The stratigraphy of pile-dwellings and other archaeological sites such as the Roman ones on the shores of Ljubljansko barje, revealed that the peat was formed after the period of pile-dwellings and perhaps even during the Roman period (Pavšič 1989).

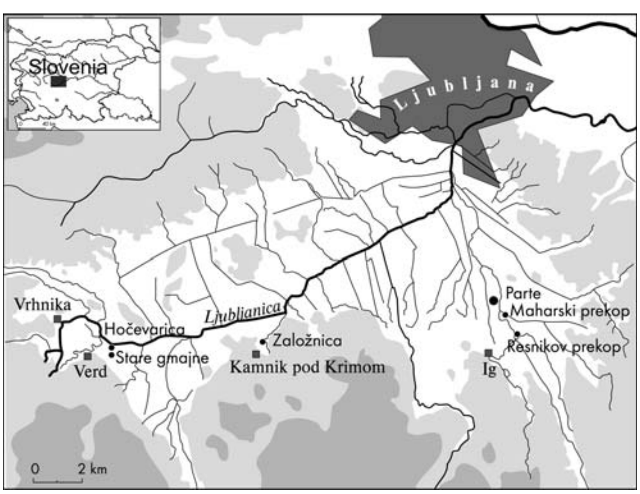

Fig. 1 Ljubljansko barje (Ljubljana Moor), Slovenia, with the most important Neolithic pile-dwelling settlements

The area of the Ljubljansko barje is rich in Neolithic pile-dwelling settlements (Fig. 1). Resnikov prekop (Fig. 1) is the most important site from the first half of the fifth millennium B.C. (Čufar and Korenčič 2006) (all the following dates are calibrated ^{14}C dates, partly verified by dendrochronology). It is a small, dispersed pile-dwelling settlement, which was probably located near the shore of the lake (Velušček 2004a). The next traces of human settlement in Ljubljansko barje date to the second and third quarters of the fourth millennium B.C. The Hočevarica site (Fig. 1) belongs to the second quarter of the fourth millennium B.C. (Čufar and Velušček 2004). Somewhat later settlements dating to the second half of the fourth millennium B.C. have also been discovered in other parts of Ljubljansko barje (Velušček and Greif 1998). In the middle of the fourth millennium B.C., a small pile-dwelling settlement referred to as Maharski prekop (Fig. 1) was located not far from the present-day village of Ig (Velušček 2004a). The site called Stare gmajne is a pile-dwelling settlement from a more recent period than the settlement at Maharski prekop. It is situated near the village of Verd (Fig. 1). The remains of a pile-dwelling settlement from approximately the first half of the third millennium B.C. have also been discovered. Dendrochronological research revealed that several locations on Ljubljansko barje were occupied simultaneously at that period (Velušček and Čufar 2002; Velušček and Čufar 2003; Čufar and Velušček 2004). The settlements at Parte near Ig and Založnica near Kamnik pod Krimom (Fig. 1) co-existed for about 40 years (Velušček and Čufar 2002).

In 1998, a team from the Institute of Archaeology at the Scientific Research Centre of the Slovenian Academy of Sciences and Arts re-excavated a small trench in Ljubljansko barje, in the region of the Hočevarica pile-dwelling site. The main goal of sample trenching was to use excavation methods that had already been tested on the Neolithic wetland settlements in Switzerland, Germany and France, but never used in Ljubljansko barje. The new method brought enough applicable data for a team of 16 researchers from Slovenia and abroad. For several of them, co-researching the Hočevarica site and working in the field of archaeology was a new experience, although they were well recognized professionals in their own fields (Velušček 2004b). For the first time in Slovenia, the sediment from the cultural layer was washed over through three different sieves.

More than 30,000 seeds, fruits and their fragments larger than 3 mm, and even more seeds smaller than 3 mm were found in the cultural layer of the sampled quadrants (Jeraj 2004). Generally, they were well preserved, and—with the exception of cereal grains—almost completely non-carbonised waterlogged subfossils (Jeraj 2004; Jeraj et al. 2008). In most cases, seeds with lignified tissues were preserved, and grape pips (seeds) formed some of the most frequent remains, representing 27% of the seeds in the large fraction of 3 mm and above (Jeraj 2004). Some of the grape pips were also AMS radiocarbon dated. The radiocarbon date of grape pips found at a depth between 159 and 142 cm (the latest settlement phase) (Velušček 2004c) is 4780 ± 40 B.P. (3640–3520 cal B.C., 1 σ range, 3650–3380 cal B.C., 2 σ range) (Jeraj 2004).

Since such an extensive quantity of grape pips was found, the question arises whether they belonged to the *Vitis vinifera* ssp. *sylvestris* (wild grapevine) or cultivated *V. vinifera* ssp. *vinifera*. Grape pips in the Neolithic pile-dwelling settlements in Ljubljansko barje (Fig. 1) are numerous (for example, Culiberg 1980 (Parte); Jeraj 2004 (Hočevarica); Tolar, unpublished (Stare gmajne)) and the oldest in Slovenia so far. To find out what subspecies (wild or cultivated) they belong to, we performed various biometric measurements on the earliest grape pips which have been found in Slovenia, from the Hočevarica site. In addition, measurements of the Roman grape seeds from Vrhnika, Slovenia, dated to the 1st century A.D. (Horvat and Mušič 2006), and of modern grape pips of *Vitis vinifera* varieties were taken into account.

Since the morphological identification of the wild subspecies, with morphologically rounded pips, or the cultivated subspecies with morphologically pyriform pips (Stummer 1911) is questionable (for example Facsar 1972; Jacquat and Martinoli 1999; Schlumbaum et al. 2008), we decided to additionally use molecular methods that could help resolve our questions. The DNA analysis was performed on six grape pips (three morphologically rounded and three pyriform ones) from the Stare gmajne site, since these were preserved in better condition than the seeds from the Hočevarica site (see below, methods).

The objective of this article is to present the first results of morphological measurements and preliminary testing of the DNA isolation from Neolithic and Roman grape pips of Slovenian archaeological and modern *Vitis*, to find out whether the oldest grape pips found in Slovenia belong to the wild or cultivated *V. vinifera*.

Materials and methods

Archaeobotanical methods

The excavated trench at the Hočevarica site was 2 m wide and 4 m long. It was divided into 8 quadrants of 1 × 1 m^2. The sediment from the cultural layer in the quadrants 1, 4, 5, and 8 was washed through metal sieves with 3.0, 1.0 and 0.5 mm mesh sizes (Velušček 2004c). The collected fractions were dried, and the plant remains were sorted (Jeraj 2004; for a critical view of the methodology applied, see Jeraj et al. 2008).

The Stare gmajne site is located about 250 m south of the Hočevarica site (Fig. 1) and dates to the second half of the fourth millennium B.C. (Velušček 2004a). In 2006 and 2007, a team of experts from the Institute of Archaeology excavated two trenches of 15 m^2. Similarly to the Hočevarica site (see above), each excavated trench at Stare gmajne was divided into 15 quadrants of 1 × 1 m^2. The whole cultural layer was wet-sieved, and the collected sieve samples were dried in order for the inorganic materials (pottery, bones and stones) to be sorted. For analysing the organic remains (mostly non-carbonised plant remains), special archaeobotanical samples were taken for the first time: two samples of 2 l each were taken randomly from the trench in 2006; 15 samples of 2 l each were taken systematically from quadrants 1, 9 and 13 (horizontal sampling) and separately from each of the five layers (vertical sampling) from the trench in 2007. Afterwards, the samples were washed over (for example Hosch and Zibulski 2003) and hand sorted in water. Grape pips were found in these very rich macrobotanical samples as well, but not as many as at the Hočevarica site (Tolar, unpublished). All macrobotanical remains larger than 0.355 mm from Stare gmajne are stored under wet conditions to prevent decay and deformation.

Morphological material

For this study, 142 grape pips from the Neolithic pile-dwelling settlement of Hočevarica in Ljubljansko barje, approximately 5,600 years old (Fig. 2a) and 73 grape seeds from the Roman settlement at Vrhnika, approximately 2,000 years old (Fig. 2b) were measured. All remains come from waterlogged layers, and their preservation is as

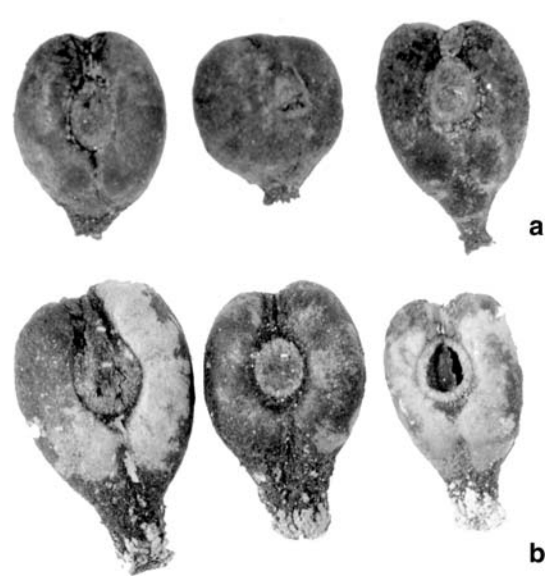

Fig. 2 Subfossil non-carbonised grape pips. **a** Neolithic, **b** Roman

non-carbonised subfossils, but they were dried after sieving (see above; Fig. 2; Jeraj 2004; Jeraj et al. 2008).

The cultivated modern populations of *Vitis vinifera* that were used for comparison comprised 50 seeds from five varieties (Fig. 3) used for wine making in Slovenia: Vrtovka (a seedling of unknown *Vinifera* origin) (Fig. 3a), an American variety originated from *V. labrusca* (Fig. 3b), Rebula (a local variety from Primorje) (Fig. 3c), Štajerska belina (Heunisch) (Fig. 3d) and Laški rizling (Welschriesling) (Fig. 3e).

Seeds of various shapes (more rounded, pyriform and in between) were taken at random from each group of Neolithic, Roman and modern (Figs. 2, 3).

Morphological methods

Identification of either wild or cultivated grape pips was first based on morphological features (Fig. 4; Stummer 1911; Terpo 1976, 1977; Mangafa and Kotsakis 1996; Jacquat and Martinoli 1999; Kohler-Schneider 2001).

Since the work of Stummer (1911), it has been generally accepted that domestication has caused changes in the dimensions and other morphological characteristics of the seeds. Therefore, it is generally possible to distinguish cultivated from wild grape pips. Stummer (1911) described the pips of wild vines as small, robust and with a rounded outline, or cordate with short stalks. On the other hand, the pips of domesticated vines were described as large, elongated, oval or pyriform, with a longer stalk. These distinguishing shape characteristics can be shown numerically, so the index $B/L \times 100$, also called the Stummer index, shows that wild grape seeds have larger Stummer indices because of their rounded shape, which is the result of their greater breath and shorter length.

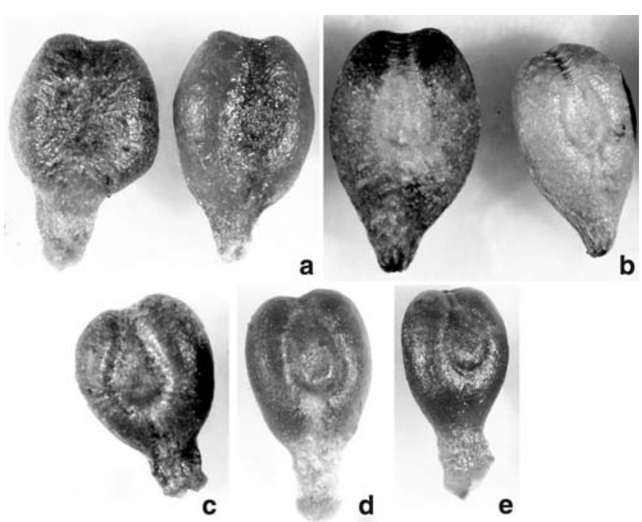

Fig. 3 Pips of modern cultivated *Vitis vinifera*. **a**Vrtovka, **b** *Vitis labrusca*, **c** Rebula, **d** Štajerska belina, **e** Laški rizling

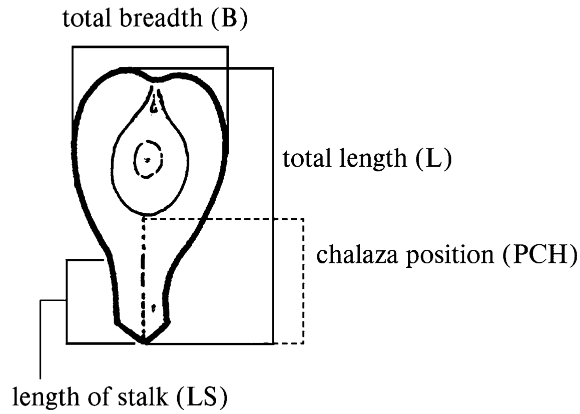

Fig. 4 Morphology measurements of the *Vitis* seed

Stummer index:

$$SI = B/L \times 100$$

Because many grape pips do not clearly fit into one of the two typical Stummer groups (for example Logothetis 1970, 1974; Smith and Jones 1990; Mangafa and Kotsakis 1996; Jacquat and Martinoli 1999; Kohler-Schneider 2001; see also the results section), several other indices and formulae for determining the *Vitis* subspecies were developed (Mangafa and Kotsakis 1996), the key to the codes used is below:

Formula 1:

$$-0.3801 + (-30.2\mathrm{LS}/L + 0.4564\mathrm{PCH} - 1.386L + 2.88\mathrm{PCH}/L + 9.4239\mathrm{LS})$$

Formula 2:

$$0.2951 + (-12.64\mathrm{PCH}/L - 1.6416L + 4.513\mathrm{PCH} + 9.63\mathrm{LS}/L)$$

Formula 3:

$$-7.491 + (1.7715\mathrm{PCH} + 0.49\mathrm{PCH}/L + 9.56\mathrm{LS}/L)$$

Formula 4:

$$0.7509 + (-1.5748L + 5.297\mathrm{PCH} - 14.47\mathrm{PCH}/L)$$

The morphological method (O. I. V. descriptors: OIV 241–OIV 244), which requires precise measuring of morphological characteristics of the grape pip, was used for our material (OIV Descriptions 1984). The following measurements were taken (Fig. 4): length (L), breadth (B), thickness (T), length of stalk (from the tip of the stalk to its base—edge of the seed corpus) (LS), placement of the chalaza (the distance from the base of the chalaza to the tip of the stalk) (PCH) (Terpo 1977; Mangafa and Kotsakis 1996; Jacquat and Martinoli 1999; Kohler-Schneider 2001).

For precise measurements, we used an *Olympus PROVIS* stereomicroscope connected to the computer and the computer programme *analysis by Soft Imaging System GmbH*.

The following indices were calculated from the measurements: breadth to length ($B/L \times 100$), thickness to breadth ($T/B \times 100$), thickness to length ($T/L \times 100$), length of stalk to total length ($LS/L \times 100$), placement of chalaza to total length ($PCH/L \times 100$) (Terpo 1977; Mangafa and Kotsakis 1996; Jacquat and Martinoli 1999; Kohler-Schneider 2001), and an attempt to distinguish between wild or cultivated *Vitis* was made using the Stummer (1911) index and the four Mangafa and Kotsakis (1996) formulae (see above).

The four Mangafa and Kotsakis formulae are for charred grape seeds, since the archaeological finds are often carbonised. The effects of carbonisation on the dimensions of the grape seeds were investigated several times by experimental charring of seeds (Logothetis 1970, 1974; Rivera Nunez and Walker 1989; Smith and Jones 1990). The investigations revealed that after carbonisation, the seeds of both wild and cultivated subspecies became smaller and more rounded, since length decreases more than breadth which, in turn, decreases more than thickness. Consequently, the B:L indices (Stummer index) increased (Logothetis 1970, 1974; Rivera Nunez and Walker 1989; Smith and Jones 1990). For that reason, Mangafa and Kotsakis (1996) suggest that the second and the third formulae (see above) are more appropriate for the identification of charred archaeological grape pips, because fewer simple size variables are used with less contribution than ratio variables.

Material for our research was not charred, but preserved wet and subsequently dried. We are aware of the possible problems in treatment of our material (Jeraj et al. 2008), but we do not know if drying of wet archaeological seeds may cause damage and changes in the dimensions of grape seeds and DNA degradation. After having seen many *Vitis* pips, we assume that drying does not influence their shape (B:L indices) (see also Rivera Nunez and Walker 1989), because the outer tissue of grape pips is highly lignified and rigid. Therefore, we calculated all known indices and formulae (Stummer; Mangafa and Kotsakis) for distinguishing wild subspecies from the cultivated ones. The results are shown in Table 1.

Since there are no pure natural wild populations of *Vitis vinifera* ssp. *sylvestris* any more in Slovenia, Table 1 also includes the results of Kohler-Schneider (2001), who measured modern cultivated *Vitis* pips from France and wild ones from the Danube floodplains in Niederösterreich (Lower Austria).

The morphological characteristics of *Vitis* seeds (both cultivated and wild) are very variable (Table 1). The seeds can be rounded, pyriform or intermediate in both groups (wild and cultivated) (Facsar 1970, 1971, 1972, 1975; Jacquat and Martinoli 1999; Kohler-Schneider 2001).

Table 1 The average of measurements and calculated formulae (indices) (according to Mangafa and Kotsakis 1996; Stummer 1911) for morphological characteristics of grape pips from the Slovenian Neolithic and Roman settlements, and modern cultivated grape pips from Slovenia, all non-carbonised but dried; and, for comparison, morphological characteristics of modern charred grape pips—cultivated ones from France and wild ones from the Danube floodplains (according to Kohler-Schneider 2001)

Measurements and indices (formulae)	Neolithic, wild	Roman, wild/cult.	Modern, cult., Slovenia	Modern, cult., France	Modern, wild, Danube
Preservation	Non-carb.	Non-carb.	Non-carb.	Carb.	Carb.
Number of measured seeds	142	76	50		
Length (L)	4.82	5.37	6.13	7.1	3.8
Length of stalk (LS)	0.73	1.31	1.76	2	0.4
Placement of chalaza (PCH)	2.35	2.82	3.17	3.9	1.7
Breadth (B)	3.7	3.45	3.6	4.9	3.3
Thickness (T)	2.73	2.58	2.71		
B/L	77.27	64.48	58.92		
T/L	57.3	47.9	44.49		
LS/L	14.85	23.9	28.71		
PCH/L	48.26	52.01	51.77		
T/B	74.23	74.66	75.87		
Formula 1 (>0.8: cultivated); (<−0.2: wild)	−2.18	0.09	1.94	3.48	−2.99
Formula 2 (>0.9: cultivated); (<−0.2: wild)	−1.73	−0.08	0.77	2.01	−2.91
Formula 3 (>0.9: cultivated); (<0: wild)	−1.67	0.08	1.13	2.38	−3.25
Formula 4 (>1.4: cultivated); (<−0.9: wild)	−1.43	−0.28	0.41	2.28	−2.7
Stummer index (44–53: cultivated); (76–83: wild)	77.27	64.48	58.92	69	87

Therefore, the identification of grape pips as far as subspecies is problematic, and morphological features (described above; Fig. 4) that are generally used to distinguish between the wild and the cultivated subspecies are not satisfactory (Jacquat and Martinoli 1999; Kohler-Schneider 2001; Schlumbaum et al. 2008). We therefore completed the classical morphological methods (described above) and used molecular DNA methods as well.

An attempt to extract and amplify ancient grape DNA was made, details (material, methods) are available as Electronic Supplementary Material (ESM).

Results and discussion

Morphological results

Both the Neolithic grape pips from the Hočevarica site and the Roman ones from the Vrhnika site were found in wet conditions, non-carbonised and later air-dried. The seeds were very well preserved. The Neolithic grape pips, despite being older, were in considerably better condition, because the Neolithic cultural layer is deeper, and the Hočevarica site is situated more in the interior of the Ljubljansko barje (Fig. 1) where the clay soil is anoxic and contains more moisture, which slows down the degradation of organic material.

The morphological characteristics such as the chalaza and stalk of all measured archaeological seeds were easily visible and recognizable (Fig. 5). The computer measurements are expressed to five decimal places.

The variable morphological characteristics in each examined group are shown in Figs. 6 and 7, where box-plot graphs show the maximum, minimum and median values (the extremes), where half of the measurements is larger and the other half is smaller than the median. The grey boxes represent two quarters (or half) of the measurements.

The majority of the measured Neolithic grape seeds (83) fall into the wild range (76–83), as proposed by Stummer (Fig. 7). The rest of the measured seeds (59) fall into the intermediate range, and none of them falls into the cultivated range (Stummer 1911). Therefore, they could be considered as representing wild grapevines. However, when we compare the graphs of all three examined groups (Fig. 7), the result is not so convincing. It suggests that the values of Stummer indices of the Neolithic group also vary, and that a quarter of the Neolithic seeds (43) fall into the range of the modern group. This is not a strong enough result for identifying the Neolithic seeds as wild *Vitis*.

Identification of the Roman grape seeds, which are assumed to be cultivated, remains uncertain after calculating the Stummer index, and the majority of their indices are somewhere in between; the range (57–70) is closer to the cultivated modern grapevines (Fig. 7). Only four of 66 measured seeds fall within the cultivated range, and six seeds within the wild range. It is possible that our sample contained two or three types of seeds, wild (rounded and

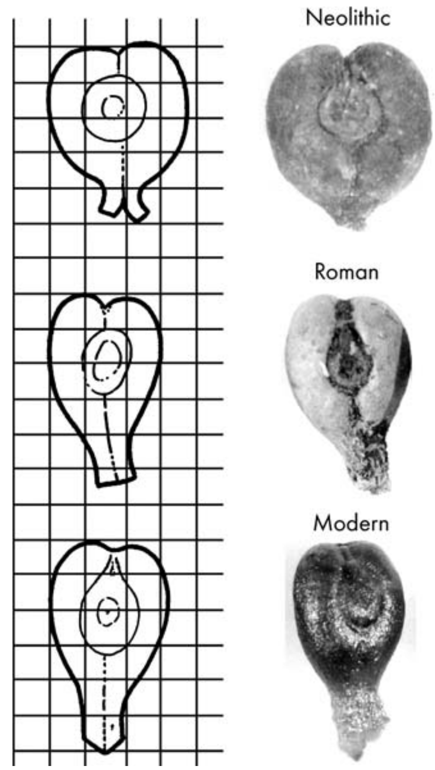

Fig. 5 Examples of Neolithic, Roman and modern grape pip

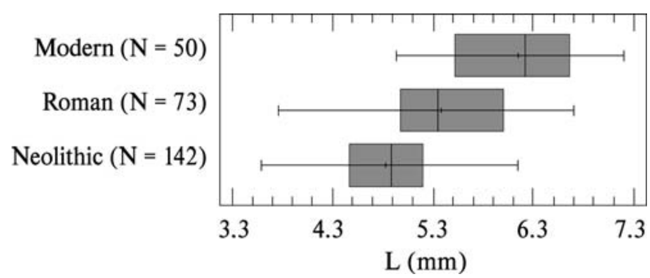

Fig. 6 Length (*L*) of grape pips (mm)

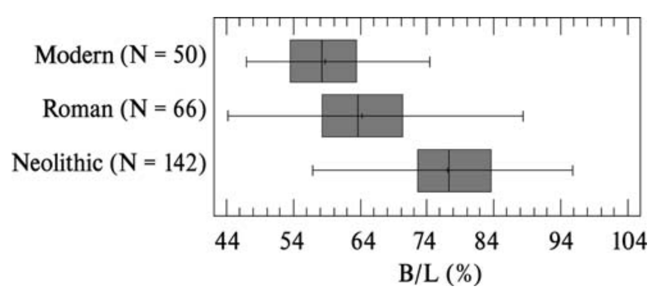

Fig. 7 Index $B/L \times 100$ of grape pips (%)

short), half-domesticated and domesticated (pyriform and elongated).

Based on the Stummer index, the majority of the modern grape seeds falls into the intermediate range too (54–64) (Fig. 7). Only 11 of the 50 measured grape seeds fall within the cultivated range (44–53), and none of them falls within the wild range. We have taken quite a diverse population of five modern varieties of *Vitis vinifera* (Fig. 3), and the variability of cultivars is notably great (Facsar 1970, 1972, 1975). So, some seeds fulfil the criteria for the cultivated range completely, but the rest to a minor extent. Moreover, in our case, it has turned out that the Stummer index is unreliable for the modern cultivated group as well. This means that there are also cultivars with round seeds (Facsar 1970, 1972), which should be, according to Stummer, a characteristic of wild grapes. Having calculated the Stummer index, we discovered that it is inconvenient (Mangafa and Kotsakis 1996; Jacquat and Martinoli 1999; Kohler-Schneider 2001), because it does not allow or consider the variability of seeds within one group (wild, cultivated or cultivars) or even within one grape (Facsar 1970, 1971, 1972; Terpo 1976, 1977). The seeds with an intermediate Stummer index (and they outnumber the other examined group) are impossible to attribute to a subspecies. Therefore, several other researchers have produced new formulae and indices for distinguishing wild from cultivated subspecies (Mangafa and Kotsakis 1996). Since we had carried out all the important measurements, we could apply them to the formulae proposed by Mangafa and Kotsakis (1996), and we compared the results obtained from these four formulae with the results of the Stummer index (Table 1). The formulae of Mangafa and Kotsakis (1996) include an error probability of imprecise measurement of the stalk length, because of difficult recognition of the stalk base or because of a frequently truncated tip, that are appropriate for dealing with archaeological seeds. The results are presented in Table 1. For comparison, we also took the average measurements and calculated indices and formulae of modern wild and cultivated grape seeds, cited in Kohler-Schneider (2001).

The values of the Mangafa and Kotsakis formulae are interpreted in the following way (according to Mangafa and Kotsakis 1996): values lower than −0.2 for Formula 1 and Formula 2, values of 0 for Formula 3, and −0.9 for Formula 4 indicate wild grape seeds. Values between −0.2 and 0 for Formula 1, between −0.2 and 0.4 for Formula 2, between 0 and 0.5 for Formula 3, and between −0.9 and 0.2 for Formula 4 indicate wild grape seeds with great probability. Values higher than 0.8 for Formula 1, 0.9 for Formula 2 and Formula 3, and 1.4 for Formula 4 indicate cultivated grape seeds. Values between 0.2 and 0.8 for Formula 1, between 0.4 and 0.9 for Formula 2, between 0.5 and 0.9 for Formula 3, and between 0.2 and 1.4 for Formula 4 indicate cultivated grape seeds with great probability.

Using all four of these formulae, it can be seen, similarly to the result obtained by the Stummer index, that the Neolithic grape pips are wild (Table 1). Only two out of the 142 measured seeds can be identified as cultivated. The values obtained using the first and the third formulae for one seed are around 0.9, and the values obtained using the second and the fourth formulae of the other seed are higher than 1, which can be considered as cultivated according to Mangafa and Kotsakis (1996). It is possible that two other seeds are cultivated according to the second (63.8% probability), third (63.3% probability) and fourth (91% probability) formulae. Since this is a common biological variability within one group, our conclusion is that the Neolithic grape pips belong to wild *Vitis*.

None of the 4 formulae can identify the Roman pips in general as either cultivated or wild (Table 1). The average calculations fall into the intermediate range with some probability of being wild. 20 out of the 66 measured Roman pips can be identified as cultivated using the first and the third formula. Using the second formula, 17 pips are identified as cultivated, and using the fourth formula, only 5 out of the 66 measured pips are identified as cultivated, and 18 other pips can be identified as belonging to cultivated vines with a 91% probability. As with the Stummer index, the four Mangafa and Kotsakis formulae cannot determine whether the Roman pips are cultivated, yet all formulae and indices show the same affinity towards the cultivated range (Table 1).

More than half the 50 measured modern cultivated grape pips can be identified as cultivated using the first three Mangafa and Kotsakis formulae. The fourth formula identifies only 8 out of the 50 measured pips as cultivated, and 19 other seeds as such with a 91% probability. The average calculations identify modern grape pips mostly or, at least, with a high probability as cultivated (Table 1).

DNA results

Fruits, seed tissues and especially archaeological samples represent unfavourable material for DNA extraction. In our first attempt at DNA extraction from recent grape seeds, a large amount of polysaccharides precipitated with the DNA and omitted the subsequent polymerase chain reaction (PCR) reactions (data not shown). After extraction, the introduction of the glass beads cleaning step to the recent seed DNA samples revealed DNA of adequate quality to be used for the PCR reaction.

Although there are numerous reports on successful extraction of amplifiable DNA from archaeological samples (Manen et al. 2004; Regner et al. 2004; Halasz et al.

2005), the first attempt at PCR (preserved chloroplast region) analysis from archaeological samples produced no successful outcome. The plastid organellar DNA exists in numerous copies per cell, and is thus more likely to survive the process of degradation and the effects of damage that follow the death of the organism, compared to single-copy nuclear sequences. Therefore, amplification of the preserved chloroplast region trnL–trnF was chosen for our analysis. The chloroplast region was successfully amplified in modern cultivated seed samples and three putative *V. sylvestris* samples, while amplification from the archaeological samples yielded no results even after several attempts under optimized conditions of amplification (data not shown). One reason for that could be the expected length of the trnL–trnF amplicon, which is up to 800 bp. Such a length can exceed the length of the archaeological DNA remains, and thus the region cannot be amplified. The problem of possible contamination of samples with modern *Vitis* DNA still remains, although several precautions were taken during extraction (see also the methods in ESM).

Conclusions

The oldest Neolithic *Vitis* pips from Slovenia are considered to be wild, according to the Stummer index (1911) and to the four formulae by Mangafa and Kotsakis (1996). The variability of the 142 grape seeds from the Neolithic pile-dwelling settlement is quite large (Fig. 7). Various results were obtained when comparing the modern wild *Vitis* pips from the Danube floodplains, measured by Kohler-Schneider (2001), with the Neolithic *Vitis* pips from Ljubljansko barje, which are considered to be wild (Table 1). The Stummer index and the four formulae show slightly smaller values for the modern wild *Vitis* seeds from the Danube floodplains, which provide more convincing evidence of their being wild.

The Roman *Vitis* seeds from Vrhnika can be determined neither as wild nor as cultivated, although the probability leans in favour of the latter option. The four formulae and indices showed that the Roman pips could not be confidently placed in either of the wild or cultivated groups, but that they were somewhere in between (Table 1). Variability among the 66 measured Roman grape seeds is also extensive (Fig. 7). It is possible that our sample contained two or three types of seeds: rounded and short wild ones, half-domesticated ones, and pyriform and elongated domesticated ones, because some individual seeds do fall into the cultivated range, according to the Stummer index and the four formulae of Mangafa and Kotsakis.

The reference seed material of modern cultivated grapes from Slovenian vineyards is considered cultivated when using the first and the third formulae by Mangafa and Kotsakis (1996), but unconvincing when using the second and the fourth formulae and the Stummer index (Table 1). However, the values obtained by using all four formulae and the Stummer index are higher in comparison to the Roman ones, which is more convincing evidence that they are cultivars.

The measurements and results of the calculated index and formulae of the modern cultivated *Vitis* seeds from France, measured by Kohler-Schneider (2001) show higher values in comparison to the modern Slovenian *Vitis* seeds; this is more convincing evidence of their being cultivars (Table 1). The great polymorphism of pips among grape varieties (Facsar 1970, 1971, 1972) can again be confirmed.

Again, the study of morphological characteristics of grape pips shows considerable variation in values within one measured group (Fig. 7). This confirms that classification into a *Vitis* subspecies based only on morphological characteristics is unsatisfactory. Therefore, an attempt to analyse the DNA was made.

PCR has transformed most areas of molecular biology; in fact, studies of DNA from archaeological remains have created an entirely new field (Pääbo 1991). We have successfully tried a method for the analysis of such remains; however, a modification of the DNA isolation procedure and PCR amplification must be carried out in order to obtain amplifiable DNA. Precautions must also be taken at the time of sample discovery to prevent them from drying out; this may be an additional reason for more rapid degradation of the DNA remains. In future, we aim to use redesigned primers to yield shorter DNA fragments to overcome the problem of ancient DNA degradation and fragmentation.

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