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Tracking the history of grape cultivation in Georgia combining geometric morphometrics and ancient DNA

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Abstract

The Near East and the Caucasus are commonly regarded as the original domestication centre of grapevine, and the region continues to be home to a high diversity of wild and cultivated grapevines, particularly within Georgia. The earliest chemical evidence for wine making was recorded in Georgian Neolithic sites (6000-5800 BC) and grape pips, possibly of the domesticated morphotype, have been reported from several sites about the same period. We performed geometric morphometric and palaeogenomic investigations of grape pip samples in order to identify the appearance of domesticated grapevine, and explore the changes in cultivated diversity in relation to modern varieties. We systematically investigated charred and uncharred grape pip samples from Georgian archaeological sites. Their chronology was thoroughly assessed by direct radiocarbon dating. More than 500 seeds from 14 sites, from the Middle Bronze Age to Modern times, were selected for Geometric Morphometric studies. The shape of ancient seeds was compared to hundreds of modern wild individuals and cultivated varieties. Degraded DNA was isolated from 3 seeds (2 sites), converted to Illumina libraries, sequenced at approximately ten thousand SNP sites, and compared to a large public database of grapevine diversity.

The most ancient seed dates from the Middle Bronze Age (1900-1500 cal BC) and the domesticated morphotype is identified from ca 1000 BC onwards. A strong diversity of domesticated shapes is regularly identified in the samples. Most are close to modern cultivars from the Caucasian, South-West Asian and Balkan areas, which suggests that the modern local diversity is deeply rooted in the early times of viticulture. DNA was successfully recovered from historic pips and genome-wide analyses found close parental relationships to modern Georgian cultivars.

Keywords

Vitis vinifera - Domestication – Diversity – Caucasus – Outline analysis - Palaeogenomics

Introduction

Grapevine (*Vitis vinifera* subsp. *vinifera*) was likely first domesticated in South-West Asia, where the most ancient archaeobotanical traces of grape cultivation are found (e.g. Zohary and Spiegel-Roy 1975; McGovern 2003; Miller 2008; Fuller and Stevens 2019). At the same time, the genetic structure of modern cultivated grapes and relatedness between cultivars and wild populations also support an origin of domesticated grape in the area from the Near East to Central Asia (Myles et al. 2011;

Bacilieri et al. 2013; Emanuelli et al. 2013, Riaz et al. 2018), while the existence of secondary domestication events in the Mediterranean basin is still debated (Grassi et al. 2003; Arroyo-García et al. 2006). More specifically, the area south of the Caucasus, between the Black and Caspian seas, was considered very early on by various scholars as the most likely place of origin of cultivated grape, due to the high local diversity of wild populations and cultivars (De Candolle 1886; Vavilov 1930; Negrul 1946; De Lorenzis *et al.* 2015). More than 500 grape varieties are considered to be native from Georgia (Maghradze et al. 2012). They comprise table and mostly wine varieties, with a large majority of white grapes. Among the most famous cultivars, ‘Rkatsiteli’, ‘Saperavi’ and ‘Chinuri’ are cultivated in Eastern Georgia, ‘Tsolikouri’ and ‘Tsitska’ in the West. Genetic investigations confirm the specificity of the Black Sea-Caucasus germplasm compared to cultivars from other regions (Imazio et al. 2013; Liang et al. 2019) and a specific subcluster gathering most of the Georgian wine cultivars can be identified (Bacilieri et al. 2013; Laucou et al. 2018).

The high diversity of Georgian germplasm known today is probably a consequence of 1) the heterogeneity of environmental conditions, from the subtropical and Mediterranean climates close to the Black Sea to continental and mountainous ones in the North-East, 2) the geographical location of the country, at the crossroads of north-south and east-west trade routes, and 3) the ancient and intensive wine growing tradition in the country. Homemade wine is still produced by most of the families in the countryside and autochthonous varieties cover 95% of the total vineyard surface in Georgia (Maghradze et al. 2012). Similar to the ancient Mediterranean practice, the typical Georgian tradition is to make wine in underground large pottery vessels, called “*kvevri*”, where the must ferments and wine is then stored (Beridze 1962; Reigniez 2016; Vigentini et al. 2016). Traditionally, grapes were simply pressed by foot in wooden containers and must was macerated in “*kvevris*” with a variable amount of skins, rachises and pedicels and for variable durations, depending on the type of wine that was to be produced. Due to its specificity and cultural significance “*kvevri*” wine tradition was recently assigned the status of National Monument of Intangible Cultural Heritage by Unesco¹. Based on linguistic, historical and archaeological data the tradition of winemaking is thought to be deeply rooted in the history of Georgia (McGovern 2003; Maghradze et al. 2012). *Kvevri*-like storage jars are commonly reported from archaeological sites in Georgia. It has been supposed that similar, moderately-sized, jars already existing in the “Kura-Arax” culture (ca. 3500-1500 cal BC) and even in the Neolithic “Shulaveri-Shomutepe culture” (SSC) (ca 6000-4000 cal BC) could have been used to make and store wine (McGovern 2003; Batiuk 2013). This hypothesis recently received a crucial support when chemical analyses of residues absorbed in pottery vessels brought evidence of winemaking at the SSC sites of Shulaveris Gora and Gadachrili Gora (5900-5500 cal BC) (McGovern et al. 2017). This result predates for at least 500 years the previous earliest evidence for wine, obtained, also by chemical analysis, at the Neolithic site of Hajji Firuz Tepe (ca 5400-5000 cal BC), in the northwestern Zagros mountains of Iran (McGovern et al. 1996), about 500 km South of Shulaveris and Gadachrili. Both concur to identify the wide area south of the Caucasus as the primary zone of emergence of winemaking.

On the other hand, archaeobotanical evidence has been repeatedly referred to to argue for an early viticulture in South Caucasus, starting from the 6th mill. BC. Sporadic finds of grape pips were reported from several Neolithic SSC archaeological sites in Georgia: Shulaveris Gora, Khramis Didi Gora, Dangreuli Gora. Additionally, grape pips were mentioned in Neolithic Chokh, in the Russian province of Dagestan, in Aratashen, Aknashen and Masi Blur, in Armenia, and in Shomu-tepe, in Azerbaijan (Lisitsina and Prishchepenko 1977; Gorgidze and Rusishvili 1984; Lisitsina 1984; Ramishvili 2001; Costantini et al. 2006; Rusishvili 2010, Hovsepyan 2015). The morphology of the pips from some of these Neolithic sites, especially Shulaveris Gora, was regarded as typical of modern cultivated grapes, so grapevine would already have been domesticated by the 6th mill cal BC in Georgia (Costantini et al. 2006).

The archaeobotanical documentation on the early history of viticulture in South Caucasus can however be considered as still limited and poorly known, first because of the small number of systematic archaeobotanical investigations and because of the restricted literature available to international readers (Costantini et al. 2006). Taphonomic issues are not fully taken into account in the publications reviewing the discoveries of grape seeds. It is sometimes difficult to understand if the pips were

¹ <https://ich.unesco.org/en/RL/ancient-georgian-traditional-qvevri-wine-making-method-00870>

preserved by charring or another process. In many cases the excavations and original archaeobotanical studies have been carried out years ago and detailed information on the archaeological context and on sample composition is not always available.

In the framework of a national Georgian research program (Maghradze et al. 2016) it was decided to systematically review the archaeobotanical grape seeds, to perform direct radiocarbon dates, and to engage geometric morphometric (GMM) (Terral et al 2010; Pagnoux et al 2015) and palaeogenomic investigations (Ramos-Madrigal et al. 2019) in order to: 1) confirm the chronology of the findings, 2) identify when domesticated grapevine occurred in the country and 3) explore how the cultivated grapes changed through time compared to the modern diversity.

Materials and methods

Vitis seed samples & radiocarbon dating

The *Vitis* seed samples available from archaeological repositories and current archaeobotanical investigations were assessed and the related information on context and preservation conditions was registered (Table 1). Carbonized grape seeds have been recovered from 8 sites, with an expected chronology according to the archaeological context ranging from the Neolithic to the Roman period (ca 6000 cal BC – 500 AD). Most of the samples (18 sites) were composed of uncharred seeds, with an expected chronology ranging from the Paleolithic until Modern times and including several Neolithic sites.

Twenty seven pips from 25 sites were selected to be radiocarbon dated. Two samples, composed of isolated pips, could not be dated. Radiocarbon dating was carried out at the D-REAMS Radiocarbon Dating Laboratory (Rehovot, Israel). Calibrated ages (95.4% probability) have been obtained by means of OxCal v. 4.2 (Bronk Ramsey 2010) and IntCal13 atmospheric curve (Reimer et al. 2013).

Geometric morphometrics (GMM)

Except the Pichori grape pip, which could not be photographed before radiocarbon dating, and the Badaani sample, only composed of broken seeds, GMM investigations were performed on all the available samples (14 sites, 15 samples, 502 pips). Through the quantitative description of seed outlines using the Elliptic Fourier Transform method, GMM allow a powerful discrimination of wild and domesticated grapevines and to characterize the changes in the cultivated diversity through time (Terral et al. 2010, Pagnoux et al. 2015). Each pip was photographed in dorsal and lateral views using a stereomicroscope (Olympus SZ-ET) and a digital camera (Olympus DP12). The photos were converted to black surfaces. The (x, y) coordinates of 360 equidistant points were sampled on each outline. Outlines were normalized before the EFT computations by centering, scaling using their centroid size, and defining the first point right above the centroid. We used only the coefficients from the 6 first harmonics (48 coefficients) in the statistical analyses. All analyses were carried out using Momocs package (Bonhomme et al. 2014) in R environment (R Development Core Team, R-3.5.3.). Pip shape variation between archaeological samples was explored using Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA), performed on the 48 shape variables. In order to identify the wild/domesticated status of the pips and the proximity between domesticated archaeological pips and modern varieties we used predictive discriminant analysis. The archaeological samples were compared to a reference collection of modern seeds from 82 wild grapevines and 280 traditional cultivars considered as typical from various areas of Europe, the Mediterranean and the Caucasus (see Pagnoux et al. 2015; S1, S2). Wild grapes were sampled by us in several countries covering most of the distribution area of *V. vinifera* subsp. *sylvestris* (France, Germany, Georgia, Greece, Italy, Spain, Switzerland and Turkey). Most of our cultivars were selected and sampled from the INRA Grape Germplasm Repository, in France (Domaine de Vassal, Marseillan-Plage) with the aim to be representative of the global diversity. Additionally, 43 autochthonous cultivars from Georgia were sampled from the Saguramo Grape Repository (Jighaura, Georgia). We have chosen wine and table varieties typical from various regions of Georgia.

The comparison of archaeological seeds to the modern collection is carried out using two nested LDAs. We first compared the archaeological seeds with modern wild (N=2430) and domesticated

(N=2430) references. Then the domesticated types were compared with modern varieties. When dealing with charred remains, we can assume that large assemblages are more likely to be well preserved compared to isolated pips, which should not be considered in cultivar level discriminant analyses (Bouby et al. 2018). In the present study, all the charred samples are composed of more than 30 seeds and were therefore all considered in the cultivar-level LDA. We consider the allocation by the LDAs reliable only when $p \geq 0.75$.

Ancient DNA

The preservation of ancient DNA (aDNA) in archaeological *Vitis* seeds has been demonstrated by conventional methods of amplification and sequencing (Manen et al. 2003, Bacilieri et al. 2017). However, more robust analyses are made possible through high throughput sequencing, where millions of DNA molecules are sequenced in parallel. This approach has been shown to be useful on ancient grape seeds (Wales et al. 2016), as it enables characterization of very short (<50bp) endogenous DNA molecules, including those containing age-related chemical damage. Following a recently established methodology for *Vitis* archaeogenetics (Ramos-Madriral et al. 2019), we performed shotgun sequencing and targeted enrichment of 10,000 SNP loci in 17 grape seeds from six archaeological sites, as summarized below.

DNA was extracted from archaeological seeds in a dedicated aDNA facility at the University of Copenhagen, using a method developed for archaeobotanical remains (Wales et al. 2014), with modifications to retain ultrashort DNA (Dabney et al. 2013). The recovered DNA was converted to double-stranded DNA libraries using the NEBnext DNA Library Preparation Master Mix Set 2 (E6070L, New England BioLabs). The libraries were quantified using real-time PCR to infer the appropriate number of PCR cycles to yield sufficient quantities of DNA for targeted enrichment experiments. Libraries were amplified with AmpliTaq Gold polymerase and sample-specific indexes, and then screened for endogenous content on an Illumina HiSeq2500. Samples with >1% grape DNA were enriched for 10,000 informative SNP loci with a custom-designed MYbaits kit (Arbor Biosciences, Ann Arbor, MI, USA), following an established protocol (Ramos-Madriral et al. 2019). Processing of sequencing data was done following the approach described in Ramos-Madriral et al. (2019). In brief, AdapterRemoval2.0 was used to trim adapter sequences (Schubert et al. 2016), reads were mapped to the grape reference genome 12X.2 (Canaguier et al. 2017) using bwa aln (Li et al. 2009) and following aDNA standard practices, PCR duplicates were removed using picard-tools, and reads with mapping qualities below 30 were excluded. The authenticity of the aDNA data was evaluated using bamdamage (Malaspinas et al. 2014) (S3). The archaeological samples were then compared to the GrapeReSeq modern reference database comprising 783 modern cultivars, 112 wild individuals and 11 other *Vitis* species (Laucou et al. 2018, Le Paslier et al 2019). A Principal Components Analysis (PCA) was performed using smartPCA lscproject (Patterson et al 2006) including the samples in the GrapeReSeq database and the archaeological samples. To account for the low coverage of the aDNA data, we sampled a random allele for both of the archaeological samples and for each site in the reference panel before performing the PCA. Identity by state pairwise distances between archaeological samples and modern accessions were calculated using plink in order to identify the closest match between the archaeological samples and the reference cultivars. Finally, we used the genotype likelihood-based approach implemented in NgsRelate (Korneliussen and Moltke 2005) to evaluate potential relatedness among the archaeological seeds as described in Ramos-Madriral et al. (2019).

Results

Authentication of archaeobotanical samples

Direct radiocarbon dating was crucial to validate the chronology of archaeological pips. The age of many samples was confirmed by radiocarbon dating but several seeds were found to be much more recent than the chronology expected according to the archaeological context (Table 1). Samples of charred plant remains were very little affected by these chronological readjustments. Many uncharred *Vitis* seeds on the other hand were given a recent age. These seeds should be regarded as

contaminations of archaeological layers by modern intrusions and cannot be taken into account in our study. Such contaminations, relatively common in archaeological layers, were not always properly taken into account in archaeobotanical investigations in the past. Most of the samples that are to be rejected come from the eastern part of the country where the climatic conditions, drier than in the western part, are probably less favorable to the preservation of waterlogged plant remains.

After the validation procedure, the remaining dataset consists of 9 charred seed samples, originating from 8 sites (N=380), and 8 uncharred seed samples from 8 sites (N=123) (Fig. 1). Intrusive pips especially impacted the supposed oldest samples. The oldest samples, only composed of a few pips, are dated from the Middle Bronze Age (1900-1500 cal BC). The Dedoplis Gora site delivered a significant sample of charred material (NB=52), dated to the Late Bronze Age (1110-940 cal BC).

The shape of the archaeological grape pips: wild and domesticated morphotypes

The first biplot of the PCA shows that the differences between the samples are weak compared to intra-sample diversity (Fig. 2). The unrooted NJ-tree realized after a LDA performed on the largest samples (N ≥ 20) nonetheless reveals a chronological trend in the organization of the samples. The existence of significant differences between the sites was checked beforehand by a MANOVA (F(336, 2996) = 5.48, p < 0.001) performed on shape descriptors and subsequent pairwise comparisons (S4). It is noteworthy that the Late Bronze Age sample from Dedoplis Gora is the only one in discordance with this temporal organization, being closer to the Roman sample from the same site than to Iron Age samples. This could potentially reflect a site-effect, a local tradition, stronger than the larger-scale chronological changes.

Preservation by charring usually causes some deformation of the pips. Experimental studies show, however, that this deformation does not prevent GMM identification of wild and domesticated morphotypes, nor, for the well preserved samples, identification of modern varieties (Ucchesu et al. 2016, Bouby et al. 2018).

In the NJ-tree the uncharred seed samples are grouped together to form the entire Medieval/Modern period group and are separated from all the charred samples, all dating from earlier periods. It is therefore difficult to assess if this separation is partly caused by deformations due to charring or if it only reflects the general chronological trend.

Following Evin et al. (2015) leave-one-out cross-validation in a LDA performed on a balanced sample of domesticated and wild grape pips randomly selected from our original modern collection, allows a very good classification of the pips into their wild/domesticated status (95.7%). The classification in the LDA of the archaeological pips allows to allocate 51.8% of the pips to the domesticated morphotype and 28.3% to the wild morphotype (threshold $p \geq 0.75$; 19.9% non-allocated). The single Middle Bronze Age sample (Dicha Gudzuba; 1746-1534 cal BC) is only composed of 3 pips allocated to the Wild-type (Fig. 3, S5). Later on, the Domesticated-type is generally dominant in the samples. The most ancient occurrence is at the Late Bronze Age site of Dedoplis Gora (1110-940 cal BC), in the Eastern part of the country (Domesticated-type=63.5%). In the western part, the oldest occurrence is in Iron Age Sukhumi (347-47 cal BC), but only few seeds are available from this region. It is of interest to underline that the Wild-type is well represented or dominant in the two Hellenistic and Roman sites. Later, during the Medieval and Modern periods, its proportions seem to decrease.

Comparison of archaeological Dom-type pips to modern varieties

The 269 seeds allocated to the Dom-type by the first LDA were then classified as additional individuals in a cultivar-level LDA. This second LDA is based on a modern collection of 280 varieties. Leave-one-out cross-validation allows a classification of 77.18% of the pips into the correct cultivar. This must be considered as a very high discrimination rate given the very large number of groups. From the 269 Domesticated-type seeds, 133 can be allocated to a specific modern cultivar with $p \geq 0.75$. This means that more than 50% of the Domesticated-type archaeological seeds cannot be attributed to our modern sample. They may correspond to cultivars not included in our comparison sample or to unknown or extinct forms.

The allocated seeds match with 65 different modern cultivars (S5). A high morphological diversity characterizes all the sites. Most cultivar-types are not detected by more than 1 or 2 seeds. The most

common morphotypes matches with 'Glycostaphyllo' (17 pips; 6 sites), 'Sliva' (8 pips; 4 sites), 'Qisi' (6 pips; 3 sites) and 'Jahafi' (5 pips; 4 sites). These morphotypes are not specific to any peculiar chronological period.

The identified morphotypes correspond to cultivars considered characteristic of different countries or large geographical areas (S6). Forty of them are regarded as typical of the Caucasus, the Near East and the Balkans, particularly of Greece. But some seeds find their best match with cultivars considered as originating from other areas of the Middle Asia, North Africa and Europe, including several Western European varieties. It should however be noted that the large majority of the pips is allocated to cultivars from the Caucasus, Near East and Balkans (Fig. 4). Moreover, when comparing the number of assigned pips to the number of pips composing each geographical group in the modern collection it is clear that the distribution of archaeological pips significantly differs from the modern sample ($Chi^2=12.584$, $p\text{-value}=0.002$, Fisher test $p\text{-value}=0.001$). The Caucasus and Near East group (EMCA) is over-represented with regard to the Central and Western European group (WCEUR). This pattern holds true regardless of the period of time the samples are dated (Fig. 5). During Antiquity (TSIK and DED2 sites) the proportion of seeds whose shape is typical of cultivars originating from Central and Western Europe is higher. This however should be regarded very cautiously as no significant difference can be detected between chronological groups using a Fisher exact test ($p\text{-value}=0.364$).

Ancient DNA affinity to autochthonous Caucasian varieties

Shotgun sequencing revealed that a majority of the archaeological seeds contained very low amounts of endogenous grape DNA (Table 2). Twelve seeds yielded a percentage of reads mapping to the grape reference genome as low as the extraction control ($\leq 0.04\%$). Since the extraction control serves as a baseline to identify erroneous mapping of short DNA to the grape genome, as well as to monitor potential contamination, we concluded that the specimens from Lagodekhi, Dedoplist Gora, Sukhimi, and Treligorebi provided no evidence for aDNA preservation. Three seeds from the most recent samples, two from Borjomi and one from Tsitsamuri, yielded $>1\%$ endogenous DNA (1.89–10.74%) and were selected for in-solution targeted enrichment so they could be compared against the modern grapevine database. As it is often observed by aDNA researchers (Carpenter et al. 2013), the fold-enrichment on the targeted SNP loci was highly variable between samples, with moderate increases for the two Borjomi samples and high enrichment for the Tsitsamuri seed.

The three enriched samples produced low to medium coverage on the targeted SNP loci, which is sufficient for conducting broad ancestry assignment analysis and evaluating potential relatedness using genotype likelihoods given a reference panel with genotype data for modern cultivars (Ramos-Madrigal et al. 2019). Although fresh seeds contain a mixture of DNA from both parents, Ramos-Madrigal et al. (2019) demonstrated archaeological grape seeds are largely composed of maternal tissue, meaning the genetic signature primarily originates from the plant carrying the grape berries. A PCA including the archaeological samples and modern accessions in the GrapeReSeq database revealed that all three archaeological seeds were most closely related to modern domesticated Georgian varieties (Fig. 6). Furthermore, when we estimate pairwise distances between the archaeological samples and the modern cultivars, the specimen with the highest coverage on the SNP loci, Tsitsamuri-3, was closest to 'Adreuli skelkana', a Georgian variety which produces white berries (Maul et al. 2019). Finally, we estimated kinship coefficients between pairs of archaeological samples using NgsRelate (S7) and found that none of the seeds show patterns consistent with highly related samples.

Discussion

The early times of grape cultivation

It is difficult to establish the time when grape cultivation started in Georgia. The oldest grape seeds dated with certainty go back to the Middle Bronze Age (1900-1500 cal BC) and belong to the wild morphotype. The domesticated morphotype is recorded, and dominant in the samples, only from the Late Bronze Age onwards (1110-940 cal BC). This most probably evidence local wine growing but it

is very young compared to what was expected and to the very early chemical traces of wine in Shulaveris Gora and Gadachrili Gora, more than 4500 years earlier. At these two sites, jar base ceramic sherds sampled from layers dated to 5900-5750 cal BC and 5700-5500 cal BC revealed the presence of wine chemical biomarkers (McGovern et al. 2017). The statement that wine was contained in the jars is not based solely on the presence of tartaric acid, which can be judged inconclusive (Stern et al. 2008, Barnard et al. 2011), but on the joint identification of a variety of organic compounds held as typical of grapes and/or wine. Tartaric, citric and malic acids can be found in high amounts in dark grapes while succinic acid is regarded as a fermentation marker (Garnier and Valamoti 2016). The combination of these different biomarkers is probably the strongest evidence that can be obtained through chemical analysis for ancient wine.

Based on the regional archaeological evidence, grapevine cultivation probably started in Georgia before the Late Bronze Age. If grape pip assemblages are more common and bigger from this period it is probably due to the intensification and spread of viticulture in the country.

In the Near East south of the Caucasus, the most ancient evidence of grape cultivation possibly dates to the 5th millennium BC, when grape seeds and pollen are recorded for the first time outside the natural range of wild grapevine (Fuller and Stevens 2019). But grape findings only become more widespread from the 4th mill BC (Fuller and Stevens 2019) and the broad cultivation of grapevine outside its natural range would have only occurred during the 3rd mill BC (Miller 2008). By the 4th mill BC grape seeds are often found with berry skins and pedicels in the sites of the Near East (Longford 2015). This suggests that grapes were not simply eaten but regularly used to make wine. In the Caucasus, a probable Chalcolithic wine making installation has been found in the cave complex of Areni-1 (Armenia). It is composed of a basin-shaped clay platform draining into a large semi-underground jar, surrounded by numerous storage vessels (Areshian et al. 2012). Desiccated grape seeds, skins, rachises and pedicels were discovered nearby. Several *Vitis* remains are dated from Late Chalcolithic times (ca 4050-3800 BC), even if other *Vitis* remains are dated from Bronze and Middle Ages (Smith et al. 2014). The hypothesis of a grape pressing and wine-making installation is corroborated by chemical results showing the presence on ceramic shreds of malvidin, an organic compound typical of red wine and pomegranate juice (Barnard et al. 2011). It is unknown if grapevine was already domesticated. No comprehensive research has been conducted on the morphology of grape pips. The results obtained from the calculation of the Stummer Index (Breadth/Length) are inconclusive (Smith et al. 2014) and this index is in any case poorly efficient when applied to modern seeds (Bouby and Marival 2001). Considering the regional context grapevine was nevertheless probably cultivated in Areni about 4000 BC, therefore possibly also in neighboring Georgia.

Wine from wild grapevines?

There is currently no archaeobotanical data to suggest that grapevine could have been domesticated as early as the beginning of the 6th millennium BC. An alternative hypothesis is that the first wines could have been made from wild grapes (Miller 2008). Microvinification experiments show that wild grapes are suitable to produce wine fermented by wild yeasts, with medium concentration of alcohol (ca 11%) and relatively high level of acidity (Arroyo-García et al. 2016). The main inconvenient is its less and irregular production.

Wild grapevine was probably already common when the first Neolithic inhabitants (SSC) settled in Georgia. The area between the Black and Caspian seas is considered as the main Quaternary glacial refugium for grapevine (Naqinezhad *et al.* 2018). Scattered charred pips have been found at several Neolithic sites in the Caucasus area (Mc Govern et al. 2017). But as far as one can tell their morphology is of the wild type. This is the case for 3 Neolithic and Chalcolithic (6th and 5th mill BC) pips in Mentesh Tepe (Decaix and Bouby, unpubl.), in Azerbaijan, where *Vitis* charcoals were also found, proving the local presence of the plant since the SSC (Decaix et al. 2016).

In Late Neolithic Dikili Tash, Northern Greece, early winemaking is suggested by the simultaneous presence of grape pressing residues (Valamoti 2015) and by chemical evidence of wine in associated vessels (Garnier and Valamoti 2016). The GMM study of these pips show that only the wild morphotype was present (Valamoti et al. 2019) and therefore that wine was produced from undomesticated grapes.

If these first Neolithic wines were produced from wild grapevines, it is quite likely that those were cultivated or managed, in order to improve and regularize their yield.

The diversity of cultivated grapevines

From the Late Bronze Age a large diversity of morphotypes of grape seeds is identified in the Georgian sites. The wild morphotype is very common until the Middle Ages. It may represent grapes collected from wild individuals growing near the settlements. People in Georgia have been reported in the recent past to regularly make wine with grapes harvested from wild plants growing on trees in the mountains (Julien 1816), even if vines deliberately cultivated on trees, a common practice in the country until recent times, could have been occasionally confused with truly wild individuals. On the other hand, the wild seed-morphotype has been found repeatedly in many Protohistoric and Historic sites in France and Greece, including wine-growing farms and urban sites, leading to the hypothesis that it represented a cultivated form (Terral et al. 2010, Bouby et al. 2013, Pagnoux et al. 2015, Valamoti et al. 2019). This wild-type would then represent, either truly wild individuals, or plants that already underwent selection for some desirable traits, but involving no identifiable change in seed morphology.

The morphology of the Dom-type pips from Georgian sites is often close to that of modern grape cultivars typical of the Caucasus and Southwest Asia. Many other seeds are similar to modern varieties from the Balkans. The identified morphological resemblance cannot be considered as direct identifications of the cultivars. Our reference collection includes only a fraction of the thousands of described varieties. However, the morphological convergences probably express a relationship between the varieties cultivated today in the region and the vines cultivated over the past 3000 years. Genetic data show that most of the Georgian modern varieties are gathered in one specific small genetic (Laucou et al. 2018). Microsatellite markers show that this group belongs to a bigger cluster mainly composed of table varieties from the Eastern Mediterranean, Western and Central Asia (Bacilieri et al. 2013). On the other hand, morphologic resemblances have long been noted between Georgian varieties and wine varieties from Asia Minor and the Balkans. Negrul (1946) considered them as two sub-groups (sub-proles *balcanica* and *georgica*) of his proles *pontica*. The predominant morphological proximities identified between ancient pips and modern cultivars from Southwest Asia and Balkan areas are therefore consistent with these relationships. Proximities identified with present varieties from other regions, such as Europe, may be explained by 1) the fact that not all oriental varieties are in our collection, 2) morphological variability within modern varieties or 3) deformation of some archaeological pips. Many of the pips allocated to European varieties are charred. Moreover, many modern cultivars are admixed and cannot be affected to any genetic group, probably as the result of long-distance exchanges along history. This is particularly true for cultivars regarded as typical of Southern Europe (Bacilieri et al. 2013, Laucou et al. 2018).

For Medieval and Modern times, direct relationships between modern and past varieties is clearly demonstrated by palaeogenomics with the archaeological seeds being most closely related to three autochthonous Georgian cultivars. Since grapevines are commonly managed through vegetative propagation, it is possible for varieties to remain genetically unchanged for centuries, and thereby lead to exact matches with archaeological pips, as observed for a 'Savagnin Blanc' grape seed from medieval Orléans, France (Ramos-Madrigal et al. 2019). One might therefore anticipate that many relatively recent archaeological specimens, such as these historic Georgian samples, would produce exact genetic matches to modern varieties. While we found that one of the archaeological seeds has a high similarity to a modern variety, our data was not sufficient to determine if they are identical. It is intriguing we did not observe more direct matches or close relationships between the other two seeds and modern varieties. A possible explanation is that the GrapeReSeq database currently includes only 20 Georgian accessions, which is a small proportion of the country's 500+ named varieties (Maghradze et al. 2012) and these relationships might only be discovered through genotyping more accessions. Another possible explanation is that Georgian varieties have remained in flux through the centuries, as recent studies demonstrate extensive gene flow between wild and domesticated populations (Riaz et al. 2018).

GMM data reveal limited changes in the diversity of grapes cultivated over time, especially in comparison to the high morphological diversity recorded at each site. Identifying the possible changes would probably require more and larger samples.

Conclusion

The combined phenotypic and genetic study of the archaeobotanical grape remains from Georgia provide evidence that grapevine is exploited and cultivated in the country at least since the Late Bronze Age. This date seems recent compared with the very early (ca 5800 BC) chemical evidence of wine making locally available and the regional archaeobotanical data showing grapevine cultivation since ca 4000 BC. This apparent contrast is probably due to the fact that recent archaeological excavations and archaeobotanical studies in Georgia are still limited in number compared to other areas south of the Caucasus. Forthcoming investigations will probably change considerably the situation. Our study provides another evidence of the need to support research based on old samples with systematic radiocarbon dating, especially when uncharred plant remains are involved. Our study combining GMM and aDNA provides the first insights into the history of grapevine diversity in a country with a very long wine-growing tradition that probably played a key role in the domestication of the species. Forthcoming archaeological excavations in the country should provide new waterlogged seed samples allowing to extend palaeogenomic research to earlier periods.

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Figure captions

Fig. 1. Location map of the investigated sites

Fig. 2. Comparison of archaeological pip samples according to seed shape (48 EFT coefficients). A. First biplot of the Principal Component Analysis performed on all the samples; B. Unrooted NJ-tree realized after a LDA performed on the largest samples ($N \geq 20$).

Fig. 3. Proportions of pips allocated by the LDA to the Domesticated and Wild morphotypes in each sample. The samples are arranged according to their chronology and location (longitude).

Fig. 4. Number of archaeological pips allocated to modern geographical groups and comparison of archaeological and modern distributions using Khi2 test (Khi2 value=12.584, DF=2, p-value=0.002).

Fig. 5. Distribution of Dom-type archaeological pips according to their date and to the geographical group of the identified cultivars. Periods: LBA=Late Bronze Age, IA=Iron Age, An=Antiquity, MA/Mo= Middle Ages/Modern times; Geographical groups: MFEAS: Middle & Far East, EMCA=Caucasus & Near East, BALK=Balkans, WCEUR=Western & Central Europe.

Fig. 6. Principal Component Analysis (PCA) biplot built using archaeological seeds and modern reference accessions from the GrapeReSeq database. a. PCA including archaeological samples, wild grapevines, and modern varieties. b. PCA including only archaeological samples and modern varieties. For both the archaeological and GrapeReSeq samples a random allele was chosen for each genomic site in the database.

Table captions

Table 1. Available samples and radiocarbon dating results.

Table 2. Archaeological grape seeds analyzed for aDNA and DNA preservation.

Supplementary information

Suppl. 1. Composition of the reference collection of modern wild grapevines (*Vitis vinifera* subsp. *sylvestris*).

Suppl. 2. Composition of the reference collection of modern grape varieties.

Suppl. 3. Authentication of ancient DNA data. Read length distribution of the mapped reads for each archaeological grape seed (left). Damage patterns observed in the sequencing data: 5' and 3' ends show an increase of C to T and G to A substitutions, respectively (right).

Suppl. 4. Pairwise comparisons of archaeological grape seed samples ($N \geq 20$) using MANOVA on shape descriptors. The p-values are provided in the table.

Suppl. 5. Detailed results of the classification of the archaeological seeds in the LDAs performed at subspecies (wild vs. domesticated) and cultivar levels.

Suppl. 6. Modern cultivars matching with the shape of archaeological pips and their main characteristics. Sex: H=Hermaphrodite, F=Female; Colour: B=Black, G=Grey, R=Red, Rs=Rose, Wh=White; Use: T=Table, W=Wine, WT=Mixt; Geographical group: BALK=Balkans, EMCA=Caucasus & Near East, IBER=Iberian Peninsula, ITAP= Italian Peninsula, MAGH=Maghreb, MFEAS= Middle & Far East, RUUK=Russia & Ukrain, WCEUR=Western & Central Europe. Geographical sub-group: BALP=Balkan Peninsula, CAUC=Caucasus, EEUR=Eastern Europe, FEAS=Far East, MEAS=Middle East, IBER=Iberian Peninsula, ITAP= Italian Peninsula,

MAG=Maghreb, RUUK=Russia & Ukrain, WEUR=Western Europe. Putative geographic origin of the cultivars according to Bacilieri et al. 2013.

Suppl. 7. Summary of the data sequenced. Number and percentage of sequenced and mapped reads obtained from the pre- and post-capture experiments. Post-capture data was used in further analyzes. Relatedness estimated on the archaeological samples. Kinship coefficients estimated between pairs of archaeological samples using genotype likelihoods (NGSrelate). Identity by descent (IBS) pairwise-distances between archaeological grape pips and modern varieties in the GrapeReSeq database.

Figures

Figure 1

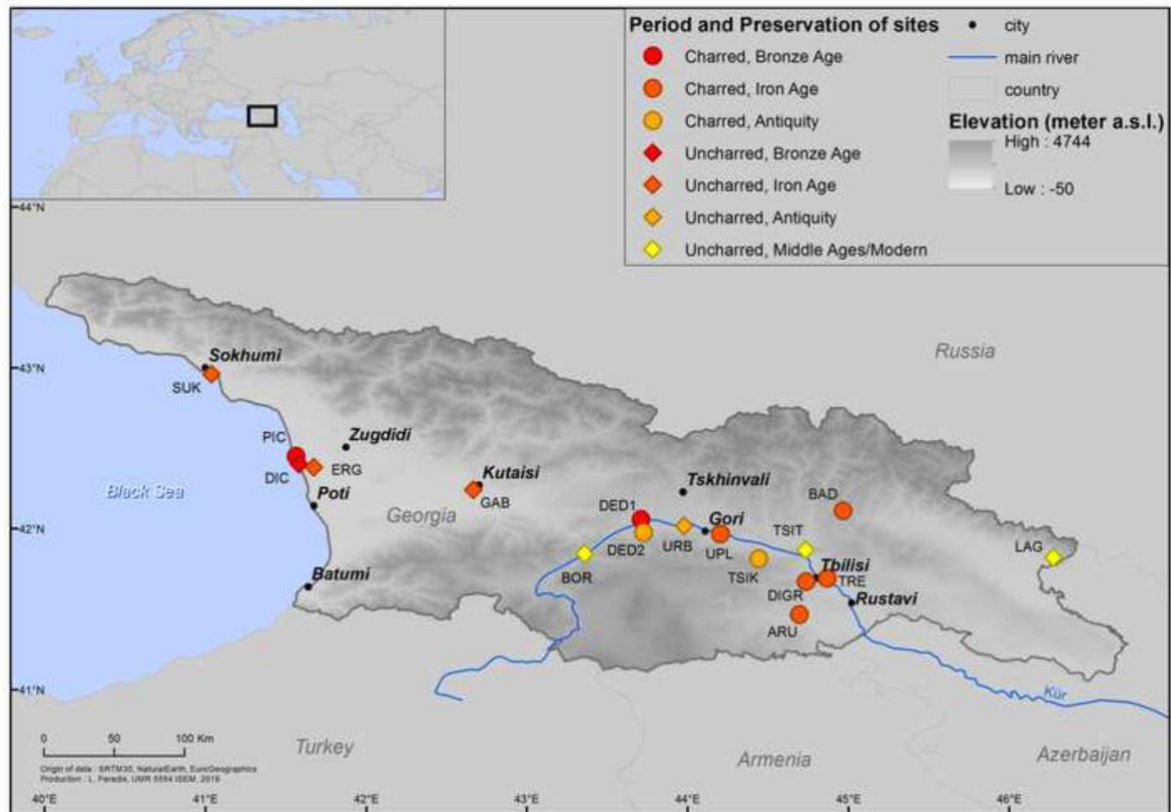


Figure2

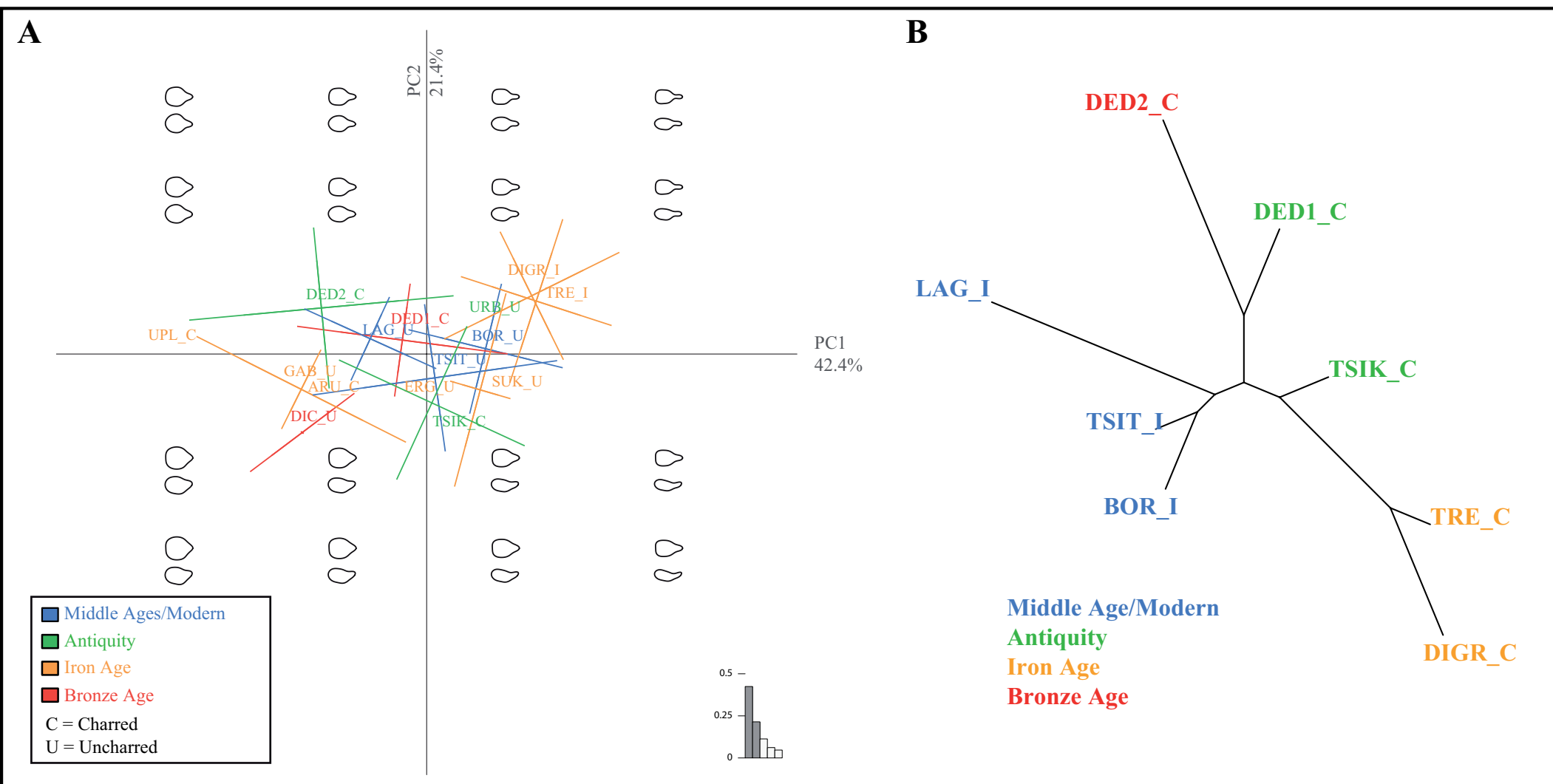


Figure3

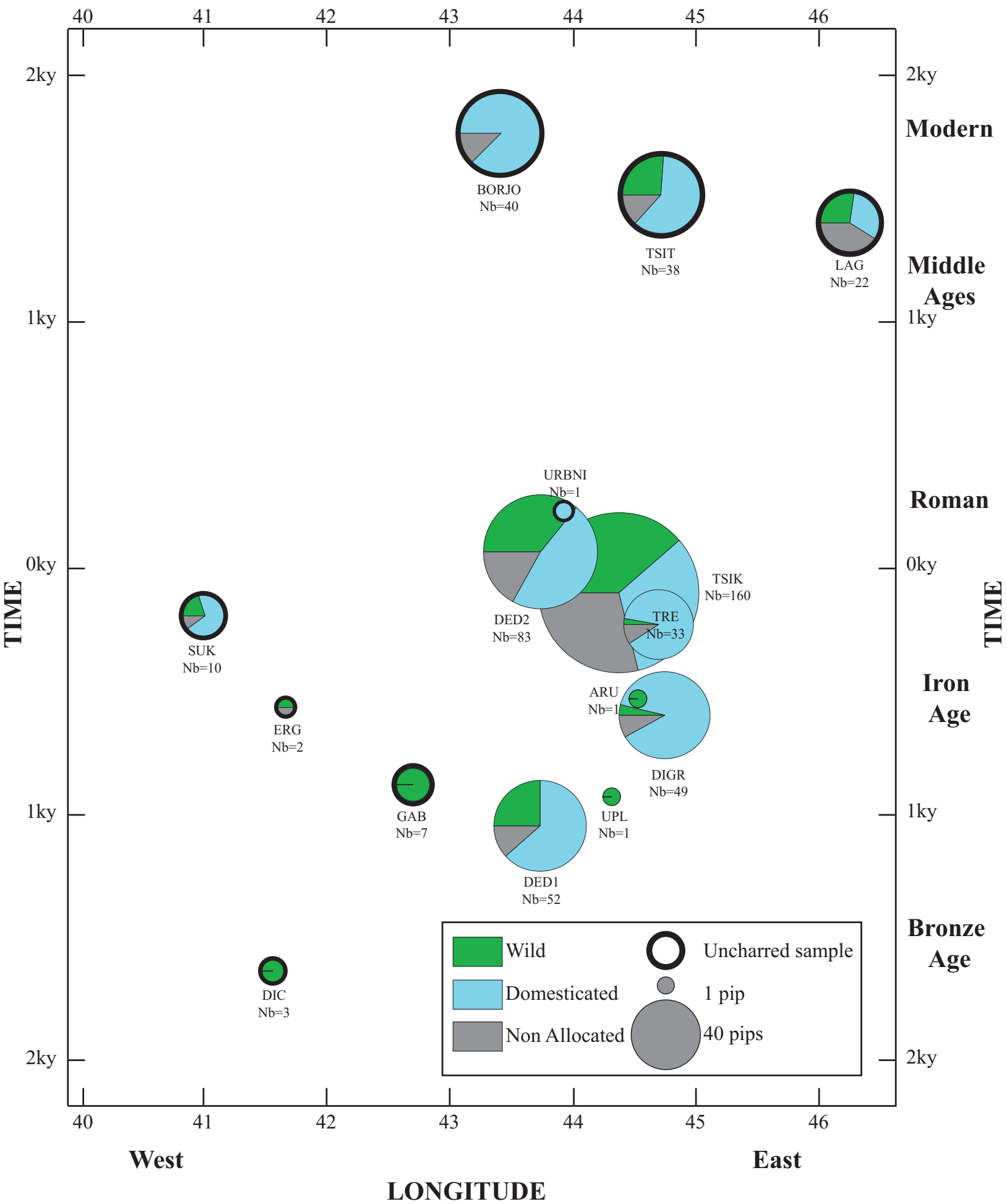


Figure4



Number of pips per group

- 10
- 20
- 30
- 40

Comparison to reference collection:
Khi2 test residual values
(red= over-represented,
blue= under-represented,
white= non-applicable)

2
1
0
-1
-2
-3

***: Fisher-test p-value < 0.05

Figure5

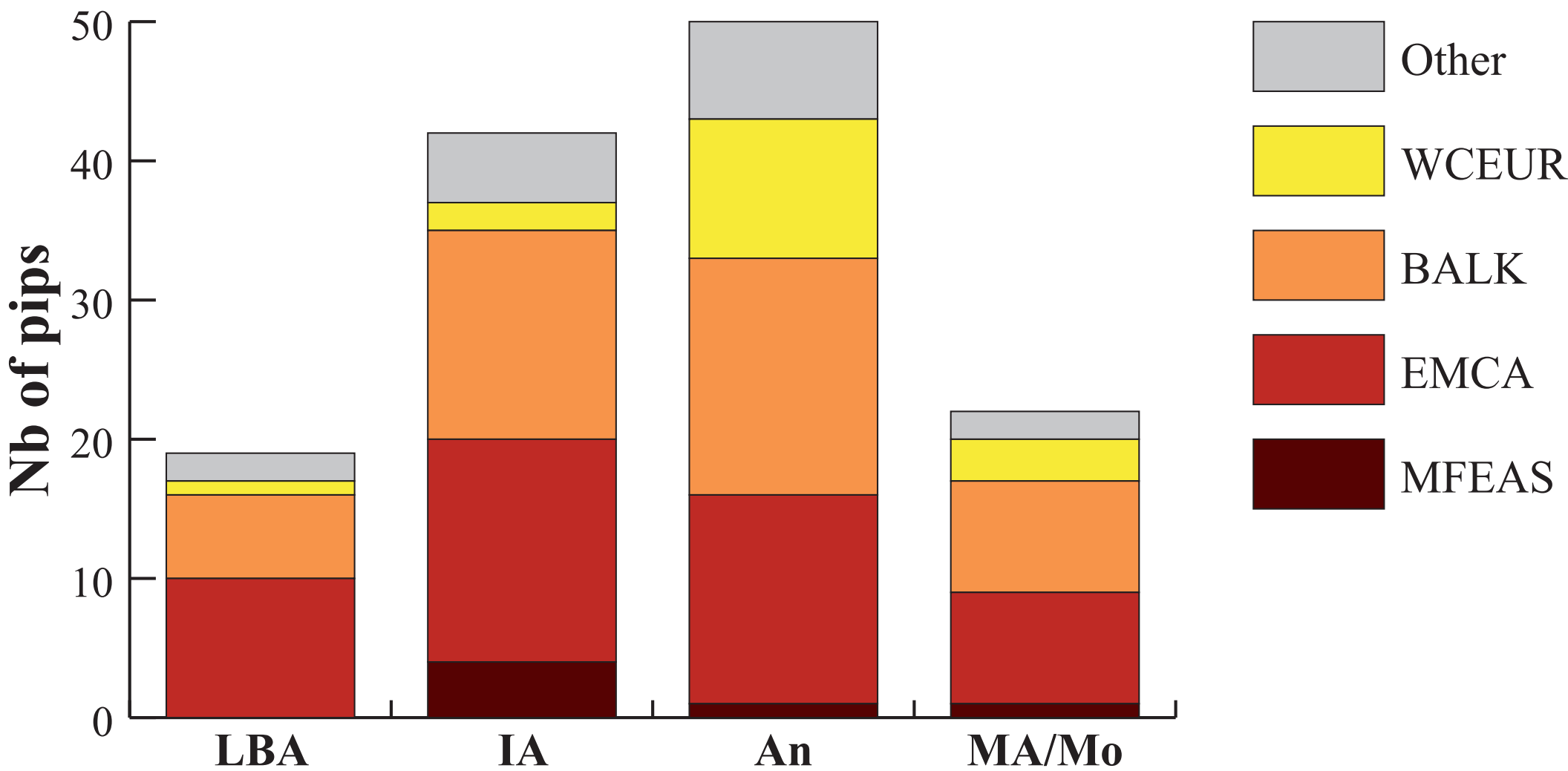
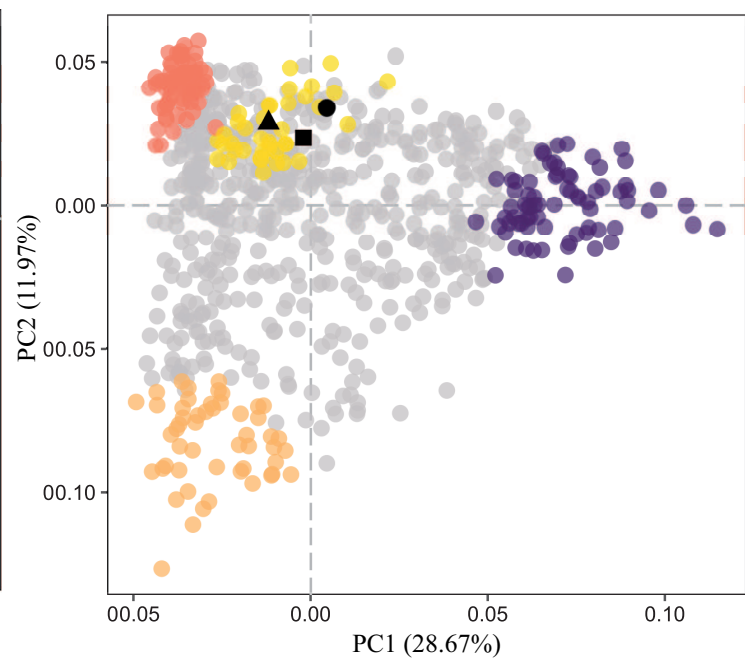
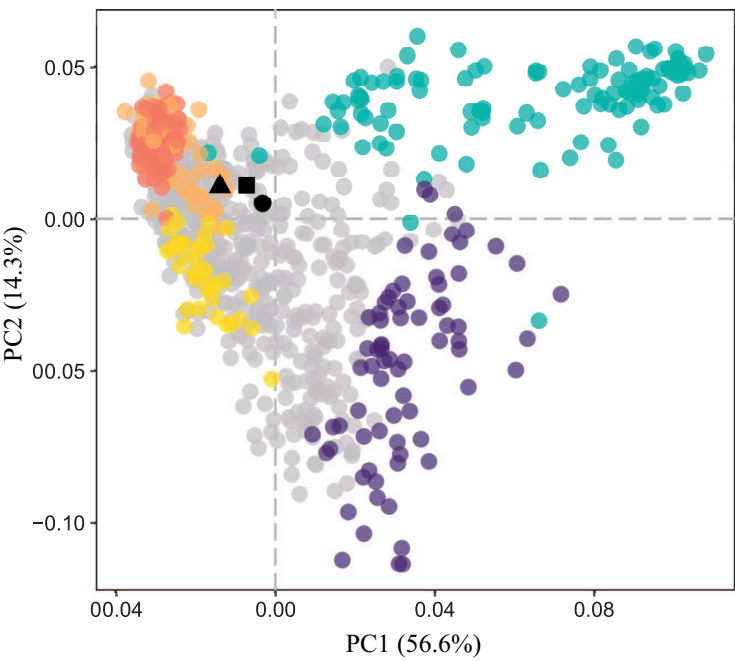


Figure6



- Wine grapes: West and Central Europe
 - Wine grapes: Iberian peninsula and Northwest Africa
 - Wine grapes: Balkans and East Europe
 - Table grapes
 - Other domesticated grapes
 - *V. vinifera* subsp. *sylvestris*
- Archaeological seeds
- Borjomi 1
 - ▲ Borjomi 2
 - Tsitsamuri 3

Tables

Table 1

Site Name	Code	Location	Longitude	Latitude	Province	Number of pips	Preservation	Expected chronology	Lab ID	C14 age ±1σ year BP	Calibrated range ± 2σ	Cultural phase
SAMPLES INCLUDED IN THE STUDY												
Pichori	PIC	Pichori	41.564626	42.450225	Samegrelo	1	Charred	2000-1500 BC	RTD 9042	3546±25	1940BC (58.5%) 1880BC 1840BC (6.6%) 1830BC 1746BC (85.2%) 1564BC	Middle Bronze Age
Dicha Gudzuba, Anaklia	DIC	Anaklia	41.58142	42.400642	Samegrelo	3	Uncharred	2000-1500 BC	RTD 7694	3369±37	1588BC (10.2%) 1534BC	Middle Bronze Age
Dedoplis Gora	DED1	Qareli District	43.71819	42.036598	Shida Kartli	52	Charred	1300-1000 BC	RTD-8892	2860±21	1110BC (90.6%) 975BC 957BC (4.8%) 940BC	Late Bronze Age
Gabashvili Datedshidzebis Gora	GAB	Kutaisi	42.72228	42.269335	Imereti	7	Uncharred	1000-700 BC	RTD-8900	2721±21	910BC (95.4%) 820 BC	Iron Age
Uplistsikhe	UPL	Uplistsikhe	44.204167	41.968333	Shida Kartli	1	Charred	1000-900 BC				Iron Age
Badaani	BAD	Tianeti District	44.966889	42.11	Mtskheta-Mtianeti	2 fgms	Charred	3000-2000 BC	RTD 7640	2520±23	789BC (26.2%) 732BC 691BC (18.2%) 661BC 651BC (51.0%) 544BC	Iron Age
Digomi Room	DIGR	Tbilisi	44.783333	41.716667	Kvemo Kartli	49	Charred	1300-1000 BC	RTD 7637	2516±24	788BC (24.3%) 730BC 692BC (17.4%) 660BC 652BC (53.7%) 543BC	Iron Age
Arukho - Layer 4	ARU	Arukho	44.694444	41.466944	Kvemo Kartli	1	Charred	6000-5000 BC	RTD 7636	2445±22	750BC (25.3%) 687BC 667BC (7.6%) 642BC 593BC (62.6%) 409BC	Iron Age
Ergeta	ERG	Kolkheti Plain	41.673068	42.382353	Samegrelo	2	Uncharred	700-500 BC	RTD 7697	2445±34	753BC (23.1%) 685BC 668BC (9.6%) 631BC 626BC (2.3%) 611BC 597BC (60.4%) 408BC	Iron Age
Treligorebi	TRE	Tbilisi	44.783333	41.716667	Tbilisi	33	Charred	800-600 BC	RTD-9425 RTD-9426	2212+33 2101+32	380BC (95.4%) 200BC 335 (0.4%) 330BC 205 (95.0%) 40BC 180BC (95.4%) 50BC	Iron Age
Sukhumi	SUK	Sukhumi	41.022675	43.004445	Abkhazia	10	Uncharred	500-300 BC	RTD-8899 RTD 7698	2096+21 2118±33	347BC (5.3%) 320BC 207BC (90.1%) 47BC	Iron Age
Tskhia Gora - Kavtskhevi	TSIK	Kaspi District	44.441542	41.81473	Shida Kartli	160	Charred	400-200 BC	RTD-7824	2107+20	193BC (84.2%) 86BC 80BC (11.2%) 55BC	Hellenistic
Dedoplis Gora	DED2	Qareli District	43.71819	42.036598	Shida Kartli	83	Charred	100-1 BC	RTD 7639	1960±29	40BC (92.1%) 87AD 105AD (3.3%) 120AD	Roman
Urbnisi cemetery	URB	Kareli District	43.977414	42.015984	Shida Kartli	1	Uncharred	1-300 AD				Roman
Lagodekhi	LAG	Lagodekhi	46.27614	41.820253	Kakheti	22	Uncharred	1000-1400 AD	RTD-7820	423±16	1436 - 1476 AD	Middle Ages/Modern
Tsistanuri	TSIT	Tsistanuri	44.73285	41.86644	Mtskheta-Mtianeti	38	Uncharred	1500-1700 AD	RTD 7701	426±31	1422AD (89.8%) 1514AD 1601AD (5.6%) 1617AD	Middle Ages/Modern
Borjomi	BOR	Borjomi	43.35825	41.843901	Imereti	40	Uncharred	1000-1300 AD	RTD 7699	247±27	1525AD (7.5%) 1558AD 1631AD (59.2%) 1678AD 1765AD (23.9%) 1800AD 1940AD (4.8%) 1955AD	Middle Ages/Modern
SAMPLES REJECTED												
Bichvinta - 23-T-1-4	BIC	Gagra District	40.42686	43.173697	Abkhazia	17	Uncharred	ca 21000 BC	RTD 7704	99.9±1.3	Modern	
Gudou River, Section 3	GD3	GUD3	40.366052	43.212541	Abkhazia	55	Uncharred	7000-3000 BC	RTD 7702	496±51	1307AD (16.5%) 1363AD 1385AD (78.9%) 1486AD	
Gudou River, section 5	GUD5	Gagra District	40.366052	43.212541	Abkhazia	80	Uncharred	7000-3000 BC	RTD-7703	189±30	1648AD (21.7%) 1694AD 1727AD (52.8%) 1813AD 1918AD (21.0%) 1955AD	
Gadachrili Gora	GAD	Marneuli Plain	44.77	41.502883	Kvemo Kartli	13	Uncharred	6000-5000 BC	RTD 7600	114.2±0.6	Modern	
Dangreuli Gora	DAN	Marneuli Plain	44.78	41.520104	Kvemo Kartli	5	Uncharred	6000-5000 BC	RTD 7647	271±19	1523AD (28.9%) 1572AD 1630AD (65.1%) 1665AD 1785AD (1.4%) 1794AD	
Shulaveris Gora	SHU	Marneuli Plain	44.77	41.502883	Kvemo Kartli	8	Uncharred	6000-5000 BC	RTD 7648	168±25	1663AD (17.3%) 1697AD 1726AD (53.3%) 1815AD 1836AD (5.4%) 1878AD 1916AD (19.4%) 1954AD	
Samtavro	SAM		44.721278	41.847656	Mtskheta-Mtianeti	50	Uncharred	1000-800 BC	RTD-7819	167±15	1666 - 1954 AD	
Nastakisi	NAS		44.564464	41.864991	Shida Kartli	12	Uncharred	1000-500 BC	RTD 7696	191±29	1650AD (22.3%) 1691AD 1728AD (53.8%) 1811AD 1923AD (19.3%) 1955AD	
Digomi Church	DIGC	Tbilisi	44.783333	41.716667	Kvemo Kartli	5	Uncharred	300-1 BC	RTD 7697	117±25	1680 (27.3%) 1740AD 1745 (0.4%) 1750AD 1750 (2.2%) 1765AD 1800 (50.3%) 1900AD 1900 (14.6%) 1940AD 1950 (0.6%) 1955AD	
Khizanaant Gora	KHI	Kareli District	43.958905	42.017095	Shida Kartli	60	Uncharred	200-400 AD	RTD 7705	99.8±1.1	Modern	

Table 2

Site	Number of seeds	Preserv.	Age	Reads mapping grape genome
Treligorebi	3	Char.	380 - 50 BC	~0.02%
Sukhumi	5	Unch.	347 - 47 BC	~0.01%; ~0.03%
Dedoplis Gora	2	Char.	40 BC - 120 AD	~0.01%
Lagodekhi	2	Unch.	1436 - 1476 AD	~0.04%
Tsitsamuri	3	Unch.	1422 - 1617 AD	1.89% ; 0.58%; 0.54%
Borjomi	2	Unch.	1525 - 1955 AD	2.11% ; 10.74%
Extraction blank	N/A	N/A	N/A	~0.04%