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Abstract The Near East and the Caucasus are commonly regarded as the original domestication centres of *Vitis vinifera* (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 of 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 grape pips from 14 sites from the Middle Bronze Age to modern times were selected for geometric morphometric studies. The shapes of the ancient pipe were compared to hundreds of modern wild individuals and cultivated varieties. Degraded DNA was isolated from three pips from two sites, converted to Illumina libraries, sequenced at approximately ten thousand SNP (Single Nucleotide Polymorphism) sites, and compared to a large public database of grapevine diversity. The most ancient pip dates from the Middle Bronze Age (1900-1500 cal BC) and the domesticated morphotype is identified from ca. 1000 BC onwards. A great diversity of domesticated shapes was regularly seen in the samples. Most are close to modern cultivars from the Caucasian, southwest Asian and Balkan areas, which suggests that the modern local vine diversity is deeply rooted in early 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

Vitis vinifera ssp. *vinifera* (grapevine) was probably first domesticated in southwest Asia, where the most ancient archaeobotanical traces of grape cultivation have been found (for example, 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 relationships 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 Sea and the Caspian Sea, 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 in 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 including 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 of the country, 2) the geographical location of Georgia, 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 local varieties cover 95% of the total area of vineyards in Georgia (Maghradze et al. 2012). Similar to the ancient Mediterranean practice, the typical Georgian tradition is to make wine in large pottery vessels set in the ground or buried, 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 the resulting 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 Unesco (https://ich.unesco.org/en/RL/ancient-georgian-traditional-qvevri-wine-making-method-00870). 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 crucial support when chemical analyses of residues absorbed in pottery vessels showed evidence of winemaking at the SSC sites of Shulaveris Gora and Gadachrili Gora (5900-5500 cal BC) (McGovern et al. 2017). This result predates by at least 500 years the previous earliest evidence for wine, also obtained by chemical analysis, from 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 in Georgia. Both agree in identifying the wide area south of the Caucasus as the primary zone of the emergence of winemaking.

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

The archaeobotanical documentation on the early history of viticulture in the southern Caucasus can however be considered as still limited and poorly known, first because of the small number of systematic archaeobotanical investigations there 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 mentioning the finds of grape pips. It is sometimes difficult to understand if the pips were preserved by charring or another process. In many cases the excavations and original archaeobotanical studies were carried out many years ago and detailed information on the archaeological contexts and on sample composition is not always available.

In the framework of a national Georgian research programme (Maghradze et al. 2016) it was decided to systematically review the archaeobotanical grape pips, to obtain direct radiocarbon dates from them and to apply 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 first occurred in the country and 3) explore how the cultivated grapes changed through time compared to the modern diversity.

Materials and methods

Vitis pip samples and radiocarbon dating

The *Vitis* pip samples available from archaeological archives and current archaeobotanical investigations were assessed and the related information on context and preservation conditions was recorded (Table 1). Carbonized grape pips have been recovered from eight sites and nine time periods, with an expected chronology according to the archaeological contexts ranging from the Neolithic to the Roman period (ca. 6000 cal BC-AD 500). Most of the samples, from 18 sites, were composed of uncharred remains, with an expected chronology ranging from the Palaeolithic until modern times and including several Neolithic sites. Radiocarbon dating was done on 27 pips selected from 25 sites. Two samples, composed of isolated pips, could not be dated. The dating was carried out at the D-REAMS radiocarbon dating laboratory at Rehovot, Israel. Calibrated ages (95.4% probability) were obtained using OxCal v. 4.2 (Bronk Ramsey 2010) and the IntCal13 atmospheric curve (Reimer et al. 2013).

Geometric morphometrics (GMM)

Except the grape pip from Pichori, which could not be photographed before radiocarbon dating, and the sample from Badaani, which was only composed of broken pips, GMM investigations were performed on all the available samples (14 sites, 15 samples, 502 pips). Through the quantitative description of pip outlines using the Elliptic Fourier Transform method, GMM allows a powerful discrimination of wild and domesticated grape pips and characterization of the changes in cultivated diversity through time (Terral et al. 2010; Pagnoux et al. 2015). Each pip was photographed in dorsal and lateral views using an Olympus SZ-ET stereomicroscope and an Olympus DP12 digital camera. The images were converted to black masks. The x and y coordinates of 360 equidistant points were sampled on each outline. Outlines were normalized before the EFT computations by centring, scaling using their centroid size and defining the first point right above the centroid. We used only the coefficients from the six first harmonics (48 coefficients) in the statistical analyses. All analyses were carried out using the Momocs outline analysis package (Bonhomme et al. 2014) in R environment (R

Development Core Team, R v. 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 or domesticated status of the pips and the closeness between domesticated archaeological pips and modern varieties we used predictive discriminant analysis. The archaeobotanical samples were compared to a reference collection of modern grape pips from 82 wild grapevines and 280 traditional cultivars considered as typical of various areas of Europe, the Mediterranean and the Caucasus (ESM 1, ESM 2: Pagnoux et al. 2015). Wild grapes were sampled by us in several countries covering most of the distribution area of *V. vinifera* ssp. *sylvestris* in France, Germany, Georgia, Greece, Italy, Spain, Switzerland and Turkey. Most of our cultivars were selected and sampled from the INRA Grape Germplasm Repository Domaine de Vassal, Marseillan-Plage, France, with the aim of being representative of the global diversity. Additionally, 43 native cultivars from Georgia were sampled from the Saguramo Grape Repository, Jighaura, Georgia. We have chosen wine and table varieties typical of various regions of Georgia.

The comparison of archaeological grape pips to the modern collection was carried out using two nested LDAs. We first compared the archaeological pips with modern wild (N=2,430) and domesticated (N=2,430) 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 pips 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* pips 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 pips (Wales et al. 2016), as it enables characterization of very short (<50bp) endogenous DNA molecules, including those with age-related chemical damage. Following a recently established methodology for *Vitis* archaeogenetics (Ramos-Madrigal et al. 2019), we performed such a shotgun sequencing and targeted enrichment of 10,000 SNP loci in 17 grape pips from six archaeological sites, as summarized below.

DNA was extracted from archaeological pips 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 polymerase chain reaction (PCR) to infer the appropriate number of PCR cycles needed 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 sequencer. Samples with >1% grape DNA were enriched for 10,000 informative single nucleotide polymorphism (SNP) loci with a custom-designed MYbaits kit (Arbor Biosciences, Ann Arbor, MI, USA), following an established protocol (Ramos-Madrigal et al. 2019).

Processing of sequencing data was done following the approach described in Ramos-Madrigal et al. (2019). In brief, AdapterRemoval 2.0 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 and Durbin 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 (ESM 3; Malaspinas et al. 2014). 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 smart PCA lsq project (Patterson et al. 2012) 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 1.9 (Chang *et al*. 2015) 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 2015) to evaluate potential relatedness among the archaeological pips as described in Ramos-Madrigal et al. (2019).

Results

Authentication of archaeobotanical samples

Direct radiocarbon dating was crucial to validate the chronology of the archaeological pips. The age of many samples was confirmed by radiocarbon dating but several pips 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* pips, on the other hand, were given a recent age. These 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 were rejected came from the eastern part of the country where the climatic conditions, drier than in the western part, are probably less favourable for the preservation of waterlogged plant remains.

After the validation procedure, the remaining dataset consisted of nine charred pip samples, originating from eight sites (N=380), and eight uncharred pip samples from eight sites (N=123) (Fig. 1). Intrusive pips especially affected the supposedly oldest samples, which were only composed of a few pips and dated to the Middle Bronze Age (1900-1500 cal BC). The Dedoplis Gora site provided 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 diversity between samples (Fig. 2). The unrooted neighbour joining 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 multivariate analysis of variance MANOVA (F (336, 2996) = 5.48, p<0.001) performed on shape descriptors and subsequent pairwise comparisons (ESM 4). It is noteworthy that the Late Bronze Age sample from Dedoplis Gora is the only one which does not fit this chronological organization, being closer to the Roman period 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 does not prevent geometric morphometric 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 neighbour joining tree the uncharred pip 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 deformation 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 wild or domesticated status (95.7%). The classification in the LDA of the archaeological pips allows allocation of 51.8% of the pips to the domesticated-type morphotype and 28.3% to the wild type morphotype (threshold p ≥0.75; 19.9% non-allocated). The single Middle Bronze Age sample (from Dicha Gudzuba; 1746-1534 cal BC) is only composed of three pips allocated to the wild type (Fig. 3, ESM 5). Later on, the domesticated-type is generally dominant in the samples. The most ancient occurrence is from the Late Bronze Age site of

Dedoplis Gora (1110-940 cal BC), in the eastern part of the country (domesticated-type, 63.5%). In western Georgia, the oldest occurrence is from Iron Age Sukhumi (347-47 cal BC), but only a few grape pips were available from this region. It is of interest to note that the wild type is well represented or dominant from the two Hellenistic and Roman sites. Later, from the medieval and modern periods, its proportions seem to decrease.

Comparison of archaeological domesticated-type pips to modern varieties

The 269 pips allocated to the domesticated-type by the first LDA were then classified as additional individuals in a cultivar level second LDA, 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 pips, 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 pips 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 pips match with 65 different modern cultivars (ESM 5). A high morphological diversity characterizes all the sites. Most cultivar types are not represented by more than one or two pips. The most common morphotypes match 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 particular chronological period.

The identified morphotypes correspond to cultivars considered characteristic of different countries or large geographical areas (ESM 6). Forty of them are regarded as typical of the Caucasus, the Near East and the Balkans, particularly Greece. But some pips find their best match with cultivars considered as originating from other areas of Western 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 composing each geographical group in the modern collection, it is clear that the distribution of archaeological pips significantly differs from the modern sample (*Chi*²12.584, p value=0.002, Fisher test p 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 date of the samples (Fig. 5). During Antiquity (TSIK and DED2 sites) the proportion of pips 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 the chronological groups using a Fisher exact test (p value=0.364).

Ancient DNA affinity to native Caucasian grape varieties

Shotgun sequencing revealed that a majority of the archaeological pips contained very low amounts of endogenous grape DNA (Table 2). Twelve pips yielded a percentage of reads mapping to the grape reference genome as low as the extraction control (≤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 Treligorebi, Sukhumi, Dedoplis Gora and Lagodekhi provided no evidence for aDNA preservation. This finding was not unexpected for the charred pips from Treligorebi and Dedoplis Gora, as previous research has revealed that high throughput sequencing of charred plant remains rarely yields useful amounts of endogenous DNA (Nistelberger et al. 2016). However given the rarity of uncharred pips from Georgia in this key chronological period, we dedicated the resources to fully explore the possibility of preserved DNA in them. The lack of endogenous DNA from the uncharred pips from Sukhumi and Lagodekhi suggests that other factors may have a significant impact on DNA preservation, such as microbial activity, age-related degradation, or specific soil chemistry.

Three pips 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 is often observed by aDNA researchers (Carpenter et al. 2015), 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 pip.

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 grape pips contain a mixture of DNA from both parents, Ramos-Madrigal et al. (2019) demonstrated that archaeological pips are largely composed of maternal tissue, meaning that the genetic signature primarily originates from the plant carrying the grapes. A PCA including the archaeological samples and modern accessions in the GrapeReSeq database revealed that all three archaeological pips 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 white grape variety (Maul et al. 2019). Finally, we estimated kinship coefficients between pairs of archaeological samples using NgsRelate and found that none of the pips showed patterns consistent with highly related samples (ESM 7).

Discussion

The beginnings of grape cultivation

It is difficult to establish the date when grapevine cultivation started in Georgia. The oldest grape pips 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 represents evidence of local vine growing, but it is very late compared to what was expected and to the very early chemical traces of wine from Shulaveris Gora and Gadachrili Gora, more than 4,500 years earlier. At these two sites, pottery jar base 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 was 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 thought to be typical of grapes and/or wine. Tartaric, citric and malic acids can be found in large 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 for ancient wine that can be obtained through chemical analysis.

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 larger from this period, it is probably due to the intensification and spread of viticulture in the country then.

In the Near East to the south of the Caucasus, the most ancient evidence of grape cultivation possibly dates to the 5th millennium BC, when grape pips and pollen are recorded for the first time outside the natural range of wild grapevine (Fuller and Stevens 2019). But grape finds only become more widespread from the 4th millennium BC (Fuller and Stevens 2019) and the general cultivation of grapevine outside its natural range would have only occurred from the 3rd millennium BC (Miller 2008). By the 4th millennium BC grape pips are often found with fruit 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 pips, fruit 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 the Bronze Age and medieval (Smith et al. 2014). The hypothesis of a grape pressing and wine-making installation is corroborated by chemical results showing the presence on potsherds of malvidin, an organic compound typical of red wine and pomegranate juice (Barnard et al. 2011). It is unknown if grapevines were already domesticated. No comprehensive research has been done on the morphology of grape pips from Armenia. 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 not very efficient when applied to modern pips (Bouby and Marinval 2001). Considering the regional context, grapevine was nevertheless probably cultivated in Areni about 4000 BC, therefore possibly also in neighbouring 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 wine could have been made from wild grapes (Miller 2008). Microvinification experiments show that wild grapes are suitable for making wine fermented by wild yeasts, with a medium concentration of alcohol (ca. 11%) and a relatively high level of acidity (Arroyo-García et al. 2006). The main disadvantage of using wild vines is their smaller and irregular production of grapes.

Wild grapevine was probably already common when the first Neolithic inhabitants of the Shulaveri-Shomutepe culture (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 (McGovern et al. 2017). But as far as one can tell their morphology is of the wild-type. This is the case for three Neolithic and Chalcolithic (6th and 5th millennium BC) pips from Mentesh Tepe, Azerbaijan (Decaix and Bouby, unpubl.), where *Vitis* charcoal was also found, proving the local presence of the vine since the SSC (Decaix et al. 2016). In Late Neolithic Dikili Tash, northern Greece, early wine making 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 shows that only the wild morphotype was present (Valamoti et al. 2020) and therefore that this wine was produced from undomesticated grapes.

If these first Neolithic wines were produced from wild grapes, it is quite likely that the vines 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 pips has been identified from the sites in Georgia. 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 gathered from wild plants growing on trees in the mountains (Julien 1816), even if vines deliberately grown up 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 pip morphotype has been found repeatedly in many protohistoric and historic period sites in France and Greece, including vineyards 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. 2020). This wild-type would then represent either truly wild individuals or plants that had already been selected for some desirable traits, but involving no identifiable change in pip morphology.

The morphology of the omesticated type pips from Georgian sites is often close to that of modern grape cultivars typical of the Caucasus and southwest Asia. Many other pips are similar to modern varieties from the Balkans. The identified morphological resemblance cannot be considered as a direct identification of the cultivars. Our reference collection includes only a fraction of the thousands of described varieties. However, the morphological similarities probably express a relationship between the varieties cultivated today in the region and the vines cultivated there over the past 3,000 years. Genetic data show that most of the modern Georgian varieties are gathered into one specific small genetic group (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, morphological resemblances have long been noted between Georgian grape 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 prole *pontica*. The predominant morphological proximities identified between ancient pips and modern cultivars from southwest Asia and the Balkans are therefore consistent with these relationships. Proximities identified with present varieties from other regions, such as the rest of Europe, may be explained by 1) the fact that not all western Asian 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 hybridised and cannot be assigned to any genetic group, probably as the result of long-distance exchanges through 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 grape pip being most closely related to three native Georgian cultivars. Since grapevines are usually propagated by vegetative means from cuttings, it is possible for varieties to remain genetically unchanged for centuries, and this could therefore have led to exact matches with archaeological pips, as observed for a 'Savagnin Blanc' grape pip 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 grape varieties. While we found that one of the archaeological pips had a close similarity to a modern variety, our data were insufficient to determine if they were identical. It is intriguing that we did not observe more direct matches or close relationships between the other two pips 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 grape varieties cultivated over time, especially in comparison to the high morphological diversity recorded at each site. Identifying these possible changes would probably require more and larger samples.

Conclusions

The combined phenotypic and genetic study of some archaeobotanical grape remains from Georgia provide evidence that grapevines were used and cultivated in the country at least since the Late Bronze Age. This date seems recent compared with the much earlier (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 the situation considerably. Our study provides further evidence for 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 pip samples allowing the extension of palaeogenomic research to earlier periods.

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Figure legends

Fig. 1 Location map of the investigated sites; abbreviations for sites in Table 1

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 neighbour joining tree realized after a LDA performed on the largest samples (N ≥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 Chi² (X^2) test (Chi² value=12.584, DF=2, p-value=0.002). Abbreviations, IBER, Iberian Peninsula; MAGH, northwest Africa; ITAP; RUUK; (others as for Fig. 5)

Fig. 5 Distribution of domesticated-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 of archaeological pips and modern reference accessions from the GrapeReSeq database. Left, PCA including archaeological samples, wild grapevines and modern varieties; on right, 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 1 Investigated samples and radiocarbon dating results. All samples analysed for radiocarbon dating consisted of a single grape pip. The archaeological information and expected age are provided together with the chemical data. Carbon percentage (C%) is the carbon measured in the sample after pre-treatment. The stable carbon isotope ratio ($\delta^{13}C$) was measured with the accelerator mass spectrometer, so it does not represent the natural/charred isotope ratio

Table 2 Archaeological grape pips analyzed for aDNA and DNA preservation

Figure1

Click here to access/download;Figure;Fig1_map.tif

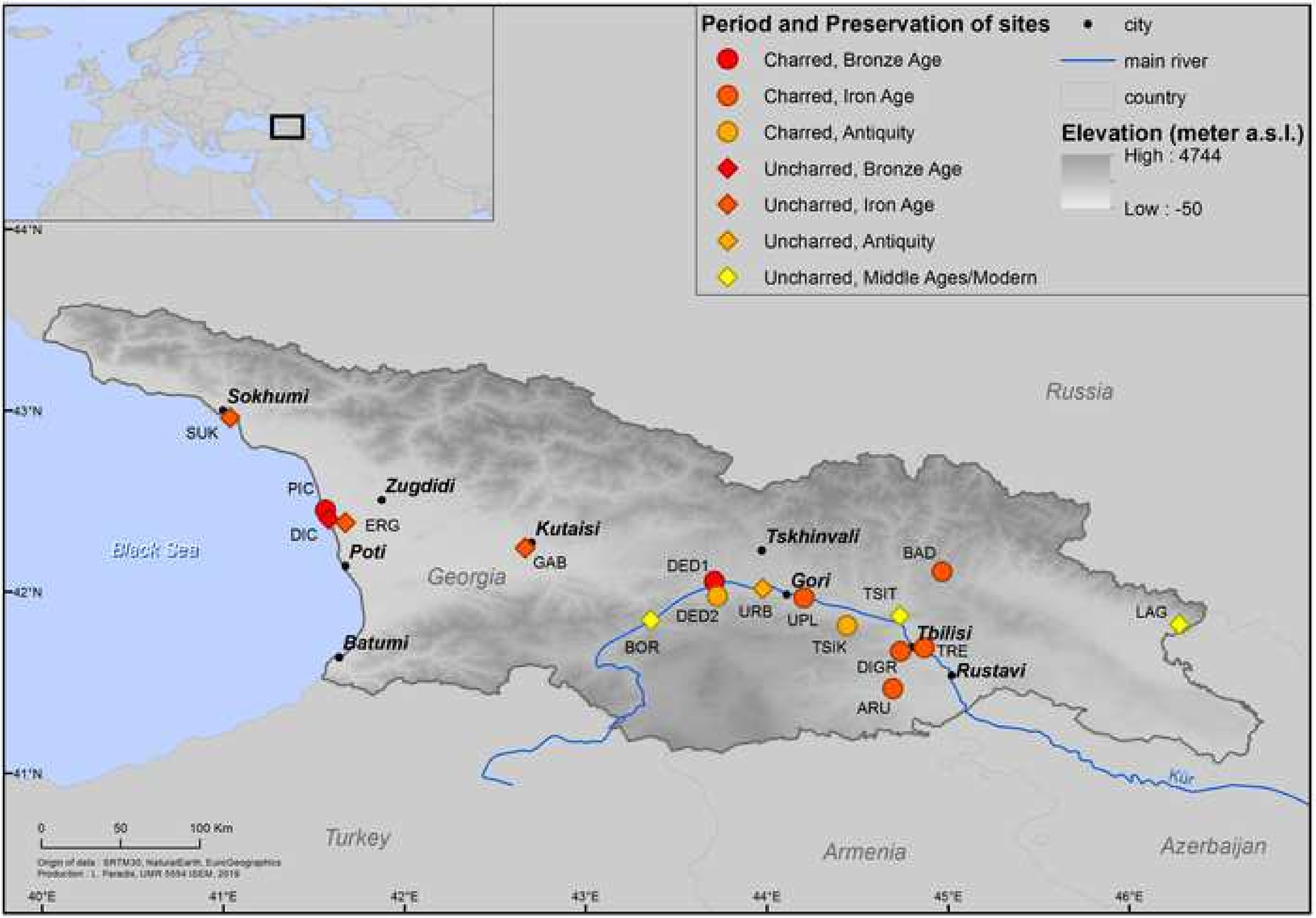
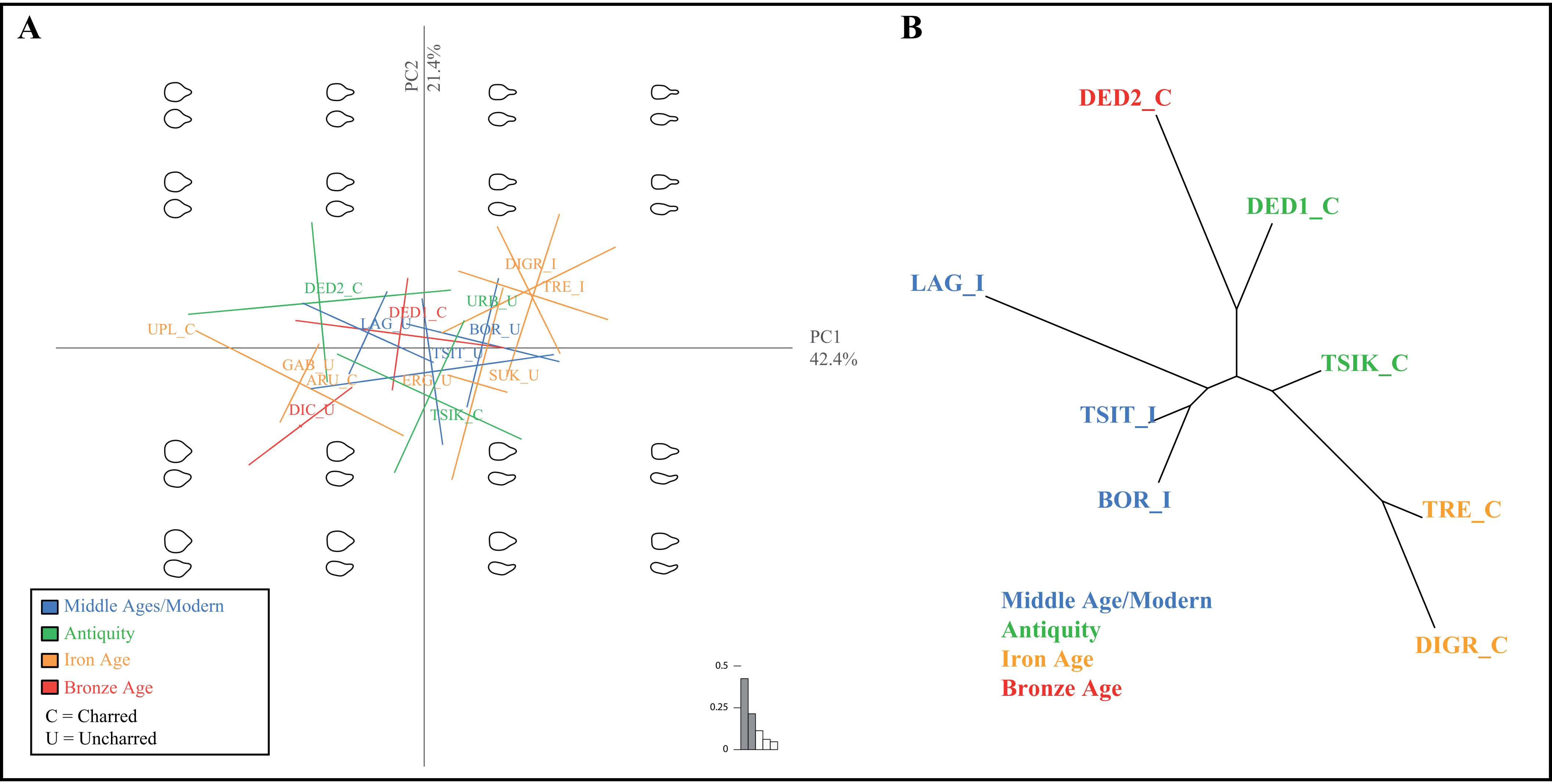


Figure2



A
PC2
21.4%
PC1
42.4%
DIGR_I
TRE_I
DED2_C
URB_U
DED1_C
LAG_U
BOR_U
UPL_C
TSIT_U
GAB_U
ARU_C
ERG_U
SUK_U
DIC_U
TSIK_C
Middle Ages/Modern
Antiquity
Iron Age
Bronze Age
C = Charred
U = Uncharred
0.5
0.25
0
B
DED2_C
DED1_C
LAG_I
TSIK_C
TSIT_I
BOR_I
TRE_C
DIGR_C
Middle Age/Modern
Antiquity
Iron Age
Bronze Age

Figure3

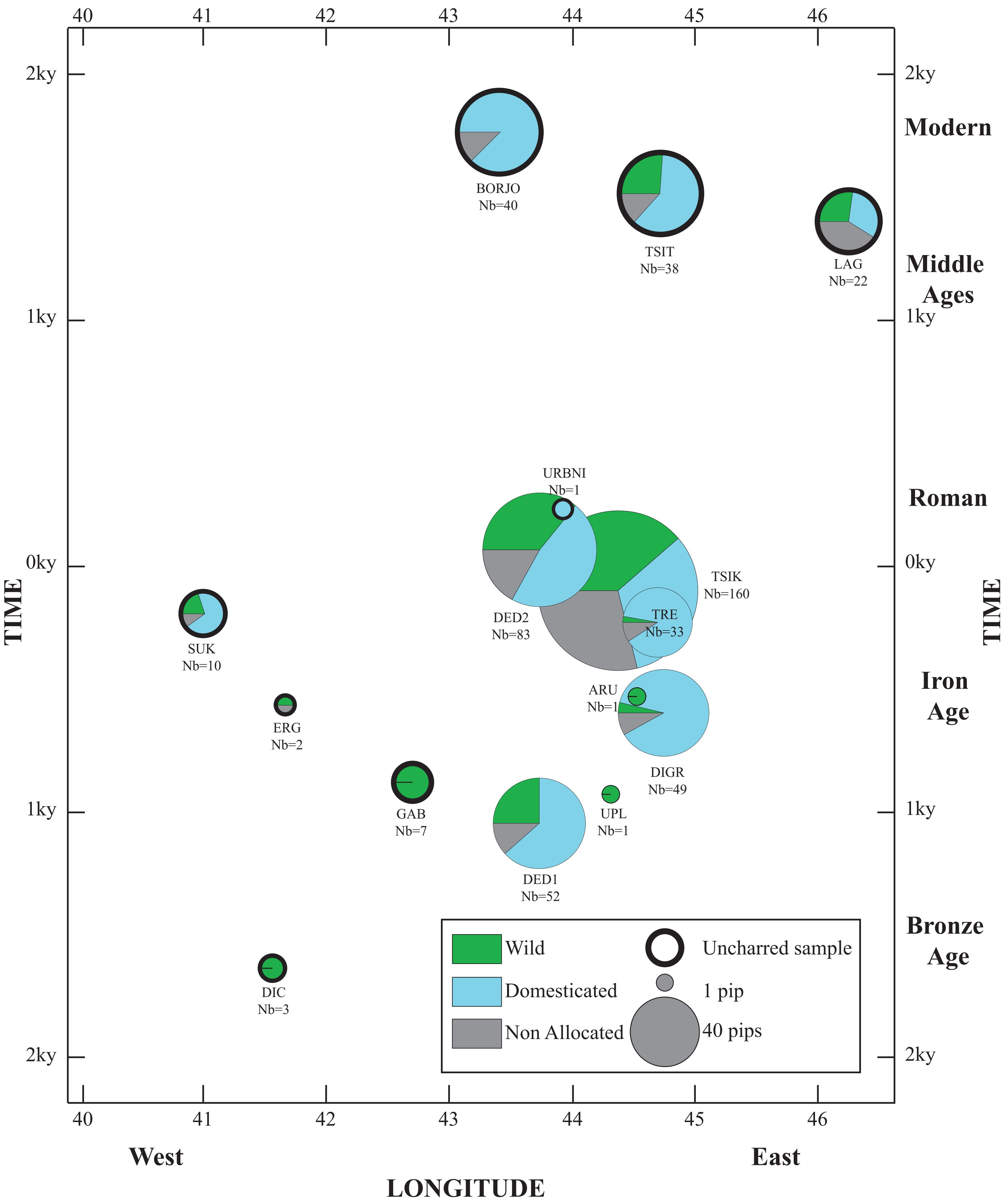

40
41
42
43
44
45
46
2ky
1ky
0ky
1ky
2ky
Modern
Middle Ages
Roman
Iron Age
Bronze Age
TIME
BORJO
Nb=40
TSIT
Nb=38
LAG
Nb=22
URBNI
Nb=1
TSIK
Nb=160
DED2
Nb=83
TRE
Nb=33
SUK
Nb=10
ARU
Nb=1
ERG
Nb=2
DIGR
Nb=49
GAB
Nb=7
UPL
Nb=1
DED1
Nb=52
DIC
Nb=3
Wild
Domesticated
Non Allocated
Uncharred sample
1 pip
40 pips
West
East
LONGITUDE

Figure4

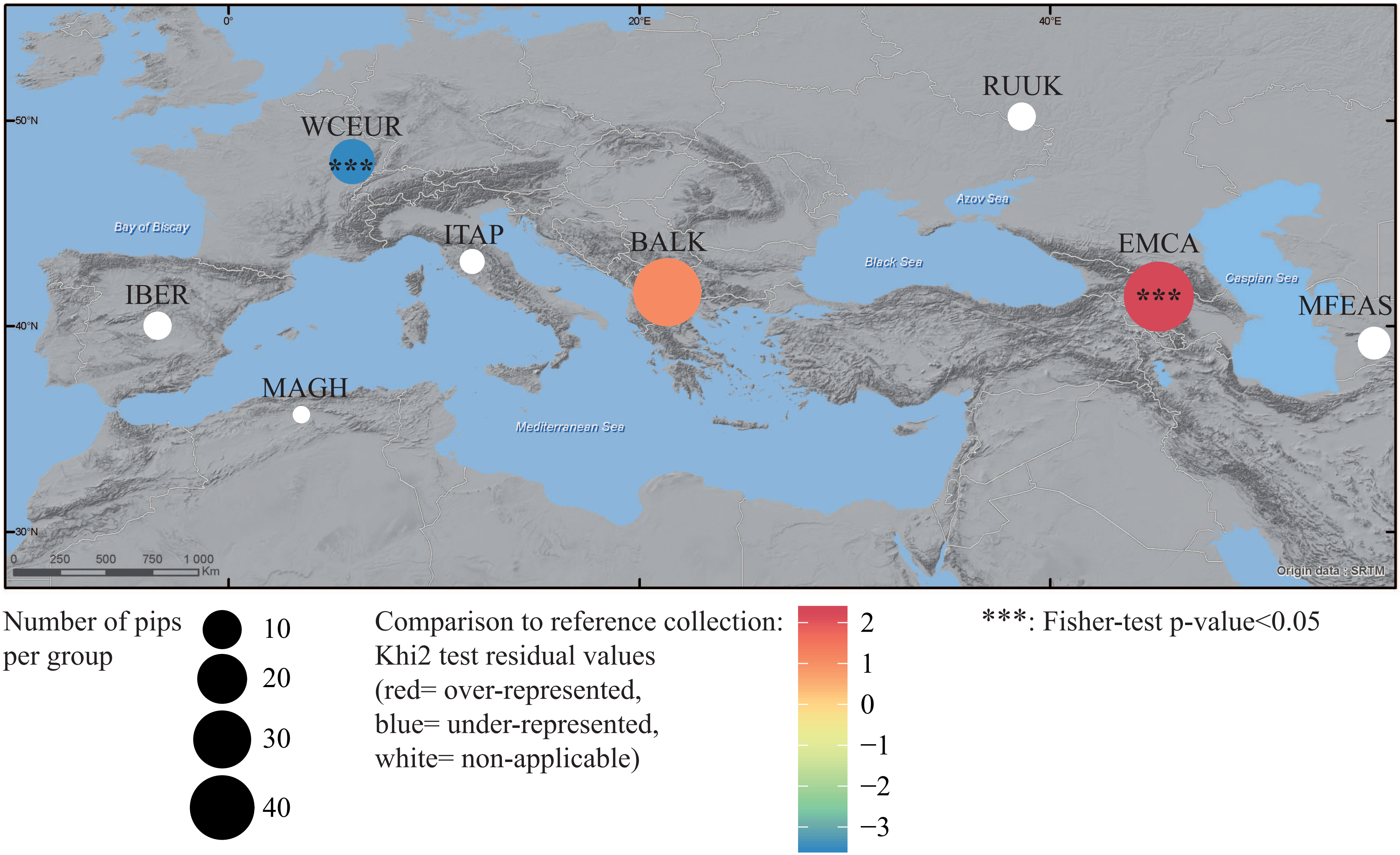

0°
20°E
40°E
50°N
40°N
30°N
RUUK
WCEUR
***
Azov Sea
Bay of Biscay
ITAP
BALK
EMCA
***
Black Sea
Caspian Sea
IBER
MFEAS
MAGH
Mediterranean Sea
0 250 500 750 1 000
Km
Origin data : SRTM
Number of pips per group
10
20
30
40
Comparison to reference collection: Khi2 test residual values (red= over-represented, blue= under-represented, white= non-applicable)
2
1
0
−1
−2
−3
***: Fisher-test p-value<0.05

Figure5

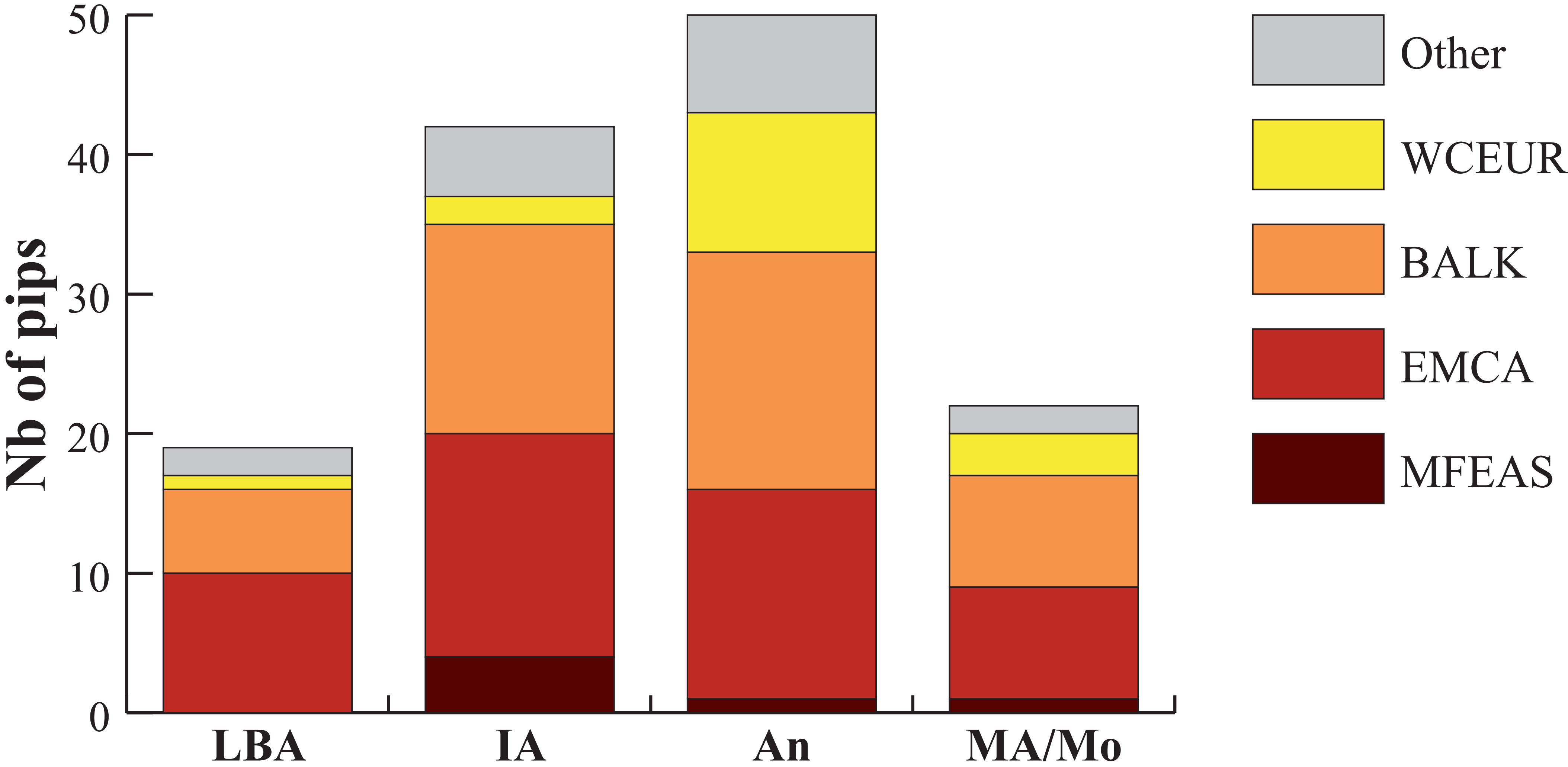
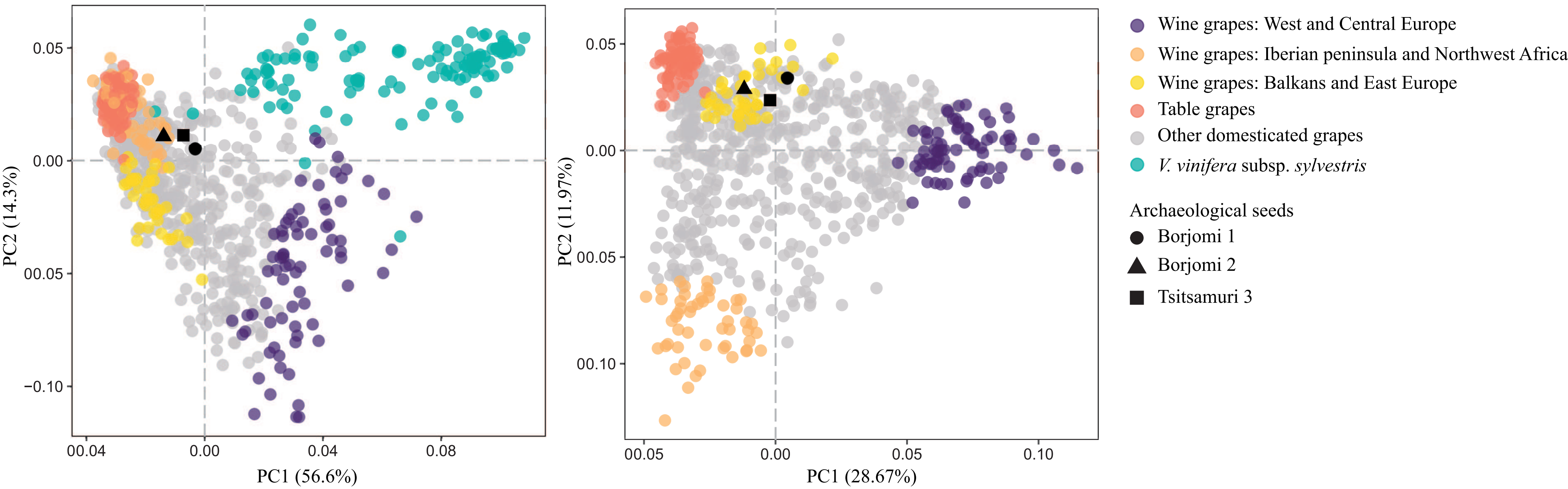

Nb of pips
50
40
30
20
10
0
LBA
IA
An
MA/Mo
Other
WCEUR
BALK
EMCA
MFEAS

Figure6



PC2 (14.3%)
PC1 (56.6%)
PC2 (11.97%)
PC1 (28.67%)
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

Table 1 Revised

Click here to access/download;Table;Tab1_Sites_Rev.xls

Site Name	Code	Location	Longitude	Latitude	Province	Number of pips	Preservation	Expected chronology	Lab ID	C %	$\delta^{13}C$ AMS ‰	^{14}C age ±1σ year BP	Calibrated range ± 2 σ	Cultural phase
SAMPLES INCLUDED IN THE STUDY														
Pichori	PIC	Pichori	41.564626	42.450225	Samegrelo	1	Charred	2000-1500 BC	RTD 9042	52.0	-26.2	3546±25	1940BC (58.5%) 1880BC 1840BC (6.6%) 1830BC	Middle Bronze Age
Dicha Gudzuba, Anaklia	DIC	Anaklia	41.58142	42.400642	Samegrelo	3	Uncharred	2000-1500 BC	RTD 7694	40.6	-28.4	3369±37	1746BC (85.2%) 1604BC 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	68.8	-23.9	2860±21	1110BC (90.6%) 975BC 957BC (4.8%) 940BC	Late Bronze Age
Gabashvili Dateshidzeebis Gora	GAB	Kutaisi	42.72228	42.269335	Imereti	7	Uncharred	1000-700 BC	RTD-8900	58.7	-26.3	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 fgmts	Charred	3000-2000 BC	RTD 7640	65.7	-21.8	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	70.2	-20.7	2516±24	788BC (24.3%) 730BC 692BC (17.4%) 660BC 652BC (53.7%) 543BC	Iron Age
Arukhlo - Layer 4	ARU	Arukhlo	44.694444	41.466944	Kvemo Kartli	1	Charred	6000-5000 BC	RTD 7636	66.4	-21.6	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	41.0	-26.9	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 RTD-8899	64.7 65.6 77.0	-25.24 -27.21 -26.2	2212±33 2101±32 2096±21	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 7698	55.5	-27.4	2118±33	347BC (5.3%) 320BC 207BC (90.1%) 47BC	Iron Age
Tsikhia Gora - Kavtiskhevi	TSIK	Kaspi District	44.441542	41.81473	Shida Kartli	160	Charred	400-200 BC	RTD-7824	70.3	-24.9	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	70.4	-20.4	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	54.3	-27.3	423±16	1436 - 1476 AD	Middle Ages/Modern
Tsistamuri	TSIT	Tsistamuri	44.73285	41.86644	Mtskheta-Mtianeti	38	Uncharred	1500-1700 AD	RTD 7701	48.4	-20.6	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	44.8	-19.8	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	54.4	-21.3	99.9±1.3	Modern	
Gudou River, Section 3	GUD3		40.366052	43.212541	Abkhazia	55	Uncharred	7000-3000 BC	RTD 7702	55.6	-15.5	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	29.0	-20.5	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	58.7	-23.5	114.2±0.6	Modern	
Dangreuli Gora	DAN	Marneuli Plain	44.78	41.520104	Kvemo Kartli	5	Uncharred	6000-5000 BC	RTD 7647	50.0	-19.5	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	52.7	-27.5	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	55.8	-23.4	167±15	1666 - 1954 AD	
Nastakisi	NAS		44.564464	41.864991	Shida Kartli	12	Uncharred	1000-500 BC	RTD 7696	43.7	-31.5	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	41.0	-26.9	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	53.1	-23.6	99.8±1.1	Modern	

Table2

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%