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Morphology and Molecular Analysis of Ancient Grape Seeds

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A B S T R A C T

The morphometry of *Vitis vinifera* seeds from different archaeological sites was studied. Preservation status differed between sites. Non-invasive and quantitative image analysis based on elliptic Fourier analysis (EFA) established different morphological variations between populations. Molecular analysis was performed to study genetic relationships, using nuclear microsatellite SSRs markers with high polymorphism. Morphometric analysis of archaeological endocarp outlines and allelic profiles of endocarp tegument delineated the general species-specific qualities of the modern cultivar.

1. Introduction

Morphometric measurements and quantitative image analysis based on the study of endocarp outline by elliptic Fourier shape analysis discriminates cultivar groups (Mangafa, Kotsakis 1996; Menesatti *et al.* 2008; Terral *et al.* 2010).

SSR markers for molecular genetic studies have produced methods for limiting contamination, for designing phylogenetic studies (Sefc *et al.* 2000), and for obtaining information from tiny quantities of archaeological material (Blatter *et al.* 2002). SSR markers have high polymorphism and have been used to determine genetic profiles of contemporary varieties of *Vitis vinifera* (Sefc *et al.* 1998; Bowers *et al.* 1999; Vignani *et al.* 2008).

The aim of the present study is to examine grape seeds from different archaeological sites. An outlines analysis (*i.e.* elliptic Fourier analysis) was applied to quantify the differences between living cultivars and observe the similarities of the archaeological samples within the morpho-space. Molecular studies with cytoplasmic markers were conducted to test for similarities and, if possible, to detect variations between individuals. By comparing morphometric analysis and molecular data we endeavoured to characterize a restricted group of cultivar.

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2. Materials and methods

2.1 Sampling and selection of archaeological material

Ancient grape seeds obtained from archaeological excavations at Shahr-I Sokhta (fragments only; Figure 1A), Poggio Bacherina (Figure 1B), Miranduolo (Figure 1C), and Florence (Figure 1D) were documented with light micrographs. Seeds from Shahr-I Sokhta (Tosi 1978) were dated to 23rd century BC, seeds from Poggio Bacherina (Paolucci 1993) were dated to the second century BC, seeds from Miranduolo castle were dated between the late 10th and early 11th century BC (Nardini, Valenti 2003), seeds from Firenze (Cantini *et al.* 2007) were dated to the 13th century BC. Samples were stored separately in sterile Falcon capsules and used, first, for the non-destructive quantitative morphometric analysis based on elliptic Fourier analysis (EFA), and then for destructive molecular DNA analysis.

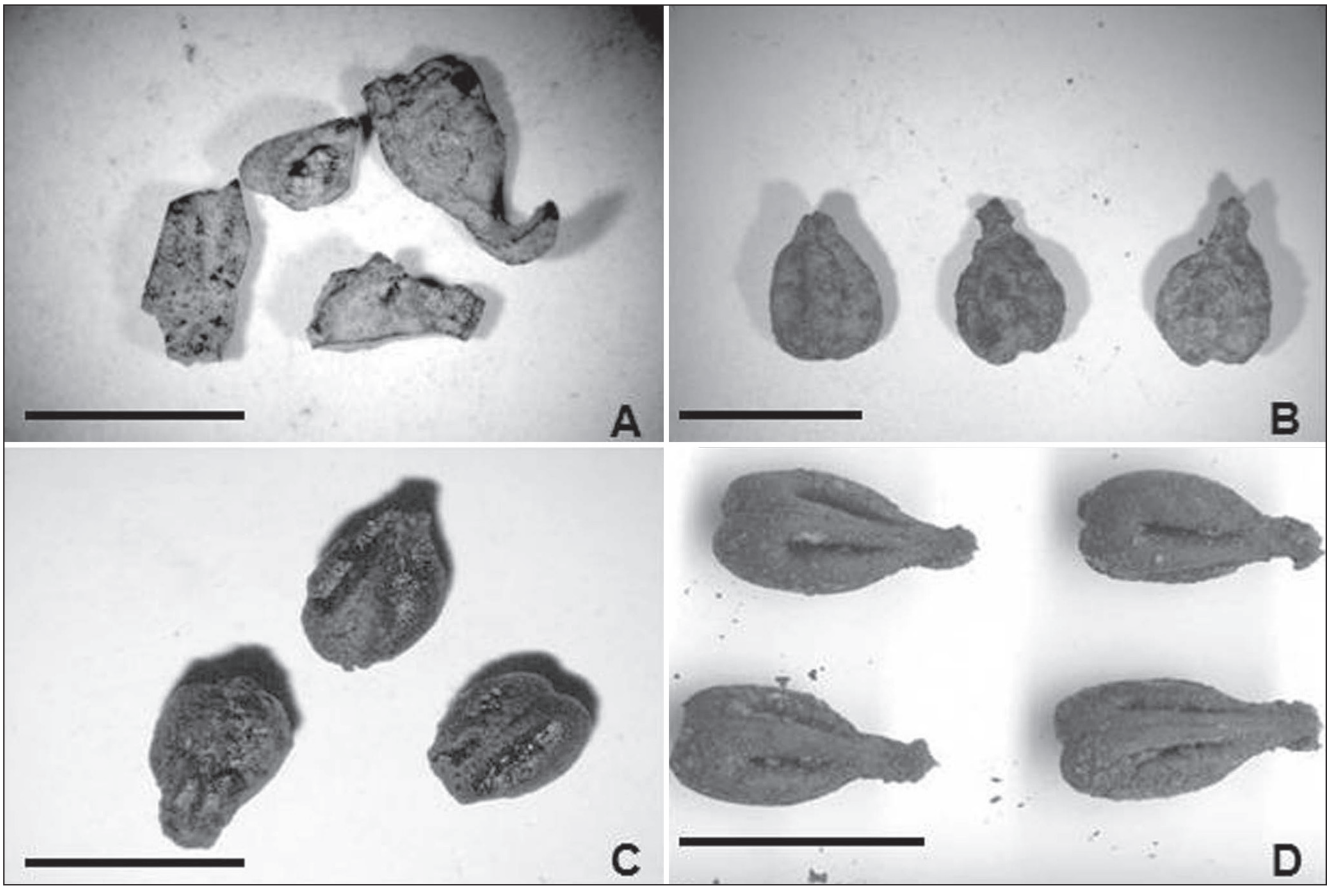

Figure 1. Light micrographs of archaeological grape seeds obtained from Shahr-I Sokhta (fragments only; Figure 1A), Poggio Bacherina (Figure 1B), Miranduolo (Figure 1C), and Florence (Figure 1D). (Magnifications Bar: A=4 cm, B=5.5 cm, C=5 cm, D=7 cm).

2.2 Morphometric analysis

The shape analysis of ancient grape seeds for each site, (Firenze, Miranduolo and Poggio Bacherina, three samples) was compared with fresh Tuscany cultivars from 11 varietals (Sangiovese Casciano, Sangiovese Montalcino, Cabernet Bossi, Merlot Bossi, Canaiolo Monti in Chianti, Canaiolo Montalbuccio, Trebbiano Montalbuccio, Trebbiano Tognazza, Malvasia Monti in Chianti, Albano Montalbuccio and Silevstris) and a total of 498 samples. The morphometric method was described in Milanesi *et al.* 2011 and Antonucci *et al.* 2012.

2.3 DNA analysis

Ancient seeds were quickly released from the matrix by HCl and HF treatment, which cleaned and removed organic matter (Milanesi *et al.* 2006), and subsequently exposed overnight to UV-light. Specimens were immediately processed for DNA extraction under controlled sterile conditions. Small pieces of archaeological and modern seeds were divided in two and processed separately for DNA extraction and PCR amplification under different sterile laminar-flow hoods. Genomic DNA was extracted using a method reported by Mulcahy *et al.* (1993). Seven different microsatellite markers (VVMD7, VVMD21, VVMD25, VVMD27, VVMD31, VVMD36, VVS2), generated by Bowers *et al.* (1999), were amplified. PCR amplification and amplicons detection were performed according to Masi *et al.* (2001). The amplified loci were cloned using the TOPO TA Clonig kit version F (INVITROGEN). Plasmids were extracted using the QIAGEN Plasmid Purification kit, and sequenced using the ALFexpress AutoRead Sequencing Kit, and analyzed on a polyacrylamide PAA gel run and the ALFexpressII semi-automated DNA sequencer. Post run data analysis was performed with the ALFwin sequence Analyzer 2.00. The generation of the consensus sequence and the pairwise sequence was carried out with DNAsys 2.10 software. The analysis was performed in order to observe VVMD7, VVMD21, VVMD25, VVMD27, VVMD31, VVMD36 and VVS2 at least three times.

3. Results

The frontal profile of endocarps was calculated on 495 fresh samples and 3 ancient seeds. The correct number of harmonics to use for computing the frontal profile of endocarps was calculated on all samples. The value selected (*i.e.* the first value exceeding 99.999%) was 27 harmonics. The mean configuration (black line) and standard deviation (gray lines) for each cultivar are shown in Figure 2. It is

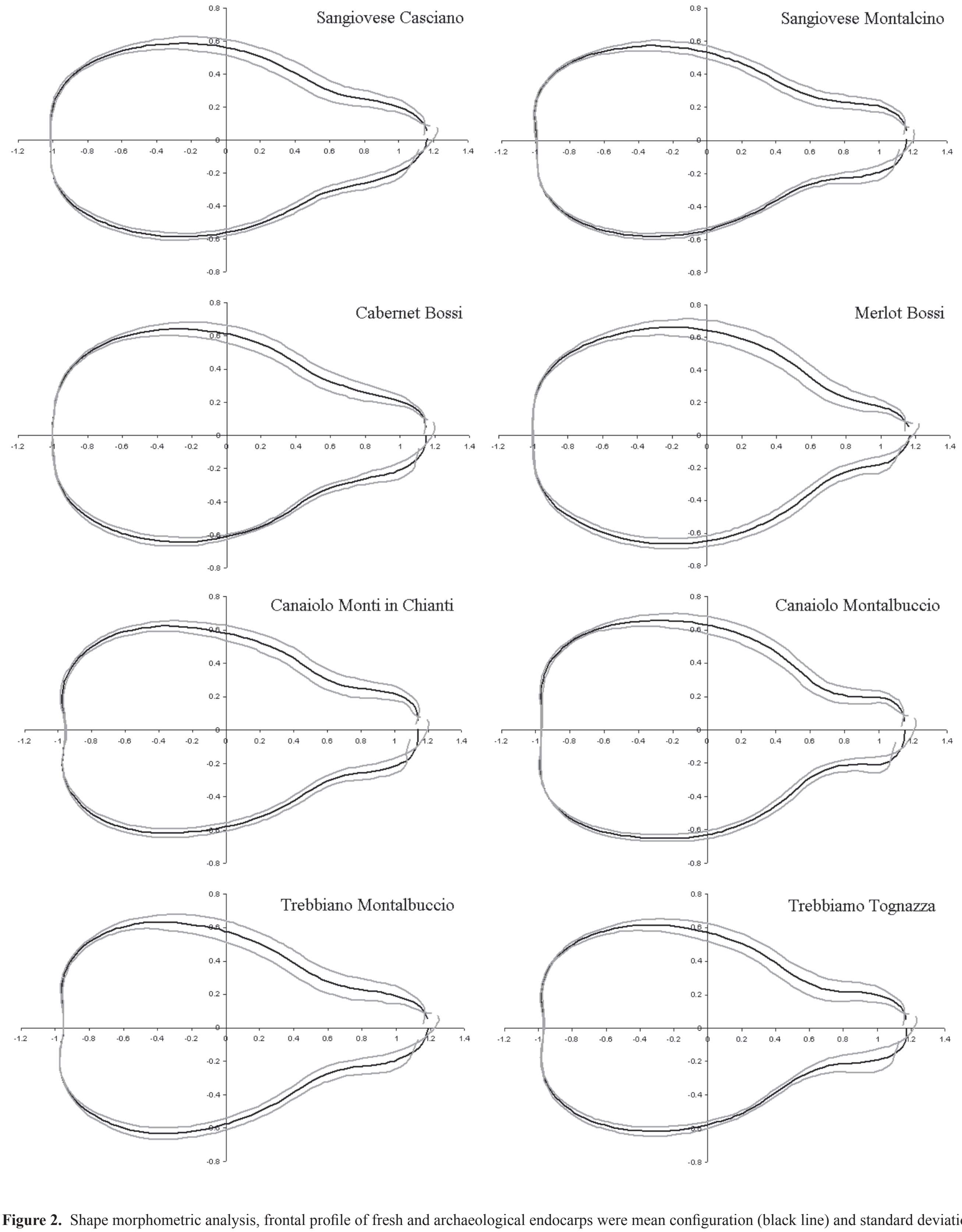

Figure 2. Shape morphometric analysis, frontal profile of fresh and archaeological endocarps were mean configuration (black line) and standard deviation (gray lines) for each cultivar are shown. It is possible to observe distinct shapes belonging to the different cultivars.

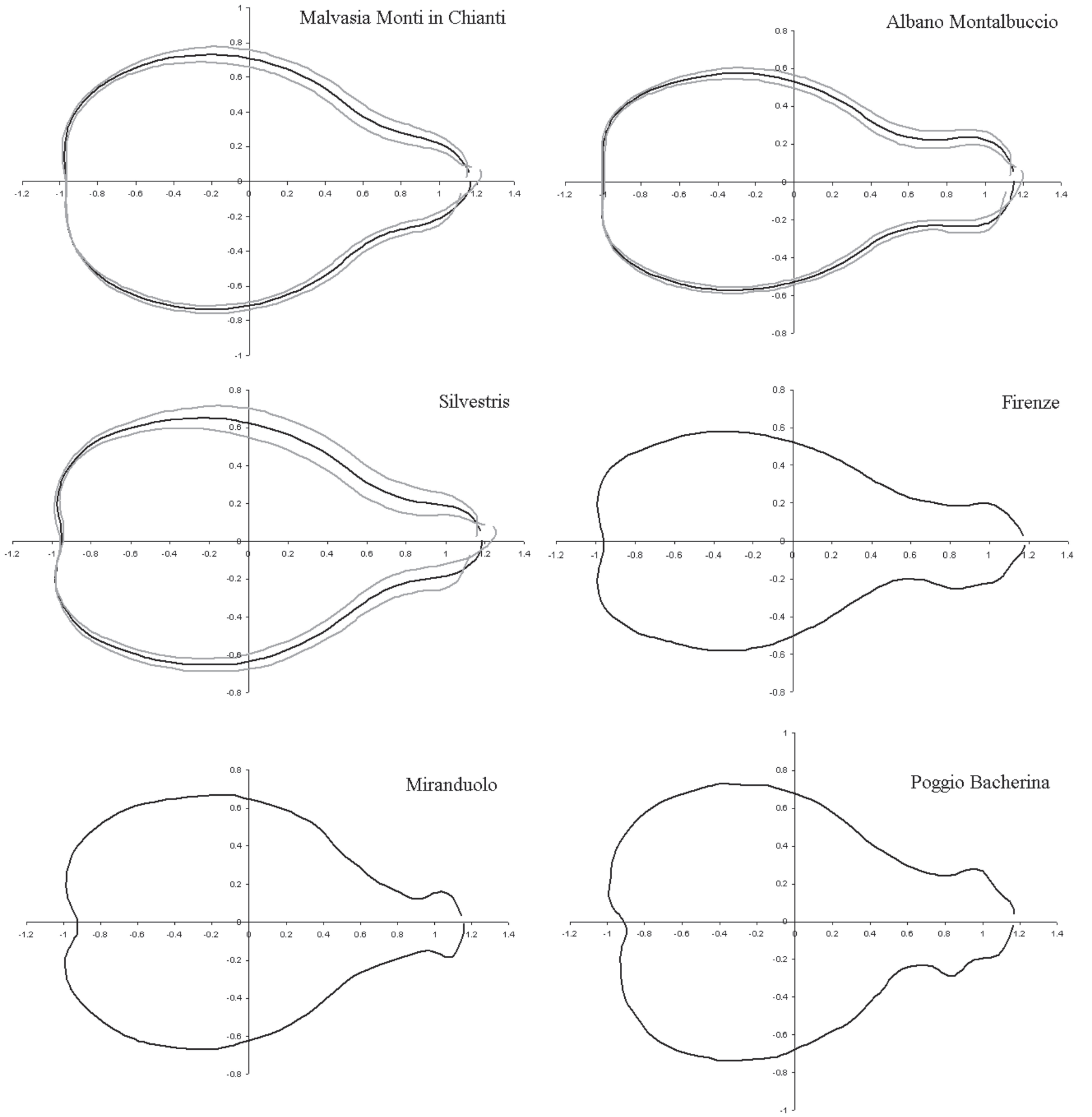

Figure 2. Shape morphometric analysis, frontal profile of fresh and archaeological endocarps were mean configuration (black line) and standard deviation (gray lines) for each cultivar are shown. It is possible to observe distinct shapes belonging to the different cultivars.

possible to observe distinct shapes belonging to the different cultivars. The cladogram, built on Euclidean distances between the mean shape configurations of each cultivar, calculated with the complete linkage algorithm using the EFA harmonic coefficients, appears in Figure 3. At a linkage distance of 0.08, cluster analysis distinguished four groups. The first included Firenze, Albano di Montalbuccio and Sangiovese di Montalcino; the second Poggio Bacherina and Malvasia Monti in Chianti; the third Miranduolo, Silvestris, Canaiolo Montalbuccio and Merlot Bossi and finally the fourth included only living cultivars. The endocarp shapes of the cultivars of each group generally appeared to be similar.

All carpological samples were negative at the VVS2 nuclear microsatellite locus (Table 1). In particular, the genetic material of samples from Shahr-I Sokhta (Iranian archaeological seeds dated to 23rd century BC) proved to be of good quality. The molecular data deposited in data banks showed homology below 1% with 25 different modern cultivars. The loci VVMD27 and VVMD31 were not found in the samples from Florence. Analysis of the four alleles

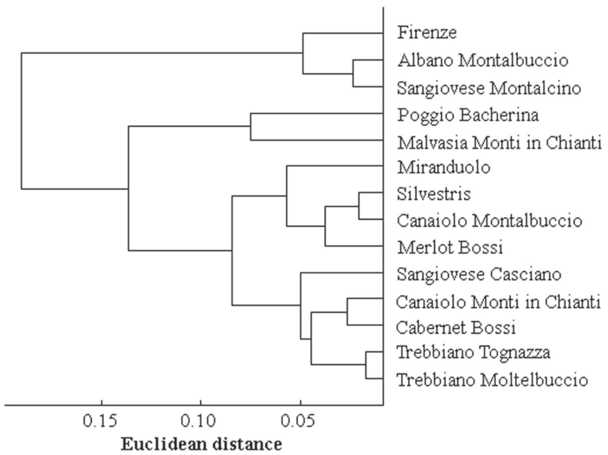

Figure 3. Cladrogram with Euclidean distances mean shape configurations of each cultivar. Cluster analysis distinguished four groups: first included Firenze, Albano di Montalbuccio, Sangiovese di Montalcino, second Poggio Bacherina, Malvasia Monti in chianti, third Miranduolo, Silvestris, Canaiolo Montalbuccio, Merlot Bossi and the fourth incuded only fresh cultivars.

obtained showed 7% homology with the Sangiovese cultivar. Etruscan seeds from Poggio Bacherina and Medieval seeds from Miranduolo castle included in the analysis were negative at every microsatellite locus, confirming the observations obtained by microanalysis and cytochemical staining.

4. Discussion

Morphometric variability in contemporary cultivars reveals phenotypic divergence in groups of individuals diversified by climatic, ecological, historical and socio-cultural factors (Terral *et al.* 2004; Terral *et al.* 2010). For shape analysis of living seeds, the high number of samples tested (nearly 500), should be considered reliable, while the few ancient seeds (3), did not allow the determination of an average ideal shape, only a direct comparison of individual characteristics. Ancient seeds are rare and a database of living seeds can be useful for other future analysis. In addition, morphometric studies are non-invasive and considered ideal for rare and valuable archaebotanical remains. A way to partially solve this issue could be to borrow macro plant remains from museum collections.

In this analysis, DNA was found in specimens from prehistoric Iranian and Florence. Mineral substrates contribute to "entombing" (Eglinton, Logan 1991) or cementing subfossil cytoplasm and preventing oxidation. Samples from the Iranian archaeological site were also subject to osmotic dehydration by salinity that transferred water by formation of anaerobic systems (Hirayama *et al.* 1995) with DNA preservation. Seeds from Florence were stored in underground tunnels. Seeds from the Florence archaeological site were in a waterlogged context, were positive to DNA microsatellite amplifications. The liquid matrix of urban sediments probably formed barriers that maintained substrate humidity, isolating and reducing exogenous degeneration (Manen *et al.* 2003). Salt dehydrated and waterlogged archaeological seeds may be a good source of archaeobotanical DNA. In Poggio Bacherina, Etruscan seeds were in aerobic conditions that lead to mineralization, loss of preservation and/or insufficient genomic DNA. In Miranduolo Medieval castle, specimens were associated with anthropomorphic impacts; seeds were charred or collapsed, and suitable only for morphometric studies (Nardini, Valenti 2003).

The molecular results were compared with national and international contemporary cultivars in data banks (Sefc *et al.* 1998; Bowers *et al.* 1999; Vouillamoz *et al.* 2006) and we submitted the data obtained with the seven

Table 1. Summarized allele size obtained with seven microsatellite markers (VVMD7, VVMD21, VVMD25, VVMD27, VVMD31, VVMD36, VVS2) from archaeological grape seeds.

Specimens seeds	1. Shahr-1 Sokhta 0%	2. Florence 7% Sangiovese	3. Poggio Bacherina	4. Miranduolo Castle	Laminar woods
VVMD7	238–244 BP	245–253 BP	–	–	1
	238–244 BP	245–253 BP	–	–	2
VVMD21	222–222 BP	225–255 BP	228–228 BP	–	1
	222–222 BP	225–255 BP	228–228 BP	–	2
VVMD25	229–262 BP	234–242 BP	234–242 BP	–	1
	229–262 BP	234–242 BP	234–242 BP	–	2
VVMD27	228–228 BP	–	–	–	1
	228–228 BP	–	–	–	2
VVMD31	226–226 BP	–	–	–	1
	226–226 BP	–	–	–	2
VVMD36	229–230 BP	255–255 BP	–	–	1
	229–230 BP	255–255 BP	–	–	2
VVS2	–	–	–	–	1
	–	–	–	–	2

microsatellite markers (Vignani *et al.* 2008). We observed a degree of microsatellite oscillation that enabled us to exclude contamination (Cappellini *et al.* 2010).

5. Conclusion

Morphometric observations and genetic analysis could enable new comparisons of ancient profiles and contemporary cultivars, providing ideas for verifying hypotheses based on historical and other sources (Herodotus 484–425). In particular, the Firenze medieval archaeology seeds, show affinity with the Sangiovese and Albano while Miranduolo Etruscan seeds show affinity with Malvasia cultivar suggests trade in wine between the eastern Mediterranean and central Italy in Classical times, while certain typical varieties such as Malvasia white, Sangiovese and Albano plausibly became autochthonous varieties in the late Middle Ages, introduced into cenobite cloisters for limited production of wine and grapes by barbaric incursions.

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