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Department of Food Science and Human Nutrition

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Introduction

The variability of the geographical landscape of Greece led to the creation of isolated microenvironments, which lead to the natural appearance of a large number of indigenous grapevine varieties. Genetic identification of the grapevine varieties is an indispensable national priority in order to support and develop the wine sector. In the present work, different grapevine genotypes have been collected from the prefecture of East Macedonia-Thrace and more specifically from the wider area of Soufli. The issue of synonymy and homonymy (This et al., 2004) among the varieties may occur in this occasion. In order to accurately determine their identity it is necessary to use molecular markers, in order to indentify and critify their nature, while the technological characteristics of the wines produced by them, should be studied, in order to assist for preliminary evaluation.

The cultivation of the grapevine plants in ancient Greece was particularly widespread, not only in Athens and the Peloponnese, but on the islands, Macedonia and Thrace as well. Especially in the region of east Macedonia/ Thrace, wine has a long history.

Nowadays the Region of Eastern Macedonia and Thrace is the smallest wine-growing region of Greece. The cultivated vineyards of the region correspond to the 2% of the total vineyard cultivation in Greece (Hellenic Statistical Authority, 2016). In addition, until recently viticulture has not been the main pillar for the local economy, with the result that wine production is limited and it is consumed mainly in a local level.

Since the 1950s, a small number of vineyards in the wider region has been eroded, as they did not have any economic interest. The process of vineyard erosion was stopped in the early 1990s, with the abandonment of tobacco cultivation, so farmers switched to the cultivation of the grapevine plant again.

The aim of this study is not to prove that some regions of the East Macedonia and Thrace have the ability to produce wine, but given the ease of grapevine plant for adaptation, wine can be produced even in the most adverse conditions. For instance, in the most diverse microclimates wines could be produced with low yields, with superior quality features, thus they should be recognized and rewarded.

In conclusion, there is a need at both central and local government level to build a strategy in order to make high quality wines competitive products.

Material and Methods

Study Area: The region of the East Macedonia/ Thrace is mostly mountainous, with mountains extended into the sea. The climate differs from other parts of Greece, because of the north and northwest winds during the winter. Moreover, the temperature goes down to low temperatures in the mountainous regions, the rain level is higher than the rest of the Greek mainland (for more details please check the study of the region 3.1.), while snow happens often and does not melt before mid-spring. In coastal areas, the climate is clearly milder, typically temperate with Mediterranean features, with warm and dry summers and during winter there are several rains.

Plant material: A total of 34 samples (Table 2) were collected from equal number of representative plants from Bellas vineyard (Alania area, Soufli) (Figure 1) and from the Traditional vineyard of "Stafilos" at the wider area of Soufli (Table 1) (Figure 2), Greece. It is important to mention that the samples were collected on the basis of the empirical ampelographic identification. The collection of plant material took place on Friday 13th of July, 2018.

It should be mentioned that in collaboration with the department of Hellenic Agricultural Organisation "Demeter" (HAO), leaf samples were collected from the Greek National Ampelographic Collection, and served as references for the comparison of the material from the wider area of Soufli. The Collection occupies about 70 acres at the Lykovrysi district in Attica, Greece, maintaining over 500 autochthonous accessions and representing the oldest and most important genetic grapevine reservoir of the country. The ampelographic and molecular characterization of the Collection is under way.

Row 1	Row 2	Row 3	Row 4	Row 5	Row 6	Row 7	Row 8	Row 9
Kiratsouda	Kiratsouda	Papadis	Ligaridia	Ligaridia	Leuka Feggia	Leuka Feggia	Alponoura Leuki	
Kiratsouda	Kiratsouda	Papadis	Ligaridia	Ligaridia	Leuka Feggia	Leuka Feggia	Alponoura Leuki	
Kiratsouda	Kiratsouda	Papadis	Ligaridia	Ligaridia	Leuka Feggia	Leuka Feggia	Alponoura Leuki	
Kiratsouda	Kiratsouda	Boulgaroudi	Ligaridia	Bogiatjides	Leuka Feggia	Leuka Feggia	Alponoura Leuki	
Kiratsouda	Kiratsouda	Boulgaroudi	Ligaridia	Bogiatjides	Leuka Feggia	Leuka Feggia	Alponoura Leuki	
Kiratsouda	Kiratsouda	Boulgaroudi	Ligaridia	Bogiatjides	Leuka Feggia	Leuka Feggia	Alponoura Leuki	
Nichato	Red Chavouzalis	Boulgaroudi	Ligaridia	Bogiatjides	Leuka Feggia	Leuka Feggia	Alponoura Leuki	
Nichato	Red Chavouzalis	Boulgaroudi	Karnachalas	Bogiatjides	Leuka Feggia	Leuka Feggia	Alponoura Leuki	
Nichato	Red Chavouzalis	Boulgaroudi	Karnachalas	Bogiatjides	Leuka Feggia	Leuka Feggia	Alponoura Leuki	
Nichato	Red Chavouzalis	Boulgaroudi	Karnachalas	Bogiatjides	Leuka Feggia	Leuka Feggia	Alponoura Leuki	
Nichato	Red Chavouzalis	Boulgaroudi	Karnachalas	Bogiatjides	Leuka Feggia	White Patiki	Alponoura Leuki	
Nichato	Red Chavouzalis	Boulgaroudi	Karnachalas	Bogiatjides	Leuka Feggia	White Patiki	Kolokithis	
Nichato	Red Chavouzalis	Boulgaroudi	Karnachalas	Bogiatjides	Leuka Feggia	White Patiki	Kolokithis	
Nichato	Red Chavouzalis	Boulgaroudi	Karnachalas	Bogiatjides	Sefka	White Patiki	Kolokithis	
Nichato	Red Chavouzalis	Boulgaroudi	Karnachalas	Bogiatjides	Sefka	White Patiki	Kolokithis	
Nichato	Red Chavouzalis	Boulgaroudi	Karnachalas	Bogiatjides	Sefka	White Patiki	Kolokithis	
Nichato	Korifia Rose	Boulgaroudi	Karnachalas	Bogiatjides	Sefka	White Patiki	Kolokithis	Siasla
Nichato	Korifia Rose	Boulgaroudi	Karnachalas	Bogiatjides	Sefka	White Patiki	Kolokithis	Siasla
Nichato	Korifia Rose	Boulgaroudi	Karnachalas	Bogiatjides	Sefka	White Patiki	Kolokithis	Siasla
Nichato	Korifia Rose	Boulgaroudi	3 Black Berries	Bogiatjides	Sefka	White Patiki	Kolokithis	Siasla
Nichato	Korifia Rose	Boulgaroudi	3 Black Berries	Bogiatjides	Sefka	White Patiki	kolokithis	Siasla
ROSE	Korifia Rose	Boulgaroudi	3 Black Berries	Bogiatjides	Sefka	White Patiki	Tsougianides 2	Siasla
	Korifia Rose	Boulgaroudi	3 Black Berries	Bogiatjides	Sefka	White Patiki	Tsougianides 2	Siasla
	Korifia Rose	Boulgaroudi	3 Black Berries	Bogiatjides	Sefka	Tsougianides 1	Tsougianides 2	Siasla
	Korifia Rose	Boulgaroudi	3 Black Berries	Bogiatjides	Sefka	Tsougianides 1	Tsougianides 2	Siasla
				Bogiatjides	Sefka	Tsougianides 1	Tsougianides 2	Savvatiano
				Bogiatjides	Sefka	Tsougianides 1	Tsougianides 2	Savvatiano
						Tsougianides 1	Tsougianides 2	Savvatiano
						Tsougianides 1	Tsougianides 2	Savvatiano
						Tsougianides 1	Tsougianides 1	Savvatiano
						Tsougianides 1	Tsougianides 1	Savvatiano

Table 1: Lay out of the traditional vineyard “Stafilos” at the area of Soufli

Bag number	Variety	Num. of Samples	Row	Num. of Plant	Vineyard
1	Karnachalas	1	F17	P3	Bellas Vineyard
2	Karnachalas	2	F20	P2	Bellas Vineyard
3	Karnachalas	3	F21	P4	Bellas Vineyard
4	Mpougialamas	1	F8	P13	Bellas Vineyard
5	Mpougialamas	2	F8	P20	Bellas Vineyard
6	Mpougialamas	3	F8	P22	Bellas Vineyard
7	Nichato	1	F1	P5	Stafilos Vineyard
8	Nichato	2	F1	P7	Stafilos Vineyard
9	Kiratsouda	1	F1	P17	Stafilos Vineyard
10	Chavouzalis Red	1	F2	P9	Stafilos Vineyard
11	Korifia Rose	1	F2	P4	Stafilos Vineyard
12	Korifia Rose	2	F2	P5	Stafilos Vineyard
13	Voulgaroudi	1	F3	P10	Stafilos Vineyard
14	Boulgaroudi	2	F3	P12	Stafilos Vineyard
15	Pappadis	1	F3	P19	Stafilos Vineyard
16	Pappadis	2	F3	P20	Stafilos Vineyard
17	Ligaridia	1	F4	P3	Stafilos Vineyard
18	Ligaridia	2	F4	P5	Stafilos Vineyard
19	3 Black Berries	1	F4	P5	Stafilos Vineyard
20	3 Black Berries	2	F4	P1	Stafilos Vineyard
21	Bogiatjides	1	F5	P5	Stafilos Vineyard
22	Bogiatjides	2	F5	P8	Stafilos Vineyard
23	Sefka	1	F6	P6	Stafilos Vineyard
24	Sefka	2	F6	P9	Stafilos Vineyard
25	Leuka Feggia	1	F6	P21	Stafilos Vineyard
26	Leuka Feggia	2	F6	P22	Stafilos Vineyard
27	Tsougianides	1	F7	P1	Stafilos Vineyard
28	White Patiki		F7	P10	Stafilos Vineyard
29	White Patiki		F7	P11	Stafilos Vineyard
30	Tsougianides 2	1	F8	P1	Stafilos Vineyard
31	Kolokithis	1	F8	P8	Stafilos Vineyard
32	Kolokithis	2	F8	P9	Stafilos Vineyard
33	Alponoura Leuki	1	F8	P5	Stafilos Vineyard
34	Alponoura Leuki	2	F8	P6	Stafilos Vineyard

Table 2: Leaf Samples for DNA extraction

Sampling and processing of plant material

Leaf Sampling

The collection of appropriate plant material were carried out in the wine-producing regions of the study of East Macedonia/ Thrace, therefore the results will reflect the diversity of both Greek vines in terms of varieties and more specifically from Soufli area. After selecting the vineyards, the selection of vivid, healthy plants of the varieties was necessary (Figure 4).

In the present study, in order to find the suitable leaf type for the varieties of interest, 10 healthy leaves were collected from each grapevine plant. The leaf sampling took place early in the morning. The plant material was young leaves and placed at -20 ° C in polysterene boxes and then was transferred to the Agricultural University of Athens at the Laboratory of Oenology.

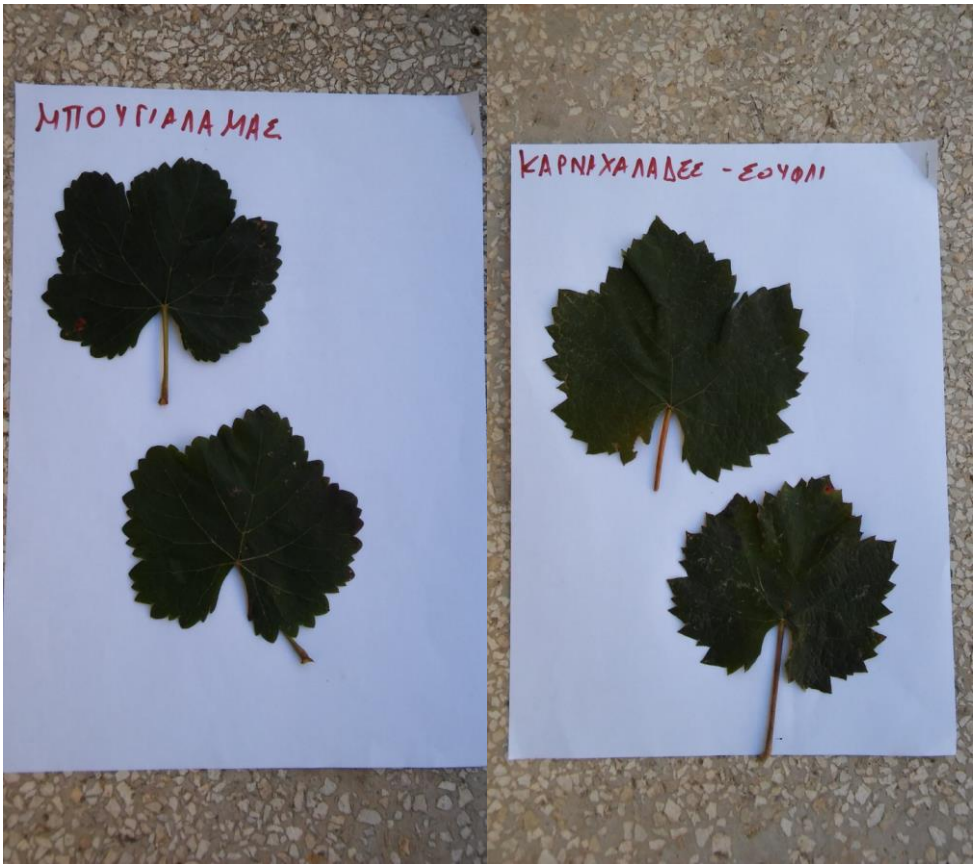

Figure 1: Leaf samples from Bellas vineyards, (Bougialamas leaves- on the left, Karnachalas leaves- on the right)

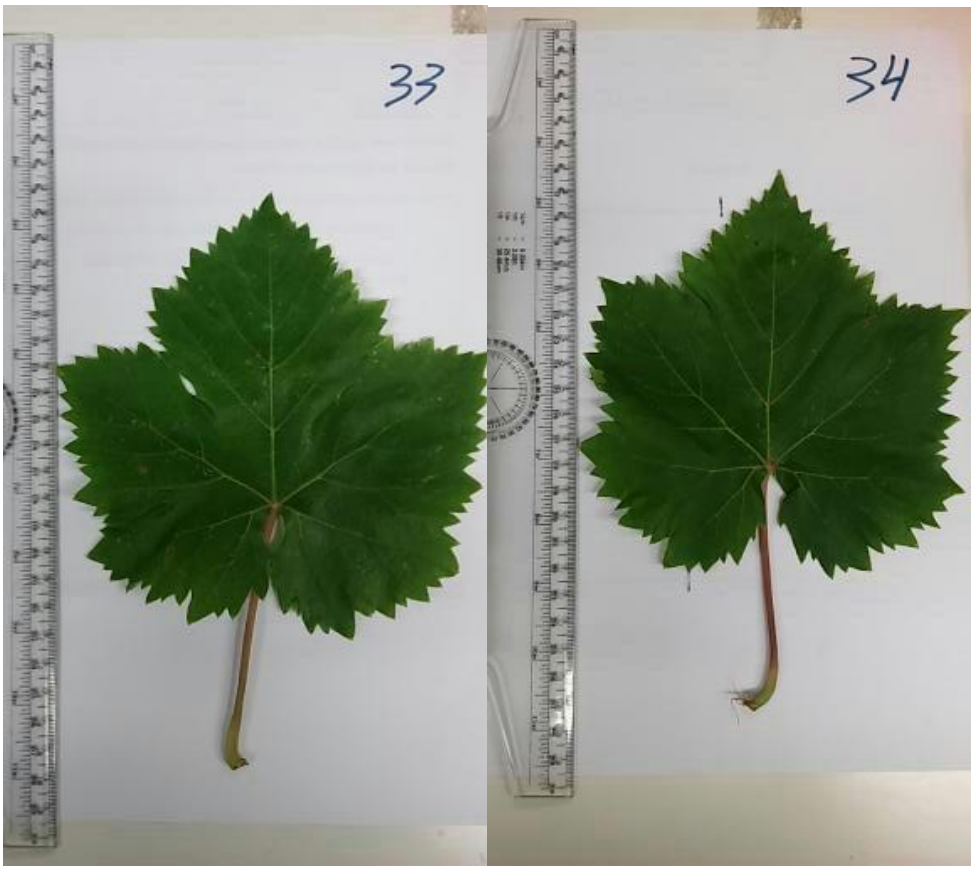

Figure 2: Leaf samples collected from the Stafilos vineyards (Alponoura Leuki leaf samples)

Berry Sampling

Collection of grape samples per different variety took place at the suitable technological maturity of the berries of each variety (1-2 baskets for micro-vinification). In the parcels, the berry samples were collected randomly, always corresponding to the totality of the vineyard. Grape berries were collected from the vine plants of the main shoots from different positions (shaded or unshaded) and from different positions of each grape bunch (top, middle, base).

At the end of sampling, all baskets with the grape berry samples were shipped to the Laboratory of Oenology at the Agricultural University of Athens.

Figure 3: Labeling the grapevine plants, Stafilos vineyard

Figure 4: Selection of healthy grapevine plants, Stafilos vineyard

Genetic Analysis: Genomic DNA was isolated from young leaves using commercially available kits following manufacturer recommendations. Quality and integrity of the DNA was evaluated by electrophoresis in agarose gels (Figure 5) whereas its concentration was estimated by the use of a NanoDrop 2000 Spectrophotometer (Thermo-Fisher Scientific) (Figure 6).

The Polymerase Chain Reactions (PCRs) that were carried out using a set of seven microsatellites (VVS2, VVMD5, VVMD7, VVMD25, VVMD28, VvZAG67, and VvZAG79) as well as the subsequent analysis and the construction of dendrograms, followed the description of Merkouropoulos *et al*. (2015). It is worth to mention that four of the microsatellites that were used in this study, have already been incorporated in the catalogue published by the Organisation Internationale de la Vigne et du Vin (OIV) as molecular descriptors (OIV, 2009).

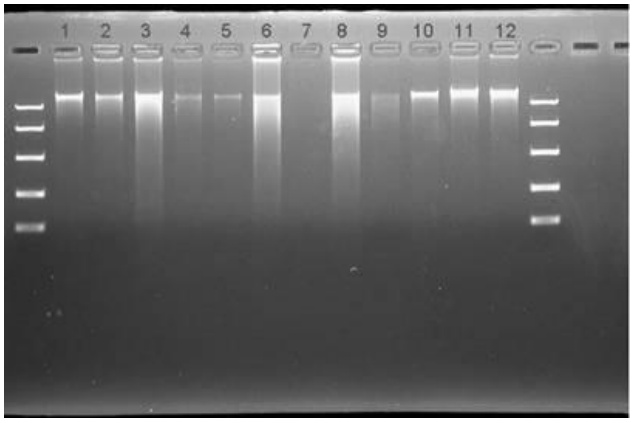

Figure 5: DNA evaluation by electrophoresis in agarose gel (position/ well 1- DNA Karnachalas, well 2- Bougialamas, well 3- Pappadis, well 4- Bogiatjides, well 5- Ligaridia, well 6- Ligaridia, well 8- White Patiki, well 9- Kiratsouda, well 10- Sefka, well 11- Leuka Feggia, well 12- marker DNA)

Figure 6: NanoDrop 2000 Spectrophotometer (Thermo-Fisher Scientific)

Figure 7: PCR thermocycler, Bio-Rad

Vinification process

Red Varieties: Karnachalas, Bougialamas

The grapes from each red variety were harvested during early hours and they were then transferred to the experimental winery of the Agricultural University of Athens where they were put in the fridge during the night in separate bins. In addition, 200 berries were frozen at -20^{o} C for further analyses (Iland, *et al*., 2004). Next morning, they were destemmed, crashed and cold pre-fermentation maceration for 24hours was performed at 5^{o} C. Potassium metabisulfite (7g/100 kg) was added. At this stage, the sugar content (Brix) was determined and classical analyses were performed (pH and total acidity), the chromatic characteristics and YAN (OIV). They were then inoculated in order to initiate alcoholic fermentation. Punching down was done three times per day. Additions of yeast nutrients (Springferm 20 gr/100 kg) was done. Brix measurement was performed daily. Six days after the initiation of the alcoholic fermentation and when the colour intensity started to be stable, the skins were separated from the must and were pressed. Malolactic fermentation occurred after inoculation with CHR Hansen bacteria and as soon as it was completed, the wines were racked and an addition of 8 g/hL of Potassium Metabisulfite was made. The wines were bottled and kept at room temperature.

Classical analyses (Brix, pH, Total Acidity, Volatile Acidity, % Alcoholic Content, Reducing Sugars), the determination of Malic acid, Total YAN and the chromatic characteristics (according the OIV guidelines) were carried upon the completion of the alcoholic and malolactic fermentation.

In addition, the following analyses were carried in the finished wines:

- Chemical age, Anthocyanes' Ionization Degree %, Total Anthocyanes, Chromatic characteristics, Total phenolics, Colour components in the presence of SO_2 (Somers el al, 1974, 1977)
- Tannins determination – BSA (Harbertson et al, 2002)
- Tannins determination – MCP (Sarneckis et al, 2006; AWRI, 2009)
- Total phenolics Folin-Ciocalteau (Waterman & Mole, 1994)

In both red and white wines, the following analyses are in the process of determination:

- Organic acids
- Anthocyanes using HPLC (Kallithraka S., et al., 2005)
- Volatile compounds using GC-MS

In both red and white grape varieties, the following analyses will be performed:

- Anthocyanes and total phenolics (Iland P., et al., 2004)
- Extractability of anthocyanins and tannins and the phenolic potential – Glories (Ribéreau-Gayon, P., et al., 2000)

Moreover, spontaneous vinifications took place, of the red grapevine varieties Bougialamas and Karnachalas, in smaller plastic bottles in order to isolate the indigenous yeasts.

Figure 8: Baskets of Bougialamas grapevine berries

Figure 9: Destemmed berries by hand

Figure 10: Destemmetion process of the Bougialamas grape berries

Figure 11: Pressing the skins after separating the skins from the must

Sensory analysis

Sensory Analysis Tests of wines produced from the grapevine varieties coming from Thrace took place in the Laboratory of Oenology at the Agricultural University of Athens. The Sensory session took place in the morning (from 11:00 a.m. to 15:00 p.m.). The samples were presented to the panel blind and coded. The panel was comprised of people recruited from members of the laboratory or external tasting specialists. In order to measure the traceability, each panel member wrote down his results on a tasting sheet (Table 3) containing the date and the assessors identification.

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Figure 12: Performing Sensory analysis Tests for the red wines

Name					
Samples					
Visual evaluation					
Color intensity (lower- medium- higher) Scale from 1 to 10					
Hue (lower- medium- higher) Scale from 1 to 10					
Olphactor evaluation					
Smell intensity (lower- medium- higher) Scale from 1 to 10					
Red Fruits (lower- medium- higher) Scale from 1 to 10					
Veggie aromas (lower- medium- higher) Scale from 1 to 10					
Spiciness (lower- medium- higher) Scale from 1 to 10					
Taste Evaluation					
Taste Balance (lower- medium- higher) Scale from 1 to 10					
Bitterness (lower- medium- higher) Scale from 1 to 10					
Astringency (lower- medium- higher) Scale from 1 to 10					
Acidity (lower- medium- higher) Scale from 1 to 10					

Table 3: Sensory sheet for the red wines

Results

The oenological evaluation of those two grapevine varieties has been conducted by carrying out the basic wine parameters, such as pH, alcoholic title, total acidity, volatile acidity according to the Compedium of International Methods of Wine and Musts (2018) (Table 4).

Karnachalas and Bougialamas were vinified at the experimental winery at the Oenology Lab of the Agricultural University of Athens during 2017 harvest. The preliminary results indicated that Bougialamas wine scored higher alcoholic strength (14.3%) than of Karnachalas wine (14,1%). Total Acidity levels of Karnachalas wine was found to be higher than Bougialamas wine (Table 4).

Furthermore, it is to be noted that Bougialamas wine is characterized by high concentration of Total Anthocyanins, (Table 13) and as presenting higher concentration of total phenolics than Karnachalas (Table 14). Moreover, according to the two methods for tannin determination, Methyl Celluse Precipitable (MCP) (Sarneckis et al., 2009) and Bovine Serum Albumin (BSA) (Habertson et al.,2002) Bougialamas (1620,9 mg/L (MCP) and 398,4 mg/L (BSA)), showed higher tannin concentration than Karnachalas (Figures 15 and 16).

In addition, higher value of colour intensity was found for Bougialamas wines than for Karnachalas wines, 17,7 and 3,57 respectively (Table 4 and Fig. 17).

Finally, according to Illand et al (2004), Bougialamas wine scored higher values in Total Phenolics and Anthocyanins in grape berries than Karnachalas wine (Fig. 19; 20;21;22)

Variety	pH	Tot. Acid. (Tartaric acid g/L)	Vol. Acid. (Acetic acid g/L)	Alcohol ethanol (%)	Res. Sugars (g/ L)	Color intensity	Hue
Karnachalas	3.48	5.3	0.33	14.1	1.23	3.57	1.05
Bougialamas	3.63	5.1	0.23	14.3	1.98	17.7	0.73

Table 4: Basic wine parameters

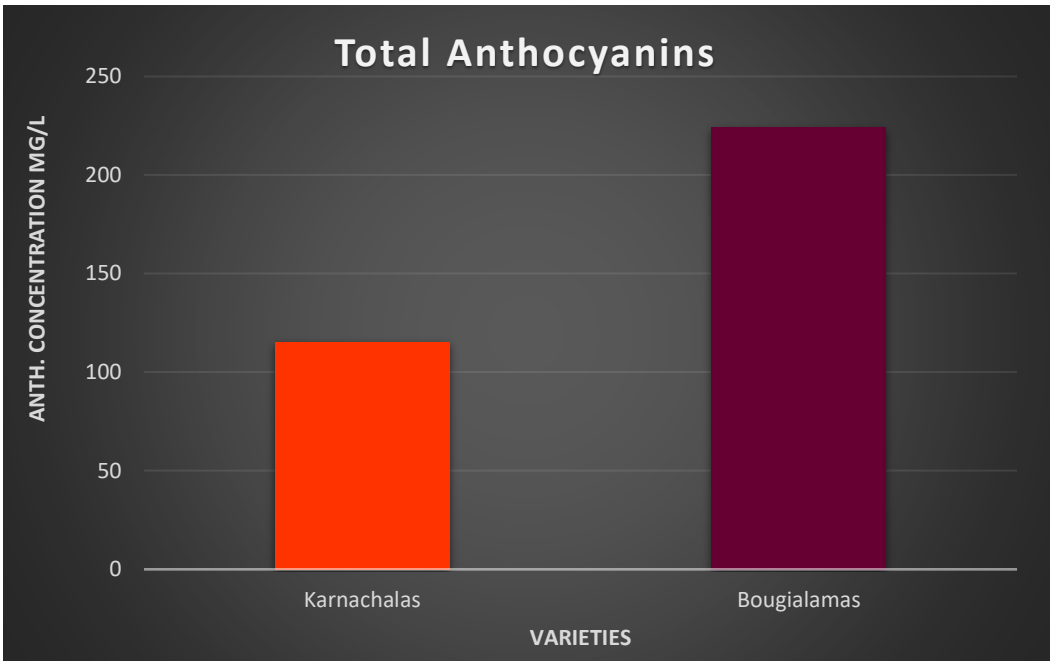

Figure 13: Total Anthocyanins in red wines mg/L

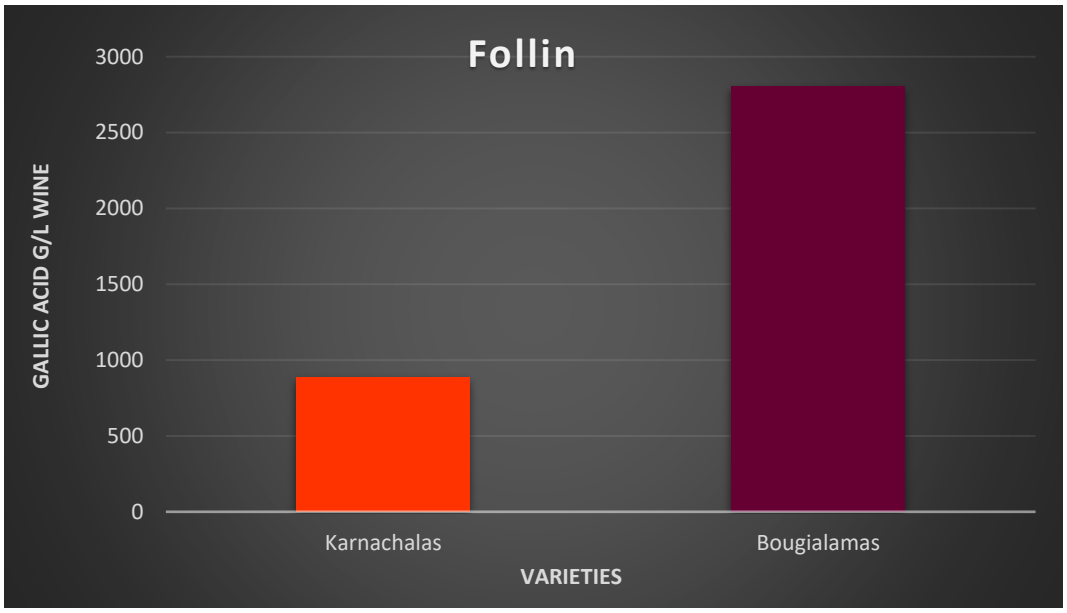

Figure 14: Total phenolics (Folin Ciocalteau) experessed in mg Gallic acid/ L of wine

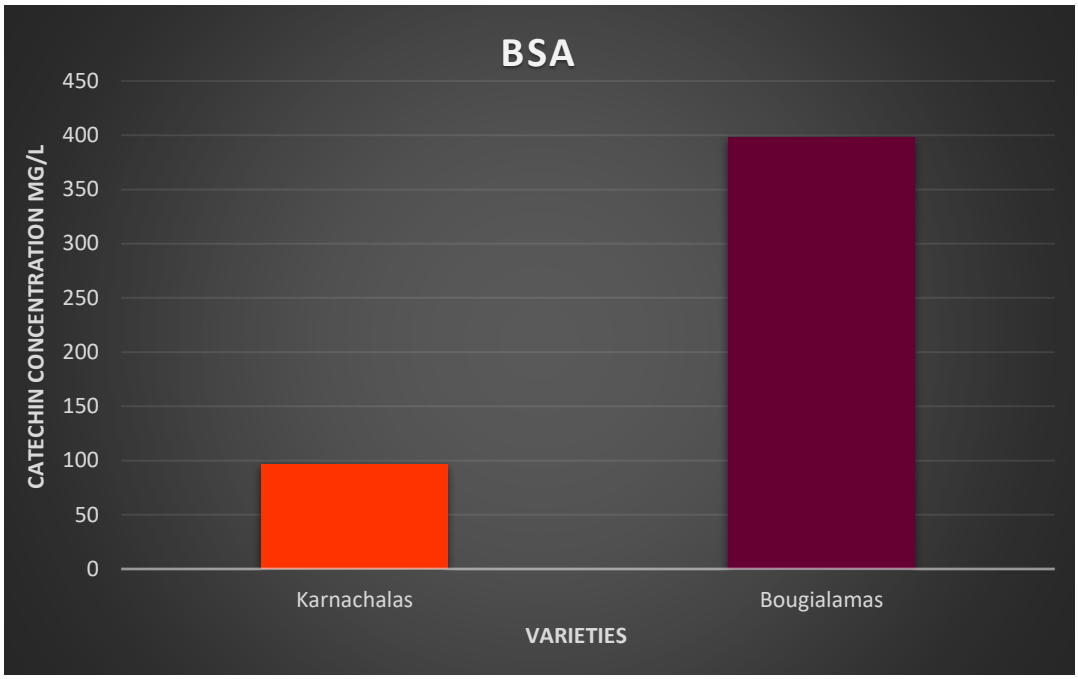

Figure 15: Tannin Determination with the method Bovine Serum Albumin (BSA) expressed in concentration of mg catechin/ L of wine

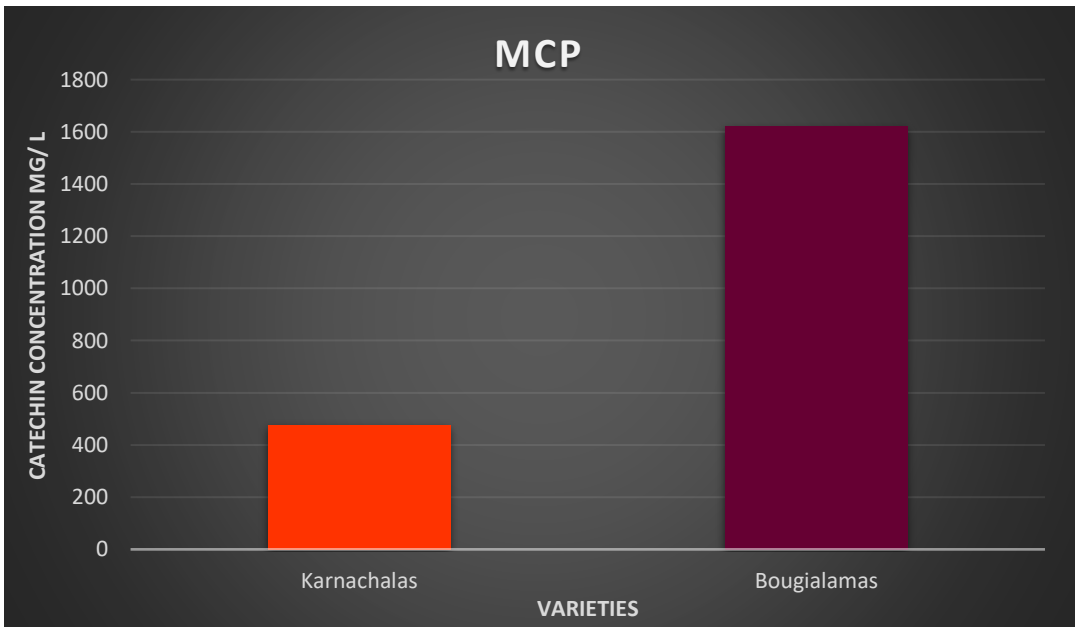

Figure 16: Tannin Determination with the method Methyl Celulose Precipitable (MCP) expressed in concentration of mg catechin/ L of wine

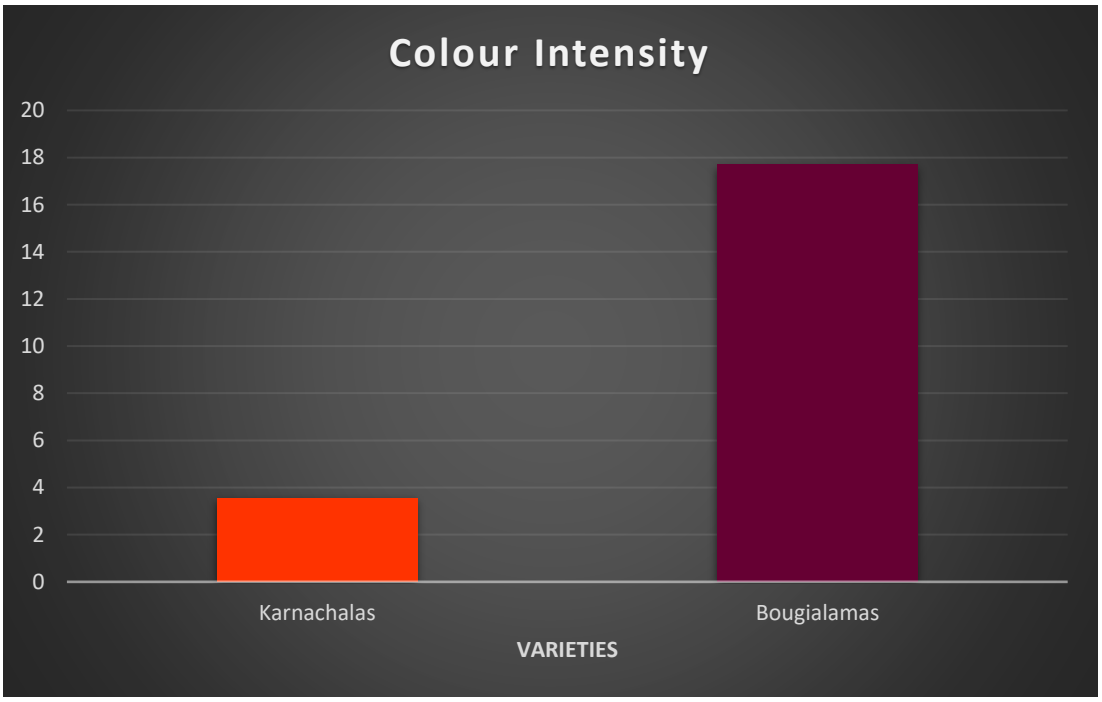

Figure 17: Red Wine Colour Intensity

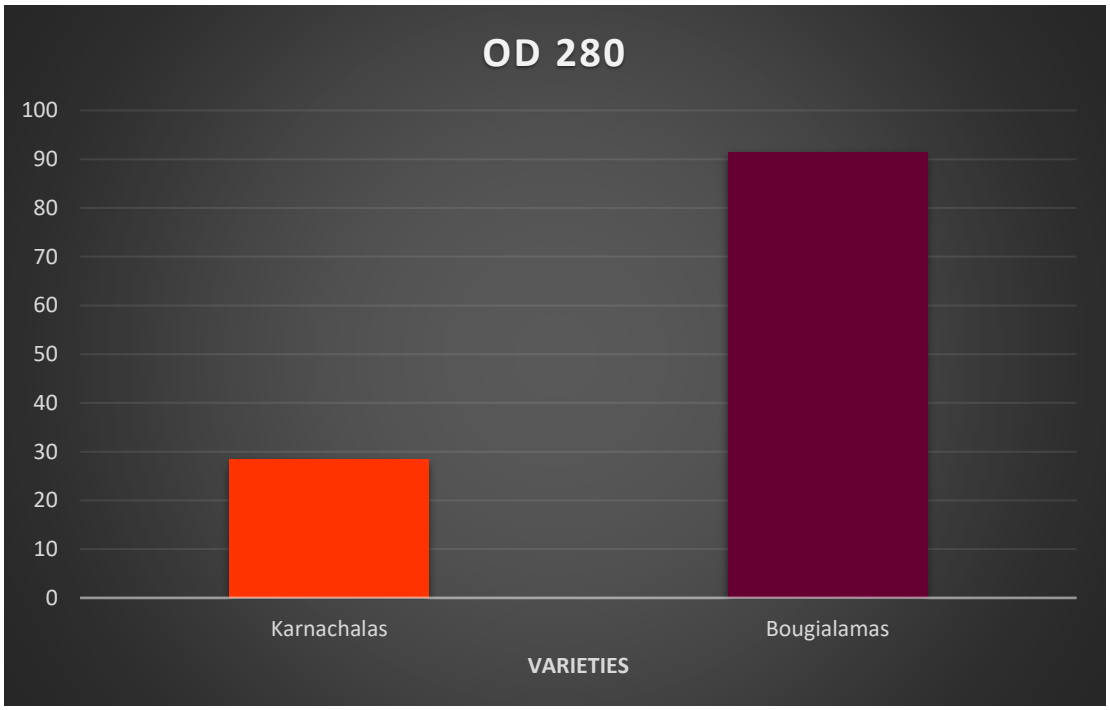

Figure 18: OD 280

Anthocyanins and total Phenolics in grape berries (Illand et al., 2004) at the red grape berries Bougialamas and Karnachalas

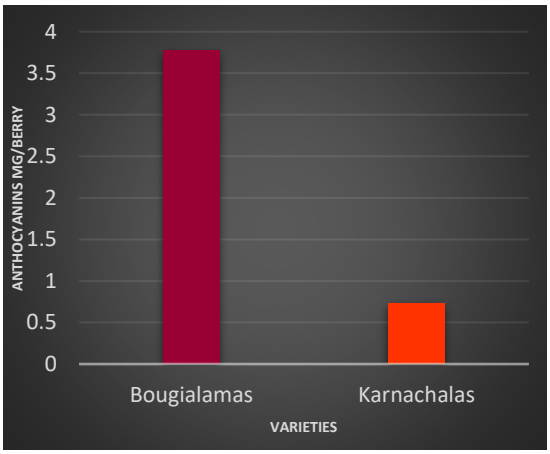

Figure 19: Anthocyanins mg/ Berry

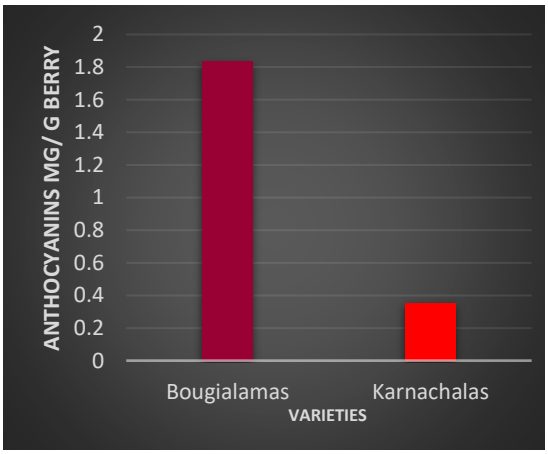

figure 20: Anthocyanins mg/ g Berry

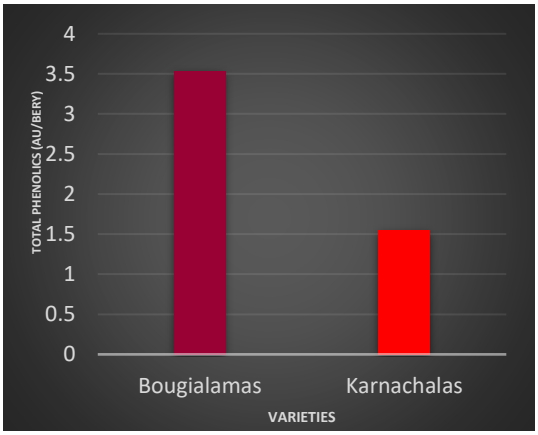

Figure 21: total Phenolics (AU/ Berry)

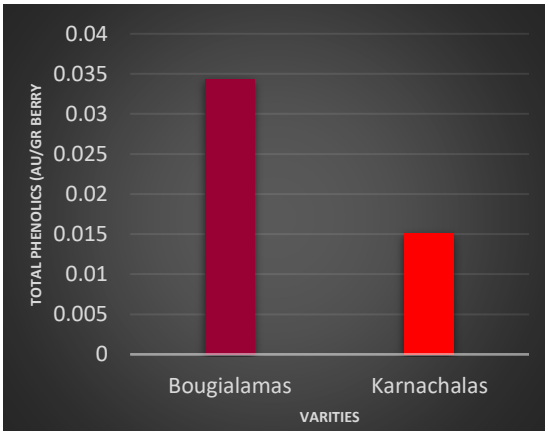

Figure 22: Total phenolics (AU/ g Berry)

Sensory analysis of the autochthonous grapevine varieties.

In concern to the sensory analysis, wines produced from the indigenous grapevines of Soufli, Bougialamas and Karnachalas, took place at the Laboratory of Oenology. According to the sensory test, Bougialamas was confirmed as a promising variety since it provides wines with high colour intensity and is characterized by spicyness (Figure 24).

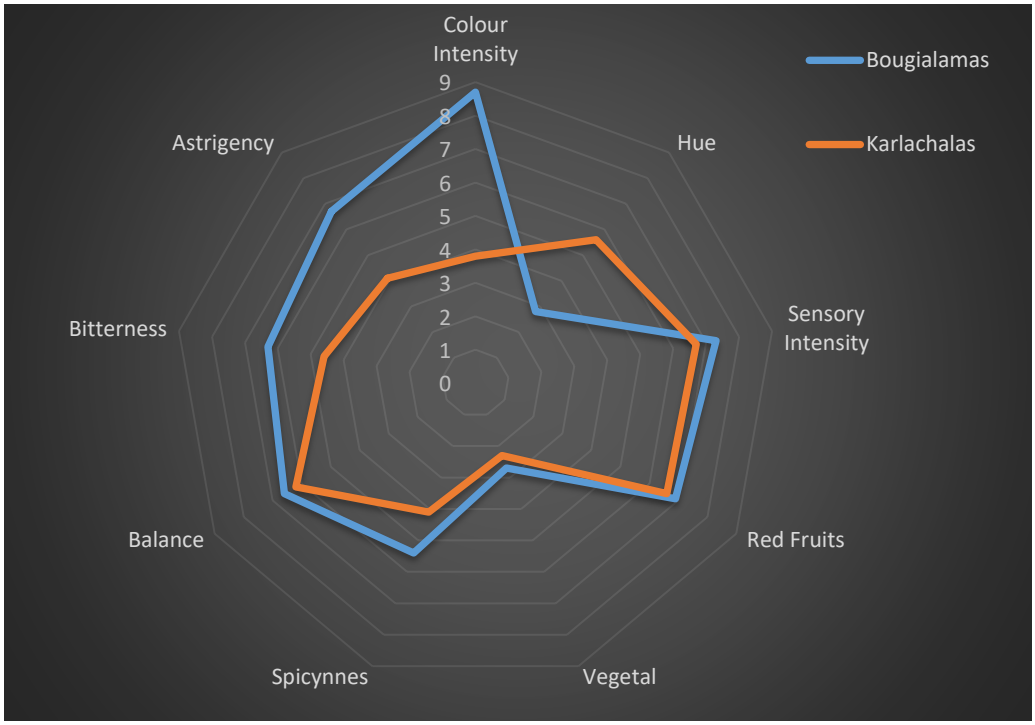

Figure 24: Sensory analysis of the red grapevine varieties Bougialamas and Karnachalas from Soufli

Figure 25: Sensory analysis Test. Bougialamas (left) and Karnachalas (right)

Molecular identification of the indigenous grapevine Varieties

PCRs were performed with the set of ten microsatellites, using the international variety "Trebbiano Toscano" (DNA was provided by the Julius Khn-Institut) as a size marker.

Hence, Genotyping is still in progress.

Conclusion

This project can be characterized as an outstanding one, due to the fact that it provides an holistic description of the varieties. It combines the molecular identification of the grapevine varieties and the oenological evaluation of the wines produced by them. It must be noted that the name of each sample is being accepted as it has been given at the collection point. However, once the Genetic Identification will be completed, we will be able to cross-check the uniqueness of the variety and the name.

Bougialamas exhibits characteristics of high quality wines, Karnachalas relatively less but is still promising for a commercial success.

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