Association Prosperity and Development in Bulgaria
Project:"SOS for endangered traditional vine varieties"
Acronym: "VineSOS"
Project No. 1829

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Interreg
Greece-Bulgaria
VINESOS
European Regional Development Fund

EUROPEAN UNION

DNA study of local vine varieties from Bulgaria and Greece

A unique DNA study of local local grapevine varieties from Bulgaria and Greece was performed as part of the "SOS project for endangered traditional vine varieties".

The aim of this study was to estimate the genetic diversity of eight Greek and nine Bulgarian local grapevine varieties with the use of seven microsatellite markers. Statistical analysis of data showed that there is high degree of genetic heterogeneity among most of the varieties studied, as well as a close genetic relationship in three variety pairs. Based on our results, we suggest the synonymy of Greek Pamid & Bulgarian Pamid and Greek Zoumiatiko & Bulgarian Dimyat. On the other hand, Greek Keratsuda and Bulgarian Keratsuda

varieties should be characterized as homonyms. Additional molecular work is needed for a thorough analysis of Greek and Bulgarian grapevine genepool.

The aim of this study was to analyze the genetic diversity and differentiation of various local Greek and Bulgarian grapevine varieties, using microsatellite genetic markers. Besides that, our goal was also to clarify the putative relationship of varieties coming from the above two countries and characterized as common

In Greece, grapevine is cultivated since antiquity (BANILAS et al., 2009; VALAMOTI 2011) and ampelographic collection accounts for 663 single cultivars, 300 of which are still cultivated for wine, table grapes and raisins (KOTINIS 1985). Grapevine cultivation and winemaking in Bulgaria dates back to the times of ancient Thrace and, nowadays, the commercial varieties consist of old native varieties, widespread European cultivars and locally selected cultivars (HVARLEVA et al., 2004). Despite the great number of varieties available around the world, the global wine market is prevailed by few such as Merlot, Syrah, Cabernet Sauvignon, Chardonnay, which has led to a significant decrease of genetic variability in source of cultivars (THIS et al., 2006).

SSR or STR markers, also known as microsatellites, have been widely used since the early 90s (THOMAS and SCOTT 1993; THOMAS et al., 1994) and, nowadays, are considered to be one of the best methods for determining cultivar identity. Due to their high polymorphism, microsatellite markers have significantly improved the robustness of DNA profiling in parentage analysis (BOWERS et al., 1996; BOWERS et al., 1999; VOUILLAMOZ et al., 2003; LACOMBE et al., 2007; VOUILLAMOZ et al., 2007; LAUCOU et al., 2008; ŠTAJNER et al., 2015; BIAGINI et al., 2016) and in molecular characterization of grape cultivars (LEFORT and ROUBELAKIS-ANGELAKIS 2002; MARTIN et al., 2003; THIS et al., 2004; VOUILLAMOZ et al., 2006; ŠTAJNER et al., 2008; LAIADI et al., 2009; POBLETE et al., 2011; AGAR et al., 2012; DE LORENZIS et al., 2013; ŽULI MIHALJEVIC et al., 2013; MERKOUROPOULOS et al., 2015; SALIMOV et al., 2015; POPESCU et al., 2017; DONG et al., 2018; JIMÉNEZ-CANTIZANO et al., 2018; POPESCU and CRESPAN 2018; MAHMOOD et al., 2019; TAHERI and DARZI RAMANDI 2020).

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Sampling, DNA extraction and genotyping

Young leaves were collected from several locations in Bulgaria and Greece (Supplemental Table 1) and immediately stored in a freezer (-20 oC) until further analysis. In total, 384 samples were collected from both countries. Genomic DNA was extracted from 20-30 mg of leaf tissue using the 'NucleoSpin Plant II' DNA extraction kit (Macherey-Nagel, Germany) and according to the manufacturer's protocol.

Multiplex PCR reaction method amplifying simultaneously seven microsatellite loci was carried out. The selected microsatellites are recommended by various studies in scientific literature and are proven to be very successful in unraveling the genetic variation and differentiation of grapevine varieties (see Introduction for related references). All multiplex PCRs were performed in 10 ul volume containing 5.5 ul of 1X KAPA2G Fast Multiplex PCR Kit (KAPABIOSYSTEMS, USA), 3.5 ul of primer mix (0.25 uM for each primer) and 1 ul (~20 ng) of template DNA. Cycling conditions for the multiplex amplification consisted of an initial 95 oC denaturation step for 3 min followed by 30 cycles of 15 s at 95 oC, 30 s at 56 oC and 30 s at 72 oC, with a final extension at 72 oC for 15 min. Fluorescently labelled PCR products were separated on an ABI 3500 Genetic Analyzer (Applied Biosystems, USA). Alleles were sized and samples genotyped using the STRand 2.4.59 software (TOONEN and HUGHES 2001). Detailed information for microsatellite markers are given in Supplemental Table 2.

Regarding Bulgarian samples, we defined populations based on producer, location and variety information. As a consequence, we had (in some cases) populations belonging to the same variety but coming from different producers and sampling areas (locations). For example, we had Tamyanka variety from BRATANOV WINERY (producer) and Shishmanovo (location) but we also had Tamyanka variety from DIMITAR DJEMPERLIEV (producer) and Dimitrovche (location). These were treated as two different populations. However, according to preliminary results (data not shown), we noticed that all populations of the same variety were genetically similar

(statistically undifferentiated) to each other, regardless of the producer and location of each population. This scenario was evident (with no exception) for all the Bulgarian varieties studied herein. Consequently, we redefined populations based exclusively on variety information (Table 1).

Genetic variation parameters (number of alleles (A), observed (Ho) and expected (He) heterozygosity, F-statistics fixation indexes) were calculated using FSTAT 2.9.3.2 (GOUDET 2003) and GENETIX 4.05.2 (BELKHIR et al., 2004) statistical packages. FSTAT software was also used to test for statistically significant differentiation between all possible pairs of the studied populations.

Graphic dispersion of varieties was carried out using Factorial Correspondence Analysis (FCA) through GENETIX 4.05.2. Finally, POPTREEW (TAKEZAKI et al., 2014) package was used to construct a UPGMA (unweighted pair group method with arithmetic mean) dendrogram based on Nei's standard genetic distance with sample size bias correction (NEI 1978). Assessment of support for clusters on the tree was performed through 1000 bootstrap resampling of loci.

The genetic analysis of Greek and Bulgarian grapevine samples, in the frame of VineSOS project, is published online https://www.mdpi.com/1424-2818/12/7/273/htm

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Table 1. Greek (1-8) and Bulgarian (9-17) populations-varieties used in the present study. Letters G and B in parentheses stand for Greek and Bulgarian, respectively.

Population	Variety	Number of samples
Population 1	Pamid(G)	14
Population 2	Keratsuda(G)	10
Population 3	Zumiatiko	10
Population 4	Limnio	10
Population 5	Sefka	10
Population 6	Mavrud(G)	9
Population 7	Bogiolamas	10
Population 8	Karnachalas	10
Population 9	Tamyanka	26

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Population 10	Mavrud(B)	26
Population 11	Rubin	20
Population 12	Pamid(B)	8
Population 13	Dimyat	19
Population 14	Ruen	20
Population 15	Shizoka Melnishkaloza	36
Population 16	Mishej Sandanski	27
Population 17	Keratsuda(B)	119
Total		384

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Supplemental Table 1. Locations and varieties of collected samples in Greece and Bulgaria.

BULGARIAN LOCATION	GRAPE VARIETY
Shishmanovo	Tamyanka, Rubin, Mavrud
Kolarovo	Pamid
Dimitrovche	Mavrud, Tamyanka, Dimyat
Vranya	Ruen, Shizoka Melnishkaloza, Keratsuda
Mitino	Shizoka Melnishkaloza
Stazchero	Mishej Sandanski
Slivnitsa	Keratsuda
Kzesha	Keratsuda
Gzadeshnitza	Keratsuda
GREEK LOCATION	**GRAPE VARIETY**
Alexandroupoli	Keratsuda
Avdira	Pamid, Zumiatiko
Soufli	Mavrud, Bogialamas, Karnachalas
Thermi	Limnio, Sefka

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Supplemental Table 2. Information for used microsatellite markers.

MARKERS	PRIMERS	FLUORESCENT DYE	REFERENCE
VVS2	F:CAGCCCGTAAATGTATCCATC R:AAATTCAAAATTCTAATCACTGG	FAM	THOMAS and SCOTT, 1993
VVMD7	F:AGAGTTGCGGAGAACAGGAT R:CGAACCTTCACACGCTTGAT	FAM	BOWERS *et al.*, 1996
VVMD25	F:TTCCGTTAAAGCAAAAGAAAAAGG R:TTGGATTTGAAATTTATTGAGGGG	HEX	BOWERS *et al.*,1999
VVMD27	F:GTACCAGATCTGAATACATCCGTAAGT R:ACGGGTATAGAGCAAACGGTGT	HEX	BOWERS *et al.*, 1999
VrZAG47	F:GTTCTTGGTCTGAATACATCCGTAAGT R:ACGGTGTGCTCTCATTGTCATTG	TAMRA	DRABEK *et al.*, 2016
VrZAG62	F:GGTGAAATGGGCACCGAACACACGC R:CCATGTCTCTCCTCAGCTTCTCAGC	FAM	SEFC *et al.*, 1999
VrZAG79	F:AGATTGTGGAGGAGGGAACAAACCG R:TGCCCCCATTTTCAAACTCCCTTCC	TAMRA	SEFC *et al.*, 1999

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Supplemental Table 3. Pairwise F_{ST} values between all possible pairs of the studied populations.

	Pamid(G)	Keratsuda(G)	Zumiatiko	Limnio	Sefka	Mavrud(G)	Bogiolamas	Karnachalas	Tamyanka	Mavrud(B)	Rubin	Pamid(B)	Dimyat	Ruen	Shizoka Melnishkaloza	Mishej Sandanski	Keratsuda(B)
Pamid(G)	**0.0000**	0.4566	0.4107	0.4940	0.5047	0.4502	0.4785	0.4866	0.4298	0.3593	0.5326	-0.0040	0.4105	0.4860	0.4979	0.4449	0.2794
Keratsuda(G)		**0.0000**	0.4118	0.6000	0.6154	0.4913	0.5627	0.4730	0.3814	0.4139	0.4988	0.4582	0.3978	0.5957	0.5838	0.5095	0.4266
Zumiatiko			**0.0000**	0.5200	0.4783	0.3374	0.5089	0.4991	0.3835	0.2871	0.5073	0.3986	0.0000	0.5241	0.5091	0.3811	0.4429
Limnio				**0.0000**	0.4286	0.3553	0.4086	0.3150	0.3617	0.2754	0.4931	0.4640	0.5205	0.5797	0.5828	0.4464	0.5398
Sefka					**0.0000**	0.3553	0.5136	0.3492	0.4132	0.2727	0.5083	0.4837	0.4783	0.5231	0.5407	0.4072	0.5407
Mavrud(G)						**0.0000**	0.3259	0.3208	0.2906	0.0175	0.3948	0.4057	0.3447	0.3987	0.3258	0.2693	0.4310
Bogiolamas							**0.0000**	0.3857	0.3660	0.2600	0.5007	0.4620	0.5045	0.5527	0.5056	0.4729	0.5158
Karnachalas								**0.0000**	0.2761	0.2784	0.3501	0.4620	0.5052	0.4844	0.5081	0.4235	0.4940
Tamyanka									**0.0000**	0.2701	0.2612	0.3977	0.3969	0.3859	0.3908	0.1835	0.4205
Mavrud(B)										**0.0000**	0.3536	0.3192	0.2987	0.3392	0.2741	0.2267	0.3720
Rubin											**0.0000**	0.5107	0.5089	0.4338	0.4813	0.3924	0.4701
Pamid(B)												**0.0000**	0.4031	0.4541	0.4647	0.4026	0.2507

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Dimyat	**0.0000**	0.5184	0.5002	0.3885	0.4412
Ruen		**0.0000**	0.2902	0.3325	0.3576
Shizoka Melnishkaloza			**0.0000**	0.2881	0.3469
Mishej Sandanski				**0.0000**	0.3787
Keratsuda(B)					**0.0000**

Supplemental Table 4. Population differentiation analysis implemented in FSTAT 2.9.3.2 software.

	Pamid(G)	Keratsuda(G)	Zumiatiko	Limnio	Sefka	Mavrud(G)	Bogiolamas	Karnachalas	Tamyanka	Mavrud(B)	Rubin	Pamid(B)	Dimyat	Ruen	Shizoka Melnishkaloza	Mishej Sandanski	Keratsuda(B)
Pamid(G)		***	***	***	***	***	***	***	***	***	***	NS	***	***	***	***	***
Keratsuda(G)			**	**	***	**	**	***	***	***	***	**	***	***	***	***	***
Zumiatiko				***	**	**	**	***	***	***	***	**	NS	***	***	***	***
Limnio					**	**	**	***	***	***	***	**	***	***	***	***	***

Sefka		**	**	***	***	***	***	**	***	***	***	***	***
Mavrud(G)			**	***	***	NS	***	**	***	***	***	***	***
Bogiolamas				**	***	***	***	**	***	***	***	***	***
Karnachalas					***	***	***	**	***	***	***	***	***
Tamyanka						***	***	***	***	***	***	***	***
Mavrud(B)							***	***	***	***	***	***	***
Rubin								***	***	***	***	***	***
Pamid(B)									***	***	***	***	**
Dimyat										***	***	***	***
Ruen											***	***	***
Shizoka	Melnishkaloza											***	***
Mishej Sandanski													***
Keratsuda(B)													

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

NS: non-significant

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Figure legends

Figure 1. Factorial Correspondence Analysis of all Greek and Bulgarian grapevine varieties under study, using Genetix 4.05.2 software (a); UPGMA dendrogram of all studied varieties constructed with POPTREEW package, comprising of two major clusters (I and II). Numbers in nodes represent bootstrap values (b).

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