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Plastid DNA sequence diversity in wild grapevine samples (*Vitis vinifera* subsp. *sylvestris*) from the Caucasus region

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Summary

DNA sequence diversity was investigated in three plastid regions (the *trnH-psbA* intergenic spacer, *accD-psaI* intergenic spacer and the *rpl*16 intron) in a group of 40 wild grape (*Vitis vinifera* subsp. *sylvestris*) samples from the South Caucasus. This group included 22 samples from Georgia, 9 samples from Azerbaijan, 2 samples from Armenia and 7 samples from Turkey. The South Caucasus region is widely believed to be the area in which grape domestication began, and the study of genetic diversity in this region is viewed as key to understanding grape domestication in general. Four plastid haplotypes are evident in the 40 samples, and are designated by their character states at each of the 4 polymorphic positions: AAAT – 22 samples, ATTT – 6 samples, GTAC – 1 sample, and ATAT – 11 samples. The AAAT haplotype is restricted to Georgia and Azerbaijan, the ATAT haplotype is distributed across the entire study area, the ATTT haplotype is distributed in the southern part of the study area from the Black Sea to the Caspian Sea. The single GTAC haplotype was only found in southwestern Georgia. The AAAT haplotype is restricted to both wild (*V. vinifera* subsp. *sylvestris*) and cultivated (*V. vinifera* subsp. *vinifera*) grape samples from the Caucasus. This observation and the presence of all other plastid haplotypes observed in a previous study of worldwide grape cultivars highlight both unique and high levels of genetic variation in wild grape (*V. vinifera* subsp. *sylvestris*) from the greater Caucasus region.

Key words: Grape DNA, *Vitis vinifera* subsp. *sylvestris*, *trnH-psbA* intergenic spacer, *accD-psaI* intergenic spacer, *rpl*16 intron, DNA sequencing.

Introduction

The geographic origins of grapevine domestication are not currently known. According to many researchers the Caucasus region (northwestern Turkey, northern Iraq, southern Russia, Azerbaijan, Armenia, Georgia) and adjacent areas (Anatolia, Syria, Lebanon, Israel), are the geographic areas where grapes were most likely first domesticated (Negrul 1946, Zhukovski 1971, Sauer 1993, Jackson 1994, Zohary and Hopf 2000, Myles *et al.* 2011). Special climate conditions in this area have favored the diversification of wild varieties from which cultivated grapes were domesticated (Negrul 1946). The wild subspecies from which grapevine was domesticated (*Vitis vinifera* subsp. *sylvestris*) is abundant in Georgia and the greater Caucasus region, and a recent archeological data from South Caucasus indicates that winemaking dates back to early 6th millennium BC in this region (McGovern 2003, Barnard *et al.* 2011).

Vitis vinifera subsp. *sylvestris* occurs in Europe, northern Africa and the Middle East, including the Mediterranean, Black, and Caspian Sea Basins from Spain to Turkmenistan (Arroyo-Garcia *et al.* 2006). The abundance of wild grapevine has been dramatically reduced, first by the arrival of North American pathogens (phylloxera, oidium, mildew) over the last 150 years, and more recently by habitat fragmentation (Grassi *et al.* 2006). In the Caucasus, and especially in Georgia, *Vitis vinifera* subsp. *sylvestris* is morphologically variable (Ekhvaia and Akhalkatsi 2010). Many intermediate wild forms were observed, including those with characters associated with cultivated grapevine (*Vitis vinifera* subsp. *vinifera*), such as white fruit, hermaphroditic flowers, and larger seeds (Negrul 1946).

The primary goal of this study was to investigate plastid DNA sequence diversity in a geographically diverse set of South Caucasian *Vitis vinifera* subsp. *sylvestris* samples. To date no study has broadly assessed DNA sequence variation in wild grapevines of the Caucasus in this way. This information is of great interest from both an ethnobotanical and crop improvement standpoint.

The South Caucasus region is widely believed to be the area in which grape domestication began, and the study of genetic diversity of wild grape samples in this region is viewed as a key to understanding grape domestication in general.

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Material and Methods

The wild grapevine samples were collected from different geographic zones of the South Caucasus region: Georgia, Armenia, Azerbaijan and Turkey. Wild populations were identified by comparing collected grape leaves to those of morphological descriptions of typical *Vitis vinifera* subsp. *sylvestris*. Total genomic DNA was extracted from young grape leaves. The leaves were ground in liquid nitrogen and the CTAB based extraction procedure was used (Lodhi *et al.* 1994). When necessary, extracted DNAs were purified with GenElute columns (Sigma-Aldrich). In certain cases DNA was extracted from silica dried leaves using a plant genomic DNA extraction miniprep system (Viogene, Qiagen).

Sequence polymorphism was investigated at three non-coding plastid DNA regions (the *trnH-psbA* intergenic spacer, the *accD-psaI* intergenic spacer and the *rpl*16 intron). The *trnH-psbA* intergenic spacer was amplified with the primers "trnH" and "psbA" (Hamilton 1999). The *rpl*16 intron was amplified with the primers "*rpl*16 internalF" and "*rpl*16 internalR" (Beridze *et al*. 2011).

The portion of *accD-psaI* intergenic spacer was amplified with the primers *accD* forward -

5' AAAGAATCAAAGGTTGCGAAT 3' and *psaI* reverse - 5'CCCGTTTTTATCTTCTAATTTCCA 3' (this study). The length of amplified region is 395 bp (it corresponds to the position 63112-63506 of published *V.vinifera* chloroplast genome (Jansen *et al.* 2006).

PCR conditions included 1 min denaturing at 94 °C, 30 cycles of 94 °C denaturing (1 min), 55 °C annealing (1 min) and 72 °C extension (2 min); followed by a final extension step at 72 °C (5 min). PCR products were purified with GenElute PCR Clean-Up Kits (Sigma-Aldrich), dye-labeled using a Big Dye Terminator Kit (Applied Biosystems) and analyzed on either an Applied Biosystems 3100 or 3700 genetic analyzers (Biology Department of Washington University, St.Louis, MO and Laboratory Services Division of the University of Guelph, ON, Canada). Sequences were manually aligned in Se-Al (Rambaut, 2002) and haplotype networks were generated using TCS 1.18 (Clement *et al.* 2000).

As shown in our earlier work (Beridze *et al.* 2011), *trnH-psbA* intergenic spacer of cultivars 'Alphonse Lavalee' and 'Yugoslavia' 360 contains 6T in a poly-T region (position 143-149). In other 111 cultivars 7T were observed. In 3 wild grape samples from Northern Turkey (samples N31,39,40 in the Table) 6T was found.

Results and Discussion

It was proposed, that for true and hybrid species (*V. riparia, V. rupestris, V. berlandieri* etc.), the most part of the wild specimens of grapevines, usually attributed to *Vitis vinifera* subsp. *sylvestris* through Europe, in reality belong to a complex group of foreign invader taxa. It deals with the naturalized forms of the rootstocks, mostly coming from North America and initially used to graft on the European grapevine, in order to defend it against the attack of the phylloxera disease (Laguna 2004). Therefore it is very important to find simple molecular test to differentiate wild grape (*Vitis vinifera* subsp.*sylvestris*) from the American species of the genus *Vitis*. In the present study, it was found, that such test can be the copy number variation inside the *accD-psaI* intergenic spacer. 5 bp sequence ACTTA (it corresponds to the position 63293-63297 of published *V. vinifera* chloroplast genome (Jansen *et al.* 2006) is doubled in the studied American species *Vitis riparia* Meissner n.1 and *Vitis rupestris* Mission.

Earlier DNA sequence diversity was investigated at two plastid regions (the *trnH-psbA* inter-genic spacer and the *rpl*16 intron) in a geographically diverse group of 113 cultivated grape samples (Schaal *et al.* 2010, Beridze *et al.* 2011). This group included 40 samples from Georgia. Four plastid haplotypes were evident in the 113 samples, and were designated by their character-states at each of three polymorphic positions, one in the *trnH-psbA* intergenic region and two in *rpl*16 intron area. All samples were classified into four haplotype groups based on their character-states at these three sites: (AAA) - 23 samples, (ATT) - 29 samples, (GTA) - 26 samples, and (ATA) - 35 samples. The AAA haplotype was only observed in Georgian samples.

In the present study 40 wild grapevine (*Vitis vinifera* subsp. *sylvestris*) samples from the South Caucasus were analyzed. This group included 22 samples from Georgia, 9 samples from Azerbaijan, 2 samples from Armenia and 7 samples from Turkey (Table). No copy number variation was detected in any 40 samples of wild grape. Plastid DNA sequence variation study revealed the presence of four polymorphic sites. The fourth polymorphic site was observed in *accD-psaI* intergenic spacer (C→T at the position 63186) in addition to three observed in earlier study.

The four haplotypes of wild grape were designated as AAAT, ATTT, ATAT and GTAC (Fig. 1). The group ATTT corresponds to chlorotype D, group ATAT to chlorotype C and group GTAC to chlorotype A in Arroyo-García *et al.* 2006 (Martinez-Zapater, pers. comm.). In the cited paper chlorotypes were identified using chloroplast microsatellites.

The geographic distributions of the four haplotype groups are presented in Fig. 2. The ATAT haplotype is distributed across the entire study area, the AAAT haplotype is restricted to Georgia and Azerbaijan, the ATTT haplotype is distributed in the southern part of the study area from the Black Sea to the Caspian Sea. The single GTAC haplotype was only found in southwestern Georgia.

Based on the sampling of this and an earlier study (Schaal *et al.* 2010, Beridze *et al.* 2011), the AAAT haplotype is globally restricted to both wild and cultivated grapevine samples from the Caucasus. This observation and the presence of all other plastid haplotypes observed in the previous study of a worldwide set of grape cultivars highlight both unique and high levels of genetic variation in wild grapevine (*V. vinifera* subsp. *sylvestris*) from the greater Caucasus region.

In south Caucasus two major centers of grape cultivar formation were deduced - Alazani river basin in the North-Eastern part of south Caucasus and the Colchis - western

Table

Sample information of the 40 sequenced samples

Sample No.	Population	Geographic Region	River Basin	GPS Coordinates
		Sequence Group AAAT		
1	v.Ağbil, Quba distr.	North-East Azerbaijan	Quruçay	41°26.037' N 48°33.491' E
2	Iori Nature Reserve, Sagarejo distr.	East Georgia	Iori	41^0 31.499' N 45^0 51.264' E
3	Sagarejo distr.	East Georgia	Iori	41°28.187' N 45°39.718' E
4	v.Susay-Qışlaq, Quba distr.	North-East Azerbaijan	Quruçay	41^0 28·023 N' 48^0 34·433' E
5	Jumaskure, Dedoplitskaro distr.	East Georgia	Alazani	41^0 21.588' N 46^0 35.934' E
6	Gardabani Reserve, Gardabani distr.	East Georgia	Mtkvari (Kura)	41^o 22.497' N 45^o 04.383' E
7	Gardabani reserve, Gardabani distr.	East Georgia	Mtkvari (Kura)	41^o 22.554' N 45^o 03.704' E
8	Korugi Reserve, Sagarejo distr.	East Georgia	Mtkvari (Kura)	41^o 39.43' N 45^o 26.17' E
9	Korugi Reserve, Sagarejo distr.	East Georgia	Mtkvari (Kura)	41°38.15' N 45°27.12' E
10	Korugi Reserve, Sagarejo distr.	East Georgia	Mtkvari (Kura)	41^o 37.46' N 45^o 27.15' E
11	Korugi Reserve, Sagarejo distr.	East Georgia	Mtkvari (Kura)	41^o 37.48' N 45^o 27.11' E
12	Borjomi distr.	South Georgia	Mtkvari (Kura)	41^0 47.8' N 43^{0}18.333' E
13	Borjomi distr.	South Georgia	Mtkvari (Kura)	41^0 54.018' N 43^0 21.036' E
14	Borjomi distr.	South Georgia	Mtkvari (Kura)	41^0 54.347' N 43^0 20.382' E
15	v.Qsovrisi, Mtskheta distr.	East Georgia	Qsani	41^o 59.383' N 44^o 31.35' E
16	v. Ananuri, Dusheti distr.	East Georgia	Aragvi	42^0 22.164' N 44^0 41.718' E
17	Dusheti distr.	East Georgia	Aragvi	42^0 10.730' N 44^0 41.043' E
18	Dusheti distr.	East Georgia	Aragvi	42^0 07.431' N 44^0 46.524' E
19	v.Meneso, Dusheti distr.	East Georgia	Aragvi	42^0 14.846' N 44^0 40.527' E
20	v.Samtavisi, Kaspi distr.	East Georgia	Lekhura	42°02.761' N 44°22.781' E
21	v.Samgereti, Tetritskaro distr.	South-East Georgia	Qcia	41°29.690' N 44°26.688' E
22	v.Pitareti, Tetritskaro distr.	South-East Georgia	Qcia	41°29.016' N 44°19.916' E
		Sequence Group ATTT		
23	Ijevan, Tavush distr.	North-East Armenia	Getik	-
24	Ijevan, Tavush distr.	North-East Armenia	Getik	-
25	Ağsu distr.	Central Azerbaijan	Girdmancay	-
26	Şavşat distr. Artvin Province	North-East Turkey	Şavşat	41°15.665' N 42°18.080' E
27	Artvin distr.	North-East Turkey	Çoruh	41°14.973' N 41°46.338' E
28	Qobustan distr.	East Azerbaijan,	Gorge of Qobustan	-
		Sequence Group GTAC		
29	v.Likani, Borjomi distr.	South Georgia	Mtkvari (Kura)	41^0 56.652' N 43^0 27.702' E

Table, continued

Sample No.	Sequence Group ATAT			
	Population	Geographic Region	River Basin	GPS Coordinates
30	Vashlovani Reserve, Dedoplitskaro distr.	East Georgia	Pantishari	41⁰ 8.142' N 46⁰ 12.678' E
31	v.Işhan, Yusufeli distr., Artvin Province	North-East Turkey	Oltis	40°47.409' N 41°44.734' E
32	v.Susay-Qışlaq, Quba distr.	North-East Azerbaijan	Qusarçay	41°28.255' N 48°36.140' E
33	Ardanuç distr., Artvin Province	North-East Turkey	Çoruh	41°08.651' N 42°00.625' E
34	Yusufeli distr., Artvin Province	North-East Turkey	Çoruh	40°48.839' N 41°28.345' E
35	v. Alpakri, Quba distr.	Northern Azerbaijan	Quruçay	-
36	Balakan distr.	North-West Azerbaijan	Balakancay	-
37	Qabala distr.	Northern Azerbaijan	Turyancay	-
38	Zaqatala distr.	North-West Azerbaijan	Alazani	-
39	Şavşat distr., Artvin Province	North-East Turkey	Şavşat	41°16.262' N 42°16.432' E
40	Yusufeli distr., Artvin Province	North-East Turkey	Barhal	40°51.282' N 41°32.507' E

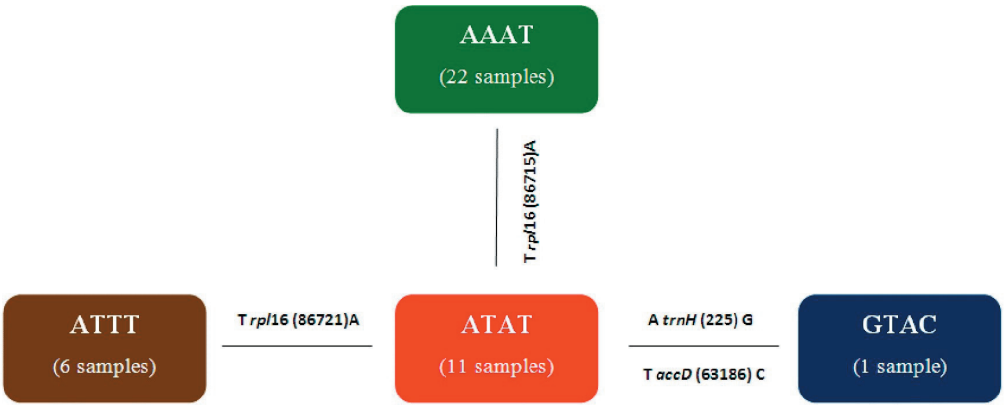

Fig. 1: Haplotype network derived from substitutions observed in the 40-sample *trnH-psbA*/*accD-psaI*/*rpl*16 dataset. The substitution type and position in the published *V.vinifera* chloroplast genome (Jansen *et al.* 2006) are indicated along each branch.

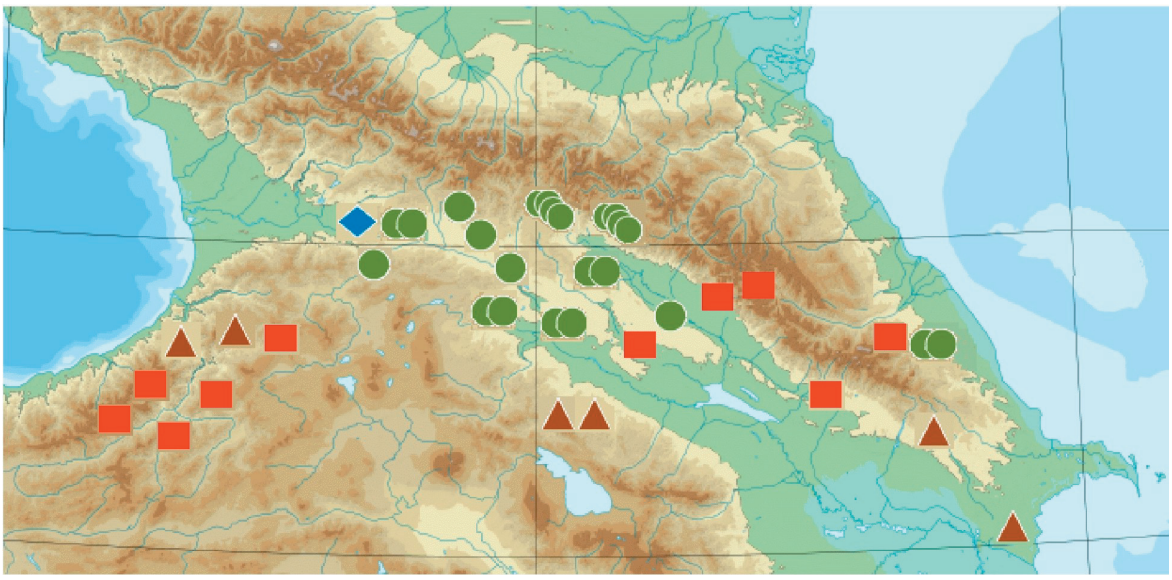

Fig. 2: Geographic location of the four plastid DNA haplotypes detected among Caucasian wild grapevine samples (*Vitis vinifera* subsp. *sylvestris*). Haplotype colour coding corresponds to that in Fig. 1.
● = Haplotype AAAT; ▲ = Haplotype ATTT; ◆ = Haplotype GTAC; ■ = Haplotype ATAT.

part of South Caucasus (Ketskhoveli *et al.* 1960). The Alazani great center of grape formation was more intensive in the lower reaches of the river. The climate in this land (temperature, high humidity) and rich soil was assisting the formation of grape varieties. According to our earlier investigation grape cultivars of the Alazani center belong to the AAAT haplotype (Beridze *et al.* 2011).

Another center of grape cultivar formation - Colchis is the area at the Black Sea coast, separated from the Alazani center by Likhi Range. The grape cultivars formed in the Colchis center belong to the GTAC haplotype (Beridze *et al.* 2011).

According to De Blij's hypothesis the grape domestication occurred in the South Caucasus and then cultivated grape was transferred by the ancient routes to Greece and Egypt followed by Greek dispersal and Roman diffusion to Europe (De Blij 1983) (Fig. 3).

During the investigation of plastid DNA sequence diversity in a worldwide set of grapevine cultivars four plastid haplotypes were evident in the 113 samples (Beridze *et al.* 2011). The AAAT haplotype was only observed in the Caucasian samples.

If de Blij's hypothesis is true it will be necessary to slightly modify his scheme. We must state that initial grape dispersal to Greece and Egypt occurred not by land but by sea, because the AAAT haplotype was formed in the Alazani hearth (far from the Black Sea) and this haplotype is absent in world-wide cultivars. Other three haplotypes (wild as well as cultivated) can be observed near the Black Sea coast. The contact of Greek sailors with Colchis has long history, described in Greek mythology as Argonauts in their guest to Colchis to find the Golden Fleece.

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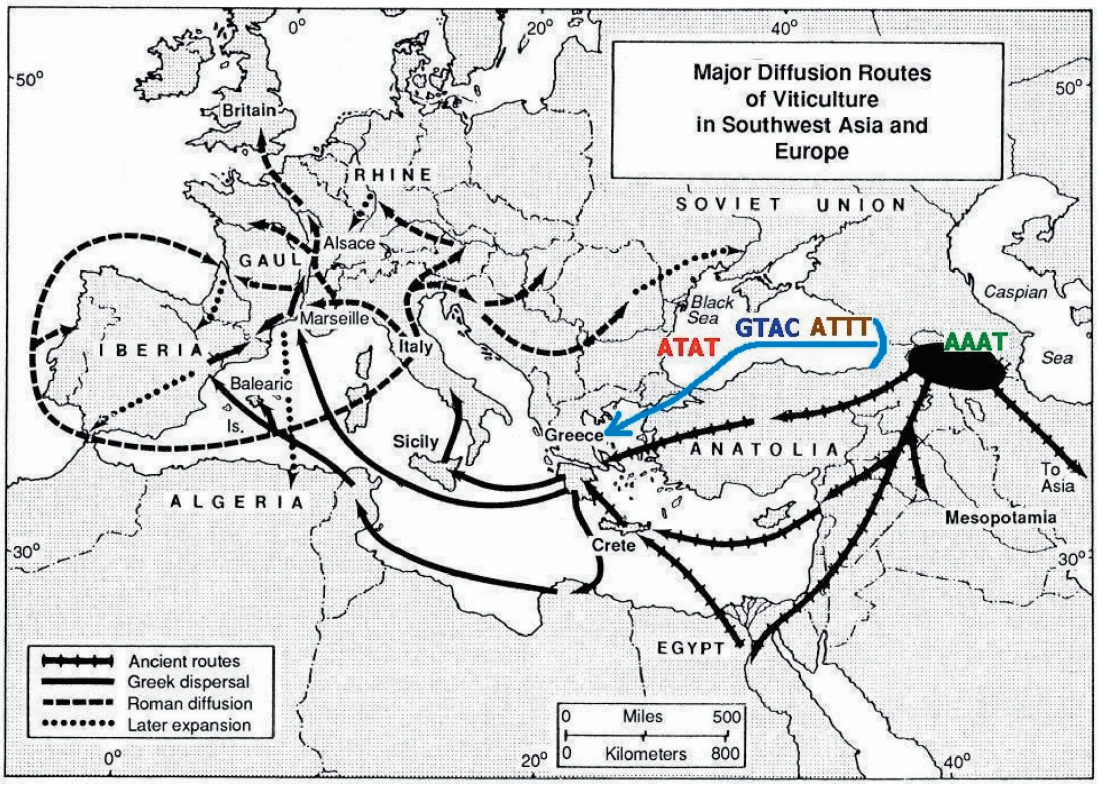

Fig. 3: Major diffusion routes of viticulture (De Blij, 1983). The route through the black sea (grey arrow) is added according to the results of this work.

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