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Nuclear SSR-based Genetic Diversity and Structure Analysis of *Vitis* Rootstocks

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Abstract

We have genetically identified 43 plants representing 28 rootstocks from three different ampelographic collections in Greece with the use of seven nuclear single sequence repeats (SSR) previously characterized. Analysis of SSR alleles and a similarity dendrogram construction was followed by a STRUCTURE and a hierarchical STRUCTURE analysis. We also compared the obtained genetic data with literature data about the origins of these rootstocks. In most cases known pedigrees of the studied rootstocks were confirmed but in some other cases microsatellite profiling did not support the pedigrees data, casting a doubt about the identity of a particular rootstock or on pedigree given by the literature. The genetic profiling of these plants enabled to reassign wrong identities in the collections and proved to be very efficient in assessing rootstock identities in a commercial dispute. The identity of an unknown rootstock was also determined. This work provides the molecular fingerprinting of 28 rootstocks and adds to their ancestry characterization.

Keywords: *Vitis*; Grape Rootstock; Simple Sequence Repeats (SSR); Molecular Identification; Structure Analysis; Genetic Diversity

Introduction

Historical evidence indicates that European grape (*Vitis vinifera* L.) was introduced from Europe to the American continent in the 16th century [1]. However, it failed to thrive due to pests and diseases, mainly phylloxera (*Phylloxera vastatrix*) and nematodes. The ability of north American *Vitis* species to resist pests and diseases was soon recognized and they were used first as phylloxera resistant hybrids with *Vitis vinifera*, then as rootstocks for the sensitive European cultivars [2]. In commercial viticultural practice, rootstocks have been in use since the middle of the 19th century to protect grapevines form soil borne pests but also to ameliorate the effect of unfavourable soil and environmental conditions, such as lime, salinity, and drought. In addition, rootstocks can affect the grapevine growth, fruit yield, and other economically important vine attributes.

From the late 1850's, grafting on various *Vitis* species or interspecific *Vitis* hybrids became a common practice in California as a consequence of the introduction of phylloxera [3]. Later, this practice was spread in virtually all viticultural regions over the world. Numerous *Vitis* species and hybrids were tested for their resistance to phylloxera [4,5] of which the most efficient have been extensively used [6-8]. In addition to phylloxera, nematodes represent another pest threatening vineyards [9]. Thus, nematode resistant rootstocks were developed from the 1930's [10-19]. Although the commercially used rootstocks are much fewer compared to grapevine cultivars, the importance of efficient and accurate genotype identification is of equal importance, if not more. There is a necessity for the grower to have an absolute certitude about the rootstocks on which his selected cultivars are grafted due to high cost for establishing a vineyard and to the long time required for full production [8].

Traditionally, the identification of *Vitis* genotypes, both cultivars and rootstocks, was based on ampelographic characters and biochemical markers, such as isoenzymic profile, until the progress in molecular biology allowed the development of numerous molecular markers for genotype identification. Among them are variable number tandem repeat (VNTR), short tandem repeat (STR), restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), sequence characterized amplified region (SCAR),

expressed sequence tag (EST), single nucleotide polymorphism (SNP), and microsatellites, single sequence repeats (SSR). In early 1990's, five RFLP probes designed from *Vitis vinifera* Chardonnay DNA were developed, which enabled to distinguish 16 rootstocks [20,21]. Concomitantly, Boursiquot and Para [22] assayed one isoenzyme system, previously characterized on grapevine cultivars for fingerprinting 30 rootstocks authorized for trade in France and were able to distinguish uniquely 5 rootstocks while others clustered in 4 phenotypic groups [23]. Walker and Liu [24] by using 5 isoenzyme systems were able to obtain a unique isoenzyme profile for each rootstock. Research for easier and more reproducible markers went on with the assessment of RAPD markers [25-27].

All the assayed markers, eventhough successful enough for providing a single identity profile for a rootstock, were dominant markers, which do not allow to differentiate between homozygotes and heterozygotes and thus to rebuild pedigrees, and consequently to carry out a follow up of selection processes, for creating and selecting new rootstocks for the future. Additionally, the methods are costly, cumbersome, affected by environmental conditions (isoenzymes) [28] or subject to low reproducibility between laboratories. These disadvantages were overcome by the availability of specific microsatellite markers developed and characterized in a large number of grapevine cultivars [29].

The use of nuclear SSR markers for genotype identification of cultivars and hybrids in *Vitis vinifera* and *Vitis* species has been developing fast and assessed for a variety of us [30,31]. The nuclear data were complemented by the use of chloroplast microsatellite [32,33]. Microsatellite profiling also opened the path for gene pools studies [34,35], molecular assisted management of genetic resources [36-42]. Furthermore a neural network algorithm and a combination of RAPD and SSR molecular markers were used for the genetic characterization of different rootstock varieties [31]. Riaz et al. [43] developed nuclear and chloroplast SSR fingerprint data from rootstocks in germplasm collections, compared them to develop a reference dataset, and carried out parentage analysis to resolve previously reported, and determine new breeding records. They refined and updated the parentage of 26 rootstocks based on 21 nuclear and 14 chloroplast markers. Results indicated that 39% of the genetic background of analyzed rootstocks originated from only three accessions of three grape species: *Vitis berlandieri* cv. Rességuier 2, *V. rupestris* cv. du Lot and *V. riparia* cv. Gloire de Montpellier. Results determined that Rességuier 2 is the maternal parent for 14 commercial rootstocks, 9 of which are full-sibs with Gloire de Montpellier as the paternal parent. Similarly, du Lot is the paternal parent of nine rootstocks.

Here we present, microsatellite profiling data of 43 plants representing 28 rootstocks from 3 ampelographic collections in Greece, in an effort to support the practical application of microsatellite profiling for chasing management mistakes, solving commercial dispute or recovering lost information. In addition, the possible pedigrees of this rootstock gene pool were assessed. The previously reported pedigree information was correct for only eight of the 28 rootstocks [43]. Additionally, misnaming of rootstocks originating from a commercial orchard was solved by applying microsatellite profiling to plants sampled in the vineyard.

Materials and Methods

Plant material

Leaves from 43 *Vitis* spp. and hybrids used as rootstocks (Table 1) were collected from the Laboratory of Plant Physiology and Biotechnology at the University of Crete, Heraklion, the Institute of Floriculture, Horticulture and Viticulture, National Agricultural Research Foundation (NAGREF), Katsabas, Heraklion and the Institute of Vine and Wine, Lykovrisi (NAGREF), Athens. Leaves were kept frozen at -80°C until used. Additionally, leaves from 12 plants in a private nursery and one from a private vineyard were sampled and stored in the same way.

Table 1: The names of the 43 *Vitis* rootstocks, the origin, the acronyms of the individuals and the acronym of the populations used for the molecular profiling of the 20 populations of *Vitis* rootstocks.

Rootstocks names	Origin	Acronyms	Populations
Aramon Rupestris Ganzin 1	4	AXR1_4	ARA
Couderc 157-11	3	Coud157-11_3	COUD
Couderc 1613	4	Coud1613_4	COUD
Couderc 161-49	1	Coud161- 49_1	COUD
Couderc 161-49	3	Coud161-49_3	COUD
Couderc 161-49	3	C161-49_3	COUD
Couderc 1616	3	Coud1616_3	COUD
Couderc 3306	3	Coud3306_3	COUD
Couderc 3309	3	Coud3309_3	COUD
Dog Ridge	4	Dogridge_4	DORI
Ecole de Montpellier 34	3	Mont34_3	ECMONT
Fercal	3	Fercal_3	FER
Fercal	3	Fercal_3	FER

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Gloire de Montpellier	3	Gloire_3	GLOMONT
Harmony	3	Harmony_3	HAR
Kobber 5BB	4	Tel5BB_4	KOBSELOPP
Kobber 5BB	3	Tel5BB_3	KOBTEL
LN33	4	LN33_4	LN
LN33	3	LN33_3	LN
Millardet et Grasset 101-14	3	101-14_3	MILGRA
Millardet et Grasset 41B	4	41B_4	MILGRA
Millardet et Grasset 41B	3	41B_3	MILGRA
Millardet et Grasset 420A	4	420A_4	MILGRA
Paulsen 1045	3	Pau1045_3	PAUL
Paulsen 1103	4	Pau1103_4	PAUL
Paulsen 1103	3	Pau1103_3	PAUL
Paulsen 1103	3	Pau1103_3	PAUL
Paulsen 1103	1	Pau1103_1	PAUL
Richter 110	3	Ri110_3	RICH
Richter 110	4	Ri110_4	RICH
Richter 31	3	Ri31_3	RICH
Richter 57	3	Ri57_3	RICH
Ruggeri 140	3	Rug140_3	RUG
Ruggeri 140	4	Rug140_4	RUG
Ruggeri 140	1	Rug140_1	RUG
Rupestris du Lot	4	Rup Lot_4	RUPE
Rupestris du Lot	3	Rup Lot_3	RUPE
Salt Creek	4	Salt Creek_4	SALTCREE
Salt Creek	3	Salt Creek_3	SALTCREE
Selektion Oppenheim 4	4	SO4_4	SEL
Selektion Oppenheim 4	3	SO4_3	SELOPPRUG
Teleki 5C	3	Tel5C_3	TEL
Teleki 8B	3	Tel5BB_3	TELKOBB

DNA extraction

DNA was extracted from 100 to 150 mg FW of leaf tissue according to a micro-method of DNA purification described elsewhere [37].

Microsatellite PCR and microsatellite profile analysis

Amplification primer sequences for 7 nuclear microsatellite loci from *Vitis* vinifera, UCH2, UCH11, UCH12, UCH19, UCH29, UCH35 and UCH40, were used for DNA amplification. PCR amplifications were carried out as described previously [38]. PCR products sizing and SSR profiles analysis were performed as described elsewhere [37,42].

Genetic Analysis and Neighbor-Joining Tree Construction

The allele sizing per locus was based on published repeat patterns. The data matrixes were produced and the genetic diversity measures were determined for each employed locus across all fingerprinted genotypes. These methods included the individual locus polymorphic information content (PIC), the observed heterozygosity (HO), and the expected heterozygosity (HE). PIC, HO, HE, estimated frequency of null alleles and probability of identity (PI) were calculated with the software CERVUS ver. 3.0.3 [44,45]. A matrix was produced employing

Nei's distance matrix within GenAlEx, version 6 [46]. Furthermore, a neighbor-joining tree was produced using MEGA 7 starting with the Nei's distance matrix [47,48]. Twenty (20) populations were formed from 43 *Vitis* rootstocks. In order to determine the divergence between the different population, pairwise Fst measurements were calculated according to Weir and Cockerham [49] using GenAlEx 6 [46]. Analysis of molecular variance (AMOVA) was also performed to assess the genetic structure of the 20 population, using GenAlEx 6.

Population Structure

The STRUCTURE 2.3.4 software was used to analyse the germplasm genetic structure. This software utilize a Bayesian clustering algorithm to identify subpopulations, assign individuals to them, and estimate population allele frequencies [50]. The analy-

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sis was carried out using a burning period of 10,000 iterations and a run length of 200,000 MCMC replications. We tested a continuous series of K, from 1 to 10, in 10 independent runs. We did not introduce any prior knowledge about the origin of the population, and assumed correlated allele frequencies and admixture [51]. For selecting the optimal value of K, ΔK values were calculated using STRUCTURE harvester [52,53]. POPHELPER, proposed by Francis (2016), was used to analyse and visualize population structure. Furthermore, a "hierarchical STRUCTURE analysis" was applied in this study by running STRUCTURE on subsequently partitioned data, as suggested by Pritchard and Falush [54].

Results and Discussion

Genetic diversity analysis of *Vitis* rootstocks

Genetic diversity is caused by multitude factors. Many evolutionary forces can change genetic frequencies of crop species, which in turn determine the genetic diversity of a population. Gene flow, genetic drift, and mutations are among the evolutionary forces, which affect gene pool of a given population [55]. Riaz et al. [43] provided valuable historical information on the diachronic efforts and the contributors worldwide in the production of the existing and extensively used grape rootstocks. In the last three decades, biochemical markers (isoenzymes), DNA-based markers, such as RAPD, RFLP, AFLP and SSR have been used to fingerprint grape rootstocks at different geographical ampelographic collections [20,24,26,36,57-60]. In addition, SSR markers were used in Spain to confirm identification and elucidate the parentage of selected rootstocks [60-62]. Some other attempts assessed the clonal variation and possible parental relations, focusing primarily on the diversity of Teleki lines and comparing them with other rootstocks [59,63]. The results revealed that the existing information in the database of many historical rootstocks is not always accurate and should be corrected De Andris et al. 2007. This work aims to add to this effort, using SSR because of their polymorphism, reproducibility, and codominant nature.

Herein, a total of 43 individuals from 20 *Vitis* rootstocks were genotyped employing 7 SSR loci (Table 1). Polymorphic fragments were reproducibly amplified at all 7 SSR loci and microsatellites profiles of these 43 plants are given in Table S1. Microsatellite profiles of 13 plants from a private nursery and a private vineyard are given in Table S2. The genetic diversity measures determined for each employed locus across all fingerprinted genotypes are shown in Table 2. The number of amplified alleles (k) by each SSR primer pair varied from eleven for UCH19 to twenty-three for UCH12 with an average number of alleles per locus of 15.857. Different amplified alleles are shown in Table S4. Observed heterozygosity (Ho) ranged from 0.721 in UCH2 and UCH35 to 0.930 in UCH19, with an average value of 0.827, while the expected heterozygosity (He) ranged from 0.783 in UCH73 to 0.941 in UCH12 with an average value of 0.8774. Bianchi et al. [64] found rootstock heterozygosity values of Ho = 0.099 and Ho = 0.734 for SNPs and SSRs, respectively, whereas SSR He value of core collection was higher than the values detected in other studies [39,58,65,66] confirming the uniqueness and preciousness of the analyzed germplasm collection. In the study by Emanuelli et al. [65] using SSR, the subset of rootstocks revealed the highest number of alleles (405) and the highest heterozygosity (0.86), in spite of their relatively small sample size.

Table 2: Genetic data at seven nuclear microsatellites of 43 plants representing 20 rootstocks from 3 different ampelographic collections obtained from the Greek *Vitis* Database [42].

Locus	k	n	Hobs	Hexp	PIC	F(Null)
UCH2	14	43	0,721	0,872	0,850	0,0998
UCH11	17	43	0,814	0,889	0,869	0,0435
UCH12	20	43	0,907	0,941	0,926	0,0129
UCH19	11	43	0,930	0,860	0,833	-0,0469
UCH29	19	43	0,907	0,925	0,908	0,0044
UCH35	14	43	0,721	0,783	0,757	0,0447
UCH40	13	43	0,791	0,871	0,847	0,043

The genotype level of polymorphism was assessed by calculating polymorphic information content (PIC) values for each of the 7 SSR loci. The average PIC was 0.8556 with a minimum value of 0.757 in UCH35 and a maximum value of 0.926 in UCH12. One of the seven loci, the UCH19 exhibited probability of null alleles, F (null), greater than 0.05. Propability Identity (PI) is the probability with which two randomly taken genotypes display the same SSR profile. The probability of obtaining an identical genotype, the cumulative probability of identity, was calculated with a value of 8.9 × 10e−12, using the 7 SSR markers in combination. This number corresponds to a statistical potential of distinguishing a large number of unrelated grapevine genotypes. AMOVA was conducted to determine the variation explained by populations. The results indicated that 30% of the genetic variation ($p < 0.0001$) resided among populations and 0% ($p < 0.0001$) resided among individuals. The remaining 70% of the total variation ($p < 0.0001$) was explained within individuals. The RST value was 0.342 ($p < 0.0001$). The genetic variation was tested using the FST statistic estimated from pairwise comparisons as a measure for genetic distance between populations and individuals. The results indicated that 24%

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of the genetic variation ($p < 0.0001$) resided among populations and 76% resided within individuals ($p < 0.0001$). The FST value was 0.287 ($p < 0.001$).

The phylogeny and genetic relations among grapevine cultivars is of great importance in genetic improvement of *Vitis* germplasm ([42,65,67]). Emanuelli et al. [65] tested the structure analysis of 111 *Vitis* rootstocks. The genetic structure of cultivated grapevine has been influenced by human selection and it can be largely understood as a complex pedigree, due to the vast number of higher order pedigree relationships [68]. An unknown relevance among the grape genotypes affect the study of the genetic structure, resulting in the overestimation of the probable subpopulation number (K) using standard methods [54,69].

STRUCTURE Analysis

The genetic structure of the germplasm collection was evaluated using STRUCTURE software. The 20 populations of the rootstocks were assigned in its ancestor population as shown in Figure 1. The analysis provided evidence for a significant population structure in this set of rootstocks. A maximum value of the rate of change in the log probability of the data was revealed at K = 2, using Evanno's method (Figure 1). The highest Delta K value was observed at K = 2 (Figure 1b). The estimated logarithm of probability of the data [L(K)] increased linearly from K = 2 up to K = 10 showing a clear point of inflection (Figure 1a). The estimated population structure inferred from the analysis identifies two genetic groups, Ancestor population (pop) A and Ancestor population (pop) B (Figure 2).

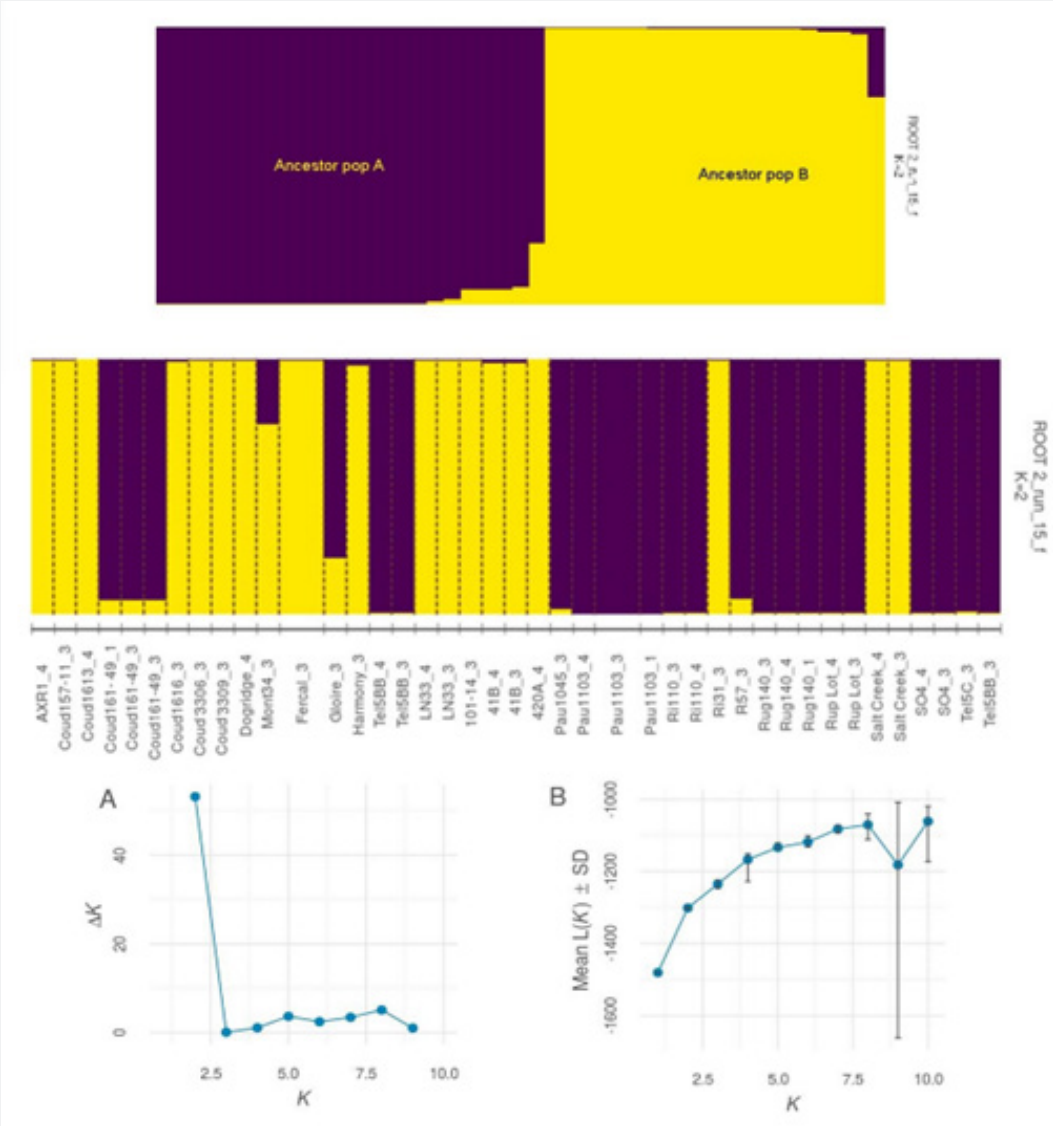

Figure 1: Genetic STRUCTURE of 43 Vitis rootstocks considering K = 2. Colors (purple and yellow) represent the two groups, defined by the K value. The assignment of the 20 populations in the ancestry groups. a) second-order rate of change of the loglikelihood of the data (ΔK) as a function of K, the number of clusters (color figure online) b) mean (± standard deviation) log-likelihood value of the data [L(K)] as a function of the value of K, the number of clusters.

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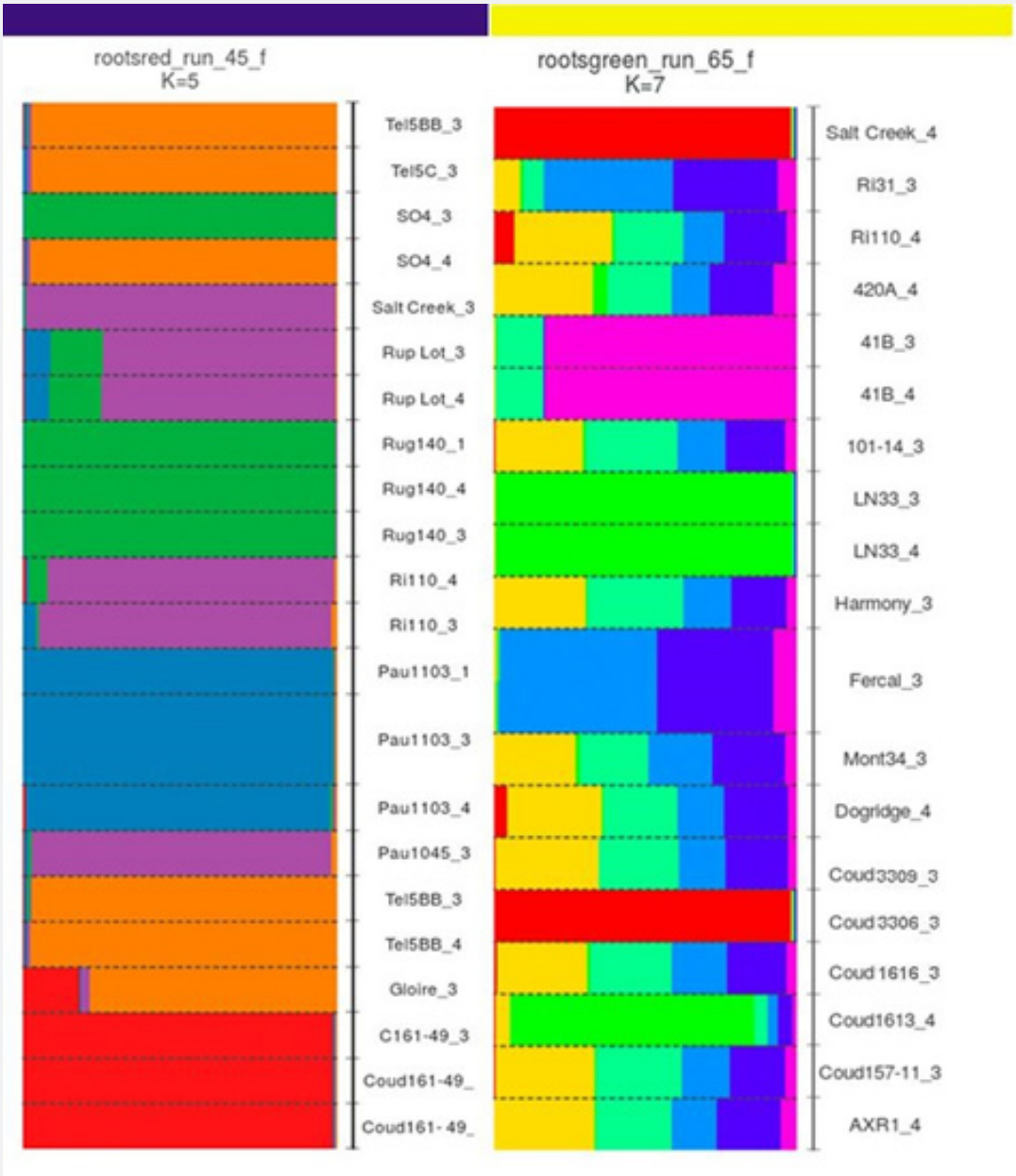

Figure 2: Flow charts of hierarchical STRUCTURE analysis of 43 Vitis rootstocks with 7 SSR markers. First round of STRUCTURE analysis: two predefined groups which are separated by color, ancestor pop A (purple) and ancestor pop B (yellow). Plots generated with POPHELPER using all the individuals used in this study. The hierarchical STRUCTURE analysis resulted in subsequent charts, where the subpopulations found are also separated by color. Second round of STRUCTURE analysis: Ancestry group A consists of 5 subgroups, Ancestry group B consists of 7 subgroups (color figure online).

Hierarchical STRUCTURE Analysis

A subsequent round (second round) of STRUCTURE revealed that the two populations A and B (K =2) were further separated as follows (Figure 2): Ancestry group A is subdivided into five distinct subgroups (K=5) (Figure 3). Twenty-three *Vitis* rootstocks are presented in the ancestry group A (Figure 2). These are Tel5C_3, Tel5BB_3, SO_4_3, SO_4_4, Salt Creek_3, Rup Lot_4, Rup Lot_3, Rug140_3, Rug140_4, Rug140_1, RI57_3, RI110_3, Pau1103_1, Pau1103_3, Pau1103_4, Pau1045_3, Tel5BB_3, Tel5BB_4, Gloire_3, Coud161-49_3, Coud161- 49_1. Interestingly some populations appear identical a) Tel5BB_3 (KOBTEL population) and Tel5BB_3 (TELKOBB population) (subgroup-orange), b) Tel5BB_4 with SO_4_4 and (subgroup-orange), c) Rug140_1,3,4 with SO_4_3 (subgroup-green). Further research is needed to verify these data. Three rootstock populations appear to have individuals in both ancestor populations (1st round) these are Richter (subgroup-purple), Couderc (subgroup-red) and Salt Creek (subgroup-purple). The remaining individuals mingle with each other according to dendrogram and their distances are higher than 0.2.

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Ancestry group B is subdivided into seven distinct subgroups (K =7) (Figure 2). Twenty *Vitis* rootstocks are presented in ancestry group B (Figure 2). These are Salt Creek_4 (subgroup-red), RI31_3 (subgroup-light blue), RI110_4 (subgroup-yellow), 420A_4 (subgroup- yellow), 41B_3 (subgroupmagenta), 41B_4 (subgroup- magenta), 101-14_3 (subgroup-light green), LN33_4 (subgroup-green), LN33_3 (subgroup-green), Harmony_3 (subgroup-light green), Fercal_3 (subgroup-light blue), Mont34_3 (subgroup-yellow), Dogridge_4 (subgroup- yellow), Coud3309_3 (subgroup- yellow), C1613_4 (subgroup-green), Coud3306_3 (subgroup-red), Coud157-11_3 (subgroup-yellow), and AXR1_4 (subgroup-yellow). Again interestingly, we can see that none of the populations are close to each other. Their distances are higher than 0.2 (Figure 3). According to Emanuelli et al. [65], the hierarchical approach needs to be applied to delve deeper into the complex relationships of the *Vitis* germplasm. Considering the relevant role of rootstocks on environmental stress tolerance, their low genetic diversity reduces the ability of grapevine cultivars to adapt to several environmental constraints. This issue can be overcome by increasing the genetic and phenotypic diversity of the breeding material, including non-conventional material in the further breeding programs.

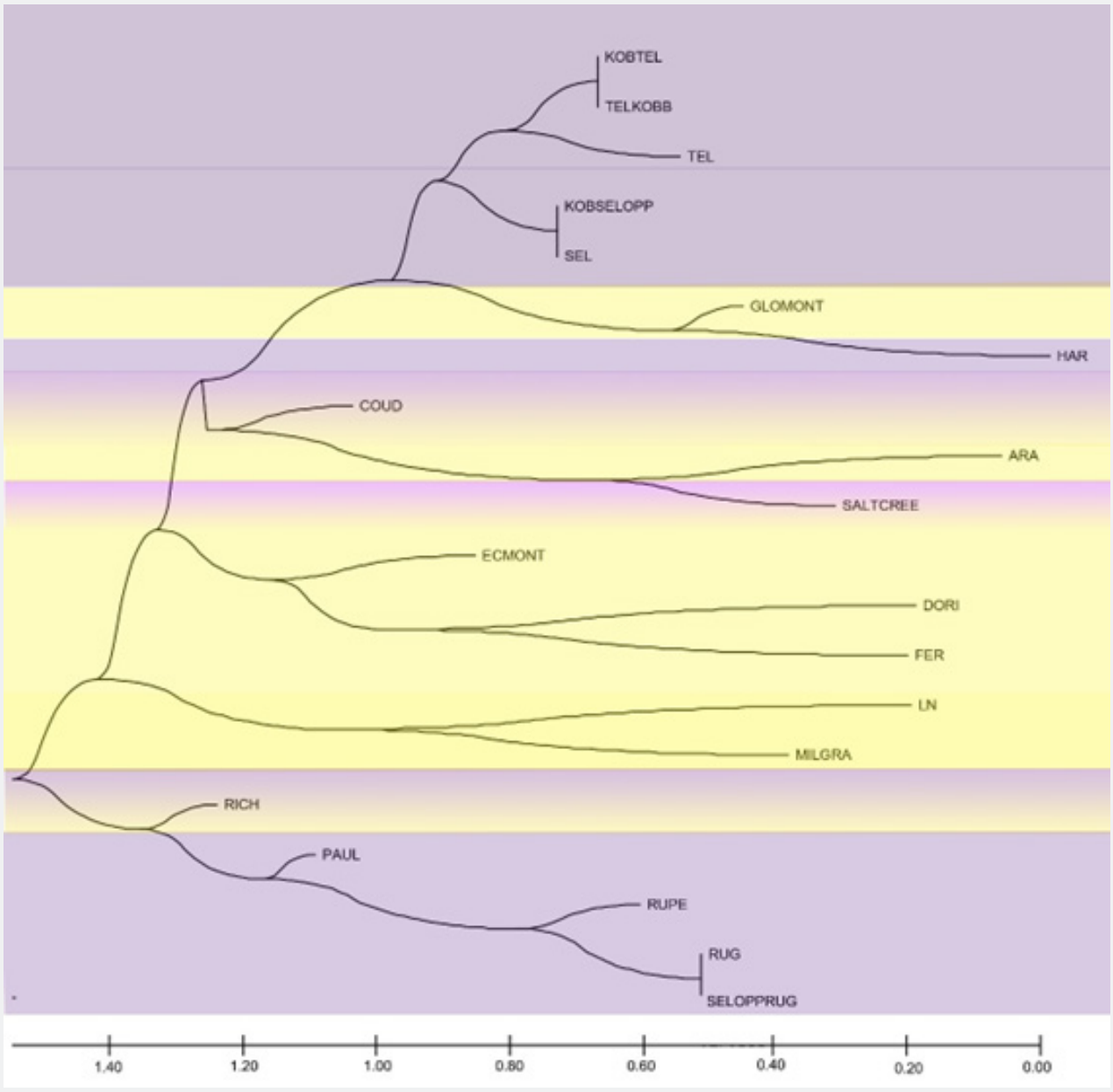

Figure 3: Neighbor-joining unweighted phylogenetic similarity tree based on Nei's distance matrix. Its color (purple-yellow, 1st round of STRUCTURE) assign its rootstock population in an ancestor population.

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Genetic Distance Analysis

A neighbor-joining tree was built based on Nei's distance matrix (Figure 3). Based on this phylogenetic tree the 43 *Vitis* rootstocks form 20 populations belonging to six major clusters.

i. KOBTEL-TELKOBB mingled with TEL and all of them mingled with KOBSELOPP-SEL, all assigned in the same ancestor population A and the same subgroup orange.

ii. GLOMONT is mingled with HAR but their big distance becomes obvious with the Structure Analysis. GLOMONT belongs to the ancestor population A and HAR to the ancestor population B.

iii. ARA, SALTCREE and COUD appear in the same cluster however many individuals belong to different ancestor populations.

iv. ECMONT, DORI and FER belong to the same ancestor population B, with ECMONT and DORI in subgroup-yellow and FER in subgroup-light blue.

v. LN and MILGRA form a cluster and belong to the same ancestor population A, LN has individuals in subgroup-green and MILGRA has some in subgroups magenta and yellow.

vi. RICH, PAUL, RUPE, RUG, SELOPPRUG form another cluster.

Solving identity ambiguities

Microsatellite profiles of 13 plants from a private nursery and a private vineyard are given in Table S2, along with profiles of reference rootstocks and cultivars (Paulsen 1103, Ruggeri 140, Couderc 3309 and Soultanina). Phenotypic discrepancies based the need for such microsatellite profiling. From the results it appeared that the allegated Paulsen 1103 was the cultivar Soultanina, that the allegated Ruggeri 140 was an unknown profile not present in the Greek *Vitis* Database and that an unknown plant from a private vineyard was in fact the rootstock Couderc 3309. This example shows the resolution of microsatellite profiling of rootstocks and cultivars to solve possible commercial disputes between nurseries and their customers.

Conclusion

The significance of rootstocks to reduce the impact of biotic and abiotic stresses on *V. vinifera* cultivars requires the increase of the genetic and phenotypic diversity of the breeding rootstock material. Most of the currently used rootstocks globally were bred nearly a century ago; however, the changing climate has prompted a renewed interest in breeding rootstocks to address current and future vineyard issues [43]. If the lineage of classic grape rootstocks is uncovered, and their inheritance is established and understood, this information can be used to broaden the genetic basis of new rootstocks. Therefore, the existing germplasm collections are valuable resources for exploring the genetic and phenotypic diversity and providing new genetic resources to support plant breeding efforts.

Acknowledgement

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