Report on the genetic analysis of 86 additional *Vitis* individuals from the Donau-Auen National Park (DANP).

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1. Introduction

The range of European wild grapevine is in constant decline in Europe. The alert was already been given by Issler in 1938. The decreasing range of this taxon is due, in large part, to the destruction of natural habitats, as well as to the spread, since 1860, of pests and diseases of the North America (phylloxera, oïdium and mildew). The genus *Vitis* is represented by several coexisting species in Europe. *Vitis vinifera* L. ssp. *sylvestris* (Gmelin) Hegi is the only existent wild European taxon.

Many spontaneous forms of grapevine cultivars are also naturalised in Europe. They belong to *V. vinifera* L. ssp. *vinifera*, introduced for at least a thousand years when domesticated forms of grapevine were spread throughout Europe. Several American and Asian *Vitis* species have been introduced during the last century as rootstock.

Nowadays taxonomic pollution represents a new threat. A large *Vitis* complex involves escaped cultivars, rootstocks and wild grapevines.

In Austria, the large number of wild grapevines in the alluvial forests around Vienna was known in the 18th century (Jacquin, 1762). In 1906, Rechinger described a large individual in the Prater in Vienna as well as presence of specimens in the alluvial forests of Morava on the Slovakian border. In 1955, Kirchheimer made an assessment of the presence of wild grapevine in Lower Austria. The previously mentioned populations were then considered missing. Ehrendorfer and Niklfeld (1972) reported wild grapes mainly located on the left bank of the Danube, and only downstream from Vienna. According to recent surveys made by the team of the Donau-Auen National Park, it is still the case, few individuals were discovered on the right bank towards Fischamend and Regelsbrunn.

In 2010, 165 samples were analysed. In 2017, 91 additional samples were analysed. The team of the Donau-Auen National Park sent us 86 samples in September 2018. A total of 86 samples were thus analysed this year.

The aim of the current project is to identify if further grapevines found in the DonauNational Park in 2018 are true wild grapevines or not.

2. Samples

Additionally to the samples received from the Donau-Auen National Park we also collected leaves from grape varieties from the Research Center of Pully as standards. Among them, we included one sample from the previous study (DA87 of 2017) as well as six grape varieties: Chasselas, Merlot, Baco Noir, Frankenthaler, Ebling, Gouais Blanc and two rootstocks: Riparia Gloire and SO4. Riparia Gloire is a crossing of a mother and a father *Vitis riparia* Michx and SO4 (Selection Oppenheim 4) is a crossing between *V. berlandieri* (Planch) (mother) and *V. riparia (*father*)*. These additional samples allowed us to standardise the data with other sets of cultivars and rootstocks previously analysed.

Packs were numbered from 1 to 9, containing each from 7 to 10 bags with dessicated leaves (Table 1 and Pictures Annexe 1).

Package	Sample Number
Vitis-Gen18-1	NPDA01
Vitis-Gen18-1	NPDA02
Vitis-Gen18-1	NPDA03
Vitis-Gen18-1	NPDA04
Vitis-Gen18-1	NPDA05
Vitis-Gen18-1	NPDA06
Vitis-Gen18-1	NPDA07

Package	Sample Number
Vitis-Gen18-5	NPDA46
Vitis-Gen18-5	NPDA47
Vitis-Gen18-5	NPDA48
Vitis-Gen18-5	NPDA49
Vitis-Gen18-6	NPDA50
Vitis-Gen18-6	NPDA51
Vitis-Gen18-6	NPDA52

Vitis-Gen18-1	NPDA08
Vitis-Gen18-1	NPDA09
Vitis-Gen18-2	NPDA10
Vitis-Gen18-2	NPDA11
Vitis-Gen18-2	NPDA12
Vitis-Gen18-2	NPDA13
Vitis-Gen18-2	NPDA14
Vitis-Gen18-2	NPDA15
Vitis-Gen18-2	NPDA16
Vitis-Gen18-2	NPDA17
Vitis-Gen18-2	NPDA18
Vitis-Gen18-2	NPDA19
Vitis-Gen18-3	NPDA20
Vitis-Gen18-3	NPDA21
Vitis-Gen18-3	NPDA22
Vitis-Gen18-3	NPDA23
Vitis-Gen18-3	NPDA24
Vitis-Gen18-3	NPDA25
Vitis-Gen18-3	NPDA26
Vitis-Gen18-3	NPDA27
Vitis-Gen18-3	NPDA28
Vitis-Gen18-3	NPDA29
Vitis-Gen18-4	NPDA30
Vitis-Gen18-4	NPDA31
Vitis-Gen18-4	NPDA32
Vitis-Gen18-4	NPDA33
Vitis-Gen18-4	NPDA34
Vitis-Gen18-4	NPDA35
Vitis-Gen18-4	NPDA36
Vitis-Gen18-4	NPDA37
Vitis-Gen18-4	NPDA38
Vitis-Gen18-4	NPDA39
Vitis-Gen18-5	NPDA40
Vitis-Gen18-5	NPDA41
Vitis-Gen18-5	NPDA42
Vitis-Gen18-5	NPDA43
Vitis-Gen18-5	NPDA44
Vitis-Gen18-5	NPDA45
Vitis-Gen18-6	NPDA53
Vitis-Gen18-6	NPDA54
Vitis-Gen18-6	NPDA55
Vitis-Gen18-6	NPDA56
Vitis-Gen18-6	NPDA57
Vitis-Gen18-6	NPDA58
Vitis-Gen18-6	NPDA59
Vitis-Gen18-7	NPDA60
Vitis-Gen18-7	NPDA61
Vitis-Gen18-7	NPDA62
Vitis-Gen18-7	NPDA63
Vitis-Gen18-7	NPDA64
Vitis-Gen18-7	NPDA65
Vitis-Gen18-7	NPDA66
Vitis-Gen18-7	NPDA67
Vitis-Gen18-7	NPDA68
Vitis-Gen18-7	NPDA69
Vitis-Gen18-8	NPDA70
Vitis-Gen18-8	NPDA71
Vitis-Gen18-8	NPDA72
Vitis-Gen18-8	NPDA73
Vitis-Gen18-8	NPDA74
Vitis-Gen18-8	NPDA75
Vitis-Gen18-8	NPDA76
Vitis-Gen18-8	NPDA77
Vitis-Gen18-8	NPDA78
Vitis-Gen18-8	NPDA79
Vitis-Gen18-9	NPDA80
Vitis-Gen18-9	NPDA81
Vitis-Gen18-9	NPDA82
Vitis-Gen18-9	NPDA83
Vitis-Gen18-9	NPDA84
Vitis-Gen18-9	NPDA85
Vitis-Gen18-9	NPDA86

Table 1. List of packs and sample names mentioned on the bags.

3. DNA Extraction

The total DNA of leaf samples was extracted in early Oktober 2018 after the delivery of the dried samples.

We used the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions.

Aliquots of purified DNA were prepared in order to have between 1ng and 1μg of DNA template in the analysis (PCR).

4. Genotyping

4.1. Nuclear molecular markers

Nuclear microsatellites markers correspond to specific and highly variable regions of DNA. They have the property of being stable inside an individual and varying greatly from one individual to another. The analysis of eight selected polymorphic microsatellites is sufficient to distinguish nearly all grape varieties in the world (Sefc et al., 2000). Markers (nSSR primers) designed in the flanking regions of these microsatellites will amplify via PCR (Polymerase Chain Reactions) these specific and variable regions of the DNA. Differences in alleles (length of fragments in basepairs) will allow to differentiate wild grapevines from cultivars and to calculate the genetic diversity of populations for example.

Nuclear DNA (nDNA) is inherited 50% from the mother and 50% from the father.

We selected 10 markers from the previous study (Arnold et al. 2017), which were normally amplifying well, were most polymorphic and were informative for differentiating wild grapevines from cultivars and rootstocks.

Methods used for PCRs, Genotyping and Statistics followed the methodology described in Arnold et al 2017.

The 10 nSSR primers amplified correctly for most samples (Fig 1).

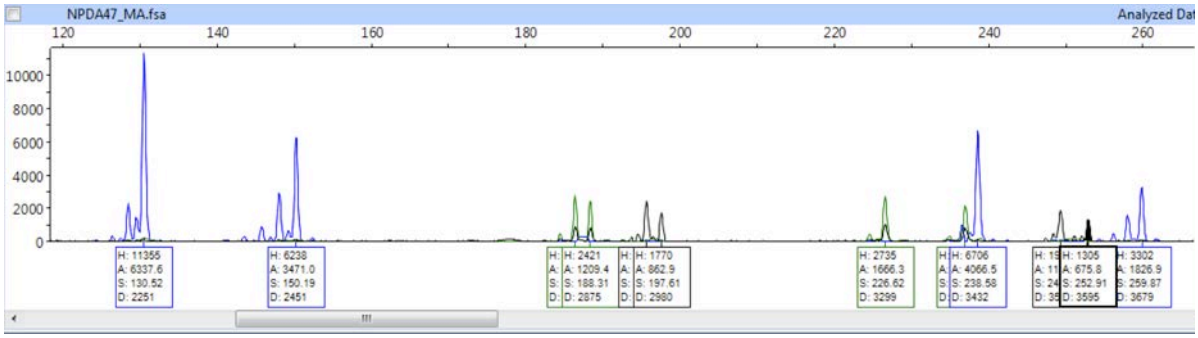

Fig 1. Example of electrophoregram of multiplex microsatellites (sample NPDA47)

The primer names, the references, the number of alleles, and the percentage of missing data is presented in Table 2.

Primer	Reference	Number of alleles	Missing Data
VVS 2	Thomas & Scott 1993	16	1.67
VVMD 5	Bowers et al. 1996	14	0
VVMD 7	Bowers et al. 1996	17	0
VVMD 25	Bowers et al. 1999	10	1.67
VVMD 27	Bowers et al. 1999	16	0
VVMD 31	Bowers et al. 1999	11	2.5
VVMD 32	Bowers et al. 1999	16	0
VrZAG 62	Sefc et al. 1999	13	0
VrZAG 79	Sefc et al. 1999	13	0
VMC 2H4	Vitis Microsatellite Consortium	21	2.5

Table 2. List of 10 nSSR primers, references, number of alleles per locus and percentage of missing data (non amplified samples).

4.2. SSR Data

Raw data were standardised. In addition to the 86 samples, we added one sample from the previous study (DA 87) as well as 21 Cultivars of *Vitis vinifera* origin and 15 Rootstocks of American *Vitis* origin.
Sample 10 did not sufficiently amplify as well as sample 52 and sample 60. They were thus removed from the data set. In total, the dataset is composed of 120 samples

4.3. Detection of suspected clones within the dataset

For this purpose we first investigated the raw data (Annexe 2), sorting data by size.

A UPGMA clustering based on the Genetic distance matrix was done (Annexe 3). A PCoA (Principal component analysis) (Fig 2) was performed in GenAlEx 6.5 (Peakall & Smouse, 2006). This allows also to confirm the clonality and the relationship.

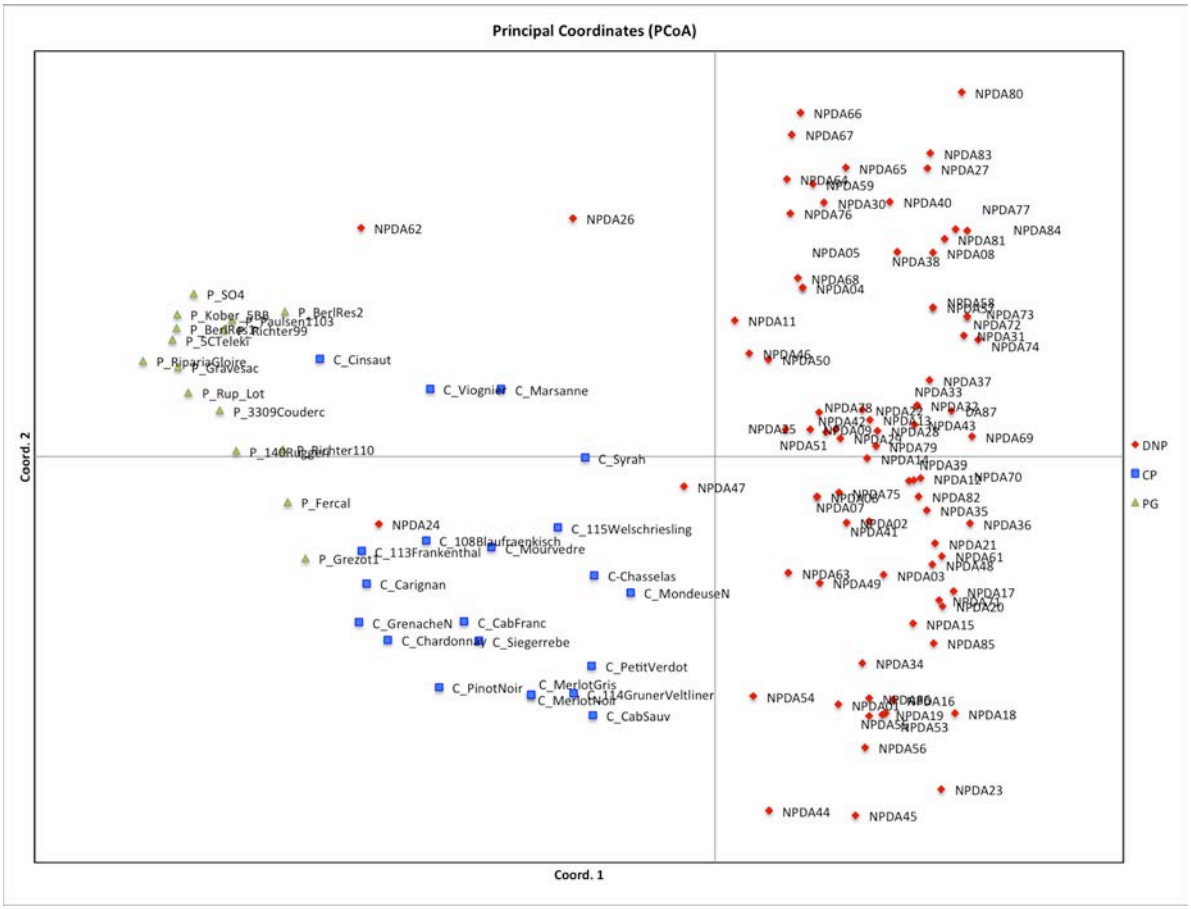

Fig 2. Plot of the first 2 axis of the PCoA performed in GenAlex via Covariance matrix with data standardization. The 83 samples from the NPDA are in red, the 21 Cultivars are in green and the 15 Rootstocks are blue.

In table 3 we regrouped individuals, which share exactly the same genotype for at least 9 of the 10 primers. One sample did not amplify for one (locus) primer but we have 6 couples of samples sharing a same genetic profile with the 10 SSR primers. However we have to remain careful as more primers need to be analysed to ensure a clonality. We see that sample numbers are following and thus it could be actually same individuals.

Suspected Clones		Remark
DA04	DA05	Identical on all primers
DA06	DA07	Identical on all primers
DA32	DA33	Identical on all primers
DA57	DA58	Identical on all primers
DA72	DA73	Identical on all primers
DA53	DA55	Identical on 10 primers VMC2H4 did not work for DA55

Table 3. Groups of samples with the same genetic profile on 10 markers.

4.4. Clustering

The UPGMA cluster allows to identify closely related individuals. Three main groups are present (Annexe 3 - in red wild grapevines, in blue cultivars and in green rootstocks). Most samples collected within the National Park are true wild grapevines. NPDA24 is located within the group of cultivars and seems to be closely related to Frankenthal ((= trollinger (in Germany), vernatsch (in South Tyrol) or schiava grossa (in Italy)) or Welschriesling. NPDA 26 and NPDA62 are located within the group of rootstocks. They seem to be closely related to *V. riparia*.

4.5. Genetic diversity and private alleles

All loci were polymorphic in the 3 populations: 1) samples from the park DANP, 2) *Vitis* cultivars and 3) rootstocks. Genetic diversity (He) is high for the cultivars (He=0.779) and rootstocks (He=0.812) (Table 4). The diversity is lower (He=0.539) in the "wild" DANP group even with the presence of hybrids samples within the analysis. A low heterozygosity can indicate a bottleneck or the presence of a large metapopulation, which is actually the case here in the DANP population. In this case, observed heterozygosity (Ho) is slightly higher than the expected heterozygosity (He). This part of the population seems to be close to the HardyWeinberg equilibrium, which was clearly not the case in the previous studies. This can just be a picture of a past genetic dynamism of the population. Compared to the previous years, the values for Ho and He are similar for wild grapevines.

Private alleles are alleles that are found only in a single population among a broader collection of populations. They were calculated using the frequency-based statistics.

Within DANP, 19 private alleles are found, in the set of cultivars used within this study there were 18 private alleles and within the set of rootstocks 36.

Pop		Na	Ne	I	Ho	He	F	No. Private Alleles
DNP	Mean	83.800	2.559	1.099	0.550	0.539	-0.018	19
	SE	0.200	0.377	0.134	0.060	0.058	0.019	
CP	Mean	20.500	4.784	1.761	0.826	0.779	-0.062	18
	SE	0.269	0.366	0.071	0.030	0.018	0.034	
PG	Mean	14.700	5.575	1.879	0.844	0.812	-0.034	36
	SE	0.213	0.393	0.061	0.074	0.014	0.089	

Table 4. Summary of genetic diversity in the 3 groups (120 total individuals). Park: DANP, Cultivars: CP and Rootstocks: PG. Na: number of alleles; Ne effective number of alleles; I Shannon's Information Index; HO and HE: observed and expected heterozygosity, respectively; F = Fixation Index; Number of private alleles.

4.6. Structure Analysis

From the structure analysis (Fig. 3) performed on the 120 grape samples, we retained K=3, separating true wild grapevines (ssp. sylvestris) from cultivars (ssp. vinifera) and hybrid rootstocks. In the rootstock clade (in green), Fercal and Grezot showed alleles of *V. vinifera*, which is normal as they have *V.vinifera* in their genealogy. Indeed Grezot has genes of Mourvèdre as shown in the UPGMA.

Among the 83 samples from the NPDA, six samples showed hybridization or introgressed patterns. A summary of these results is given in Table 5.

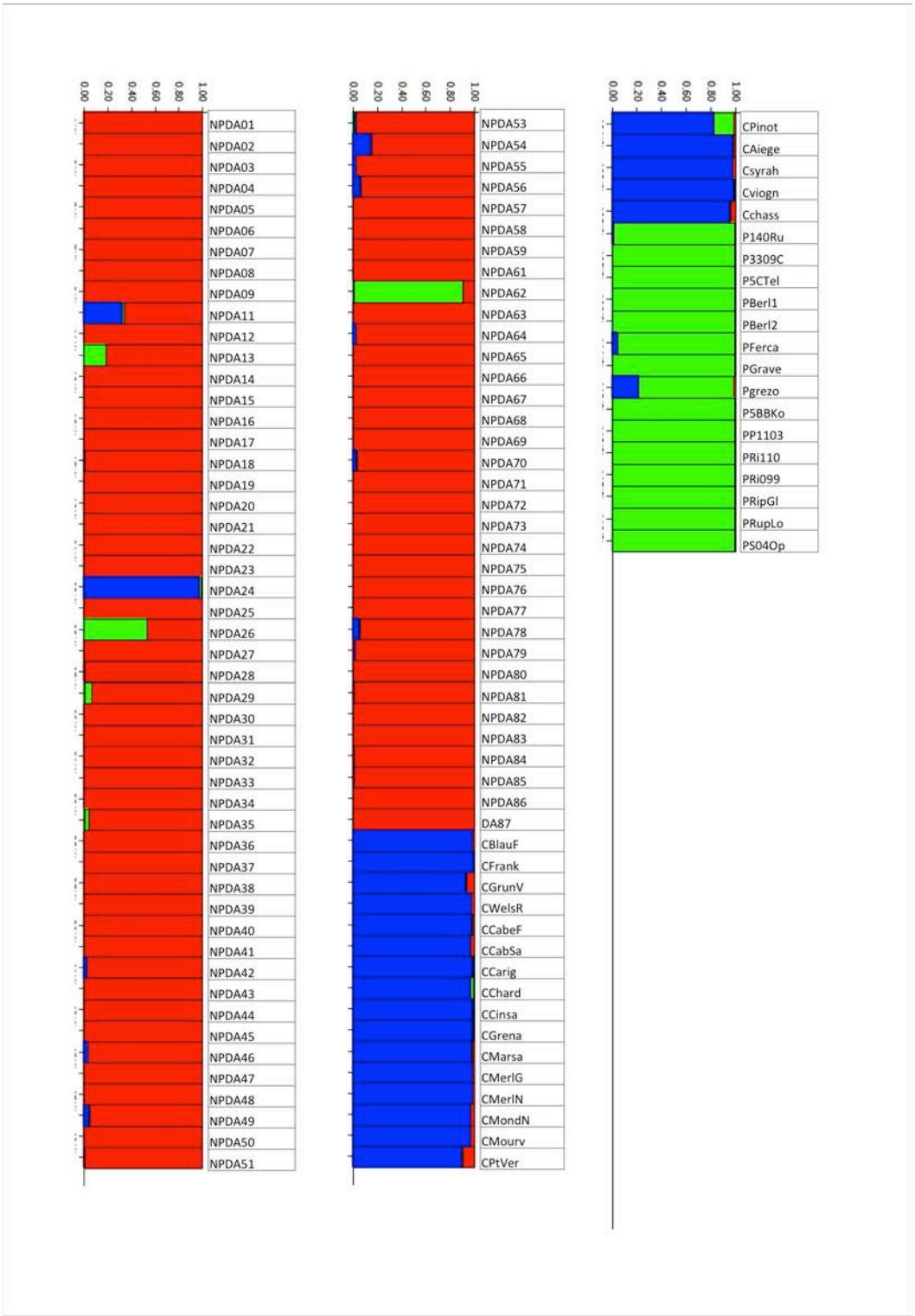

Fig. 3. Population structure of the *Vitis* complex of the Donau Auen National Park inferred with the Bayesian clustering algorithm implemented in STRUCTURE. Each individual is represented by a vertical bar, partitioned into *K* segments representing the proportions of ancestry of its genome in *K=3* clusters. Wild grapevines are in red, cultivars are in green and rootstocks are in blue.

Sample	Haplotype	Putative Crossing
NPDA11	H1	Introgression ? Wild x Cultivar
NPDA13	H1	Introgression ? Wild x Rootstock
NPDA24	H3	Cultivar x Cultivar
NPDA26	H1	Hybrid? Wild x Rootstock
NPDA54	H2	Introgression ? Wild x Cultivar
NPDA62	H1	Rootstock x Rootstock

Table 5. Haplotypes and putative origin of the crossing or introgression.

At K=3 only six samples do share alleles with rootstocks or cultivars. NPDA 24 is probably issued from a crossing between 2 cultivars. It has also a haplotype present mostly in cultivars. NPDA62 has a genetic profile of rootstock however the haplotype is typical of wild grapevines. The other samples are hybrids or introgressed with wild grapevines.

5. Chloroplast

5.1. CP Introduction

Three labelled primer pairs designed for the following chloroplastic regions were amplified by PCR: ndhF2, TrnC and TrnK2 regions (Table 6).

These 3 regions are sufficient to give information on the mother origin of grapevines. Within this sample set we identified a total of four haplotypes distributed in both wild grapevines and hybrids/introgressed. H1, which is common in the wild populations of western Europe; H2, which is common in the wild populations of eastern Europe; H3, which is similar to Chardonnay and Merlot; H4, which is similar to Chasselas and Cabernet Sauvignon. No H5 (which regrouped all the American rootstocks of various origins) was found.

CP Regions Annealing T°C	ndhF2 51°C	trnC 52°C	trnK2 49°C
H1	173	194	131
H2	172	194	131
H3	173	139	131
H4	173	139	136
H5	181	194	131

Table 6. Chloroplastic regions and length of the amplified fragments (in bp) related to the five haplotypes generally identified in wild *Vitis* in Europe.

5.2. CP Methods

Amplifications (PCR) were carried out according to the method described in Arnold et al 2017.

5.3. CP Results

94% of samples did amplify (Annexe 4) at all locus or at least sufficiently to allow an identification to the *V.vinifera* or the Rootstock group.
66 out of 86 samples are belonging to haplotype (H1) (Fig. 4).

In the previous studies performed in the DANP we did also find H1 to H4 in the true wild individuals, and the distribution of samples within each haplotype was relatively similar.

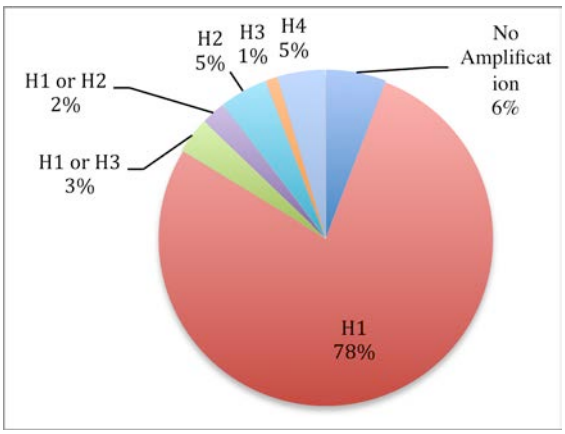

Fig. 4. Percentage of haplotypes within the DANP

6. Discussion and conclusion

As already discussed in the paper and in previous studies performed in the DANP, substantial number of propagules regularly reach the park. They are issued from vineyards and gardens surrounding the National Park. Grapevine shoots, berries, seeds or pollen are brought within the Park by flooding, mammals, birds or insects.
This occurs since ages and will continue. We noticed over the years that the success of establishment of hybrids or introgressed individuals is rather low. In this dataset, introgressed or hybrid individuals are very rare. At least less than in previous similar analysis. Maybe these samples were collected in a better-preserved part of the DANP. We do not have the ecological information that could support this hypothesis.

We found 6 couples of clones. As the sample numbers are close we suspect that you collected the same individuals. With 10 SSR markers, we can not confirm the clonality, but this is not in the aim of the current study.

As we already mentioned, a better flooding dynamic should be welcome for the true wild grapevines species. This will for sure also lead to an increasing establishment of hybrids or introgressed grapevines sharing genes with cultivars or rootstocks.

7. References

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