Report on the genetic analysis of 91 additional *Vitis* individuals from the Donau-Auen National Park (DANP).

31.10.2017

Dr. Mag. Claire Arnold
Association Nterractive and University of Lausanne
CH- 2068 Hauterive CH-1015 Lausanne

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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, 200 samples were recorded in the Donau-Auen National Park (DANP) (Fig 1). A study was performed on 165 individuals. These results were presented at the "20 Jahr Nationalpark Donau-Auen - Konferenz 18.5.2016". They were published in 2017 in Ecology and Evolution. A copy of the publication (Chapter 2) and the corresponding data (Annexe 1, 2 and 3) are included in this report.

The aim of the current project is to identify if further grapevines found in the Donau National Park in 2016 and 2017 are true wild grapevines or not.

2. Insights into the *Vitis* complex in the Danube floodplain (Austria)

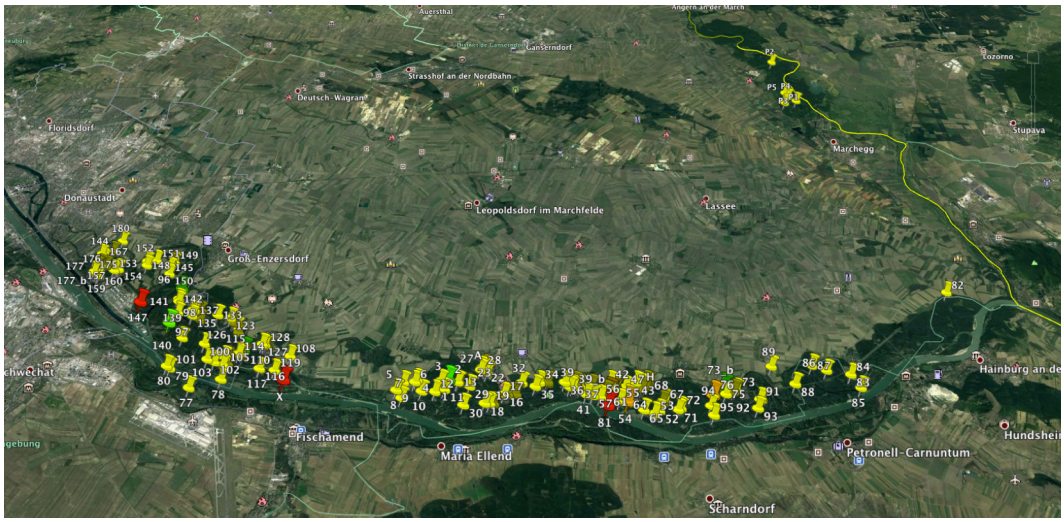

Fig. 1 Google Map of Vitis Samples in the DANP in 2011

Received: 6 March 2017 | Revised: 20 May 2017 | Accepted: 30 May 2017

DOI: 10.1002/ece3.3187

ORIGINAL RESEARCH

WILEY Ecology and Evolution Open Access

Insights into the *Vitis* complex in the Danube floodplain (Austria)

Claire Arnold[1] | Olivier Bachmann[2,3] | Annik Schnitzler[4]

[1]Unicentre, University of Lausanne, Lausanne, Switzerland

[2]Laboratory of Evolutionary Botany, University of Neuchâtel, Neuchâtel, Switzerland

[3]Laboratory of Plant Ecology, University of Basel, Basel, Switzerland

[4]Laboratoire Interdisciplinaire des Environnements Continentaux LIEC - UMR 7360 CNRS, University of Lorraine, Metz, France

Correspondence
Claire Arnold, University of Lausanne, Lausanne, Switzerland.
Email: claire.arnold@unil.ch

Funding information
Wüthrich and Matthey-Dupraz Funds

Abstract

European grapevine populations quickly disappeared from most of their range, massively killed by the spread of North American grapevine pests and diseases. Nowadays taxonomic pollution represents a new threat. A large *Vitis* complex involves escaped cultivars, rootstocks, and wild grapevines. The study aimed to provide insight into the *Vitis* complex in the Danube region through field and genetic analyses. Among the five other major rivers in Europe which still host wild grapevine populations, the Danube floodplain is the only one benefiting from an extensive protected forest area (93 km²) and an relatively active dynamic flood pulse. The Donau-Auen National Park also regroups the largest wild grapevine population in Europe. Ninety-two percent of the individuals collected in the park were true wild grapevines, and 8% were hybrids and introgressed individuals of rootstocks, wild grapevines, and cultivars. These three groups are interfertile acting either as pollen donor or receiver. Hybrids were established within and outside the dykes, mostly in anthropized forest edges. The best-developed individuals imply rootstock genes. They establish in the most erosive parts of the floodplain. 42% of the true wild grapevines lived at the edges of forest/meadow, 33.3% at the edges forest/channels, and 23.9% in forest gaps. DBH (Diameter Breast Height) varied significantly with the occurrence of flooding. Clones were found in both true wild and hybrids/introgressed grapevines. The process of cloning seemed to be prevented in places where flooding dynamics is reduced. The current global distribution of true wild grapevines shows a strong tendency toward clustering, in sites where forestry practices were the most extensive. However, the reduced flooding activity is a danger for long-term sustainability of the natural wild grapevine population.

KEYWORDS
genetic diversity, habitat fragmentation, invasion biology, river dynamics, *Vitis* complex

1 | INTRODUCTION

The Eurasian wild grapevine (*Vitis vinifera* ssp. *sylvestris* (Gmelin) Hegi) is currently distributed in a few alluvial (Figure 1) and colluvial forests around the Mediterranean basin between the 38th and 49th northern parallel, from sea level up to an altitude of 1,600 m (Arnold, 2002; Vassilczenko, 1970). These areas are refugia where grapevine pest (the homoptera *Daktulosphaira vitifoliae* Ficht traditionally called phylloxera) and fungi diseases (oïdium; mildew) have a restricted spread. These pest and diseases were imported with the American *Vitis* species at the end of the 19th century.

Phylloxera is particularly harmful for grapevine. It has been the major factor in determining the rate of decline in vineyards and wild populations worldwide since the middle of the 19th century (Arnold,

FIGURE 1 Typical habitat of wild grapevine in the Donau-Auen National Park (upper left). Wild grapevine in the canopy in autumn (right). Grape berries of a female wild grapevine (lower left) Photographs by Claire Arnold and Olivier Bachmann

2002). Grapevines survived in wet, temporarily anoxic soils of alluvial areas where this homoptera could not live (Ocete et al., 2004a; Ocete et al., 2006). River management led to the elimination of flood events, and a sinking of ground water levels. This induced among others a severe drying out of the environment. Phylloxera could then enter floodplains and killed massively grapevine populations (Arnold, 2002). For example in the Rhine upper valley, the 200 individuals recorded at the beginning of the 20th century (Issler, 1938; Kirchheimer, 1946; Schutz, 1946) had nearly disappeared a few decades later (Arnold, Schnitzler, Douard, Peter, & Gillet, 2005; Schumann, 1974). In Austria, Jacquin (1762) described forests covered with veils of grapevines. In 1955, Kirchheimer gave an update of the presence of wild grapevines in Lower Austria and considered this species in decline because of the destruction of its habitats. In 1972, wild grapevines just remained on the left riverside of the Danube and only downstream Vienna (Ehrendorfer & Niklfeld, 1972).

Recent studies have shown that wild grapevines survived as small populations in remote mountain sites, screes, floodplain forests of large rivers, their deltas, and their tributaries (Danube, Rhine, Rhône, Seine, Guadalquivir, Pô), in no-man's-lands between countries, and on islands (Corsica, Sardinia) (Anzani, Failla, Scienza, & Campostrini, 1990; Arnold, 2002; Arnold, Gillet, & Gobat, 1998; Arrigo & Arnold, 2007; Arroyo-Garcia et al., 2006; Lacombe et al., 2003; Ocete et al., 2004a,b; Terpo, 1976). In light of the ongoing threats, *V. vinifera* ssp. *sylvestris* has thus been considered as an "endangered species" since the 1980s.

Another threat has to be taken into account: taxonomic pollution through gene flows between wild grapevines and the *Vitis* taxa that escape from vineyards. The taxa may be either European cultivars (*V. vinifera* ssp. *vinifera)*, interspecific cultivars (PIWI (pilzwiderstandsfähig) (a total of 6,154 cultivars have been created in the world, OIV, 2013) or artificial polyhybrids of *Vitis* species (*Vitis aestivalis*, *V. berlandieri*, *V. cinerea*, *V. labrusca*, *V. riparia*, *V. rupestris)* that are used as rootstocks for grafting onto cultivars. Specific rootstocks are used in each viticultural region according to the local abiotic conditions, such as calcium, salt, lime, or clay content of soils. When they escape from vineyards, they rapidly invade unoccupied lands or anthropized landscapes (roadsides, channels, railroad tracks) via sexual and vegetative reproduction. These rootstocks have good rooting capacity and large leaves, and they produce a large amount of fruits. Anthropogenic populations can therefore rapidly cover large surfaces. They are also resistant but are vectors of pathogens and diseases. As all the *Vitis* species in the world seem to be interfertile and show a remarkable ability to hybridize with sister species (Arroyo-Garcia et al., 2006; Levadoux, 1956; Tröndel et al., 2010), grapevines found in the wild are considered to be a mixture of wild forms, naturalized cultivars and rootstocks, and hybrids derived from spontaneous hybridizations and introgressions among these species and forms (Arrigo & Arnold, 2007; Bodor et al., 2010; Lacombe et al., 2003; Laguna, 2003; Laguna 2004; Levadoux, 1956; Lowe & Walker, 2006; Ocete et al., 2012; This, Lacombe, & Thomas, 2006; Warwick & Stewart, 2005; Zecca et al., 2009).

Two aspects of the *Vitis* complex dynamics have not yet been investigated in-depth: (1) the contribution of parents (i.e., orientation of crossings and parentage pedigree) and (2) the role of habitat characteristics in the propagation and establishment of progenies in nature. Indeed, personal field observations in Spain, France, Austria, Croatia, and Iran suggest that hybrids/introgressed individuals are absent from well-preserved floodplain forests (i.e., natural architecture and dynamic flooding).

For this purpose, we chose the Donau-Auen National Park (DANP) as a model site. Among the five other major rivers in Europe which still host wild grapevine populations, none benefits from such an extensive protected forest area (93 km^2) and an active dynamic flood pulse

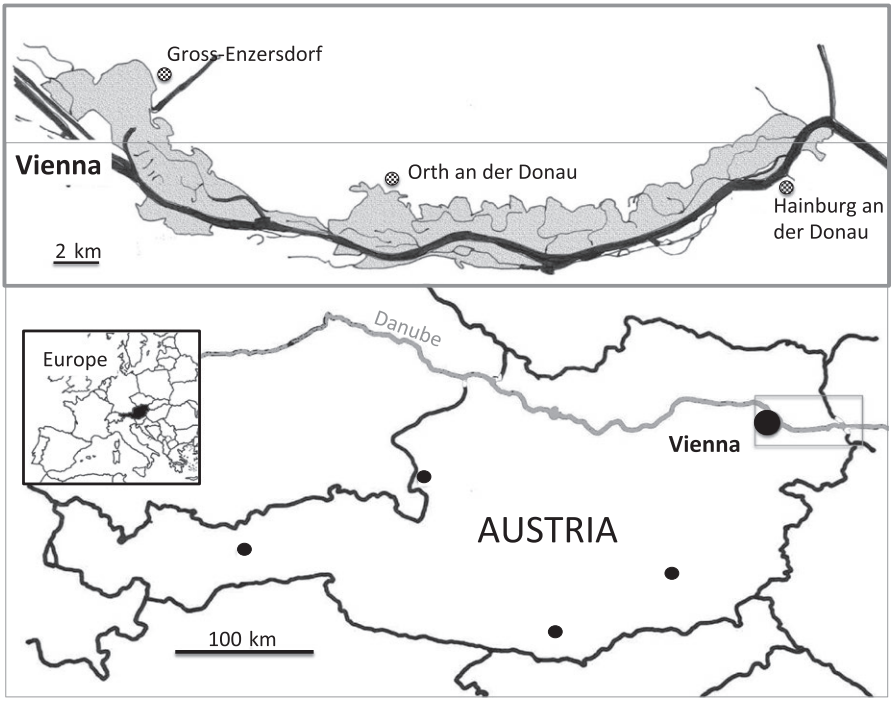

FIGURE 2 Localization of the study area Donau-Auen National Park

(Schnitzler & Carbiener, 2007). This Danube area also regroups all these parameters and contains the largest population of wild grapevine recorded in Europe (Arnold, 2002).

Since 1993, many studies have been conducted in the park on river dynamics, vegetation and target species like *Vitis*. In 2003, under the supervision of Christian Fraissl from the DANP, a comprehensive field survey was conducted in the entire protected area by Claudia Freiding, Christa Gußmark, and Ulrike Haubenwallner. From this study, we now know that there are exactly 180 grapevines in the DANP. Among them, non-native *Vitis* were recorded.

Our study aimed to provide insight into the ecology of the *Vitis* complex in this Danube region through molecular analyses of cpDNA and nSSR regions, pedigree of grapevines, morphology, and distribution of *Vitis* individuals.

2 | MATERIALS AND METHODS

2.1 | Plant material

The Eurasian wild *V. vinifera* ssp. *sylvestris* is dioecious, with either male flowers with fully developed anthers and fertile pollen and a nonfunctional ovary, or female flowers with a large, well-developed ovary and pistil associated with small anthers with sterile pollen. In male flowers, the pollen is heavy and sticky, which suggests that these plants are mainly pollinated by insects, or wind at short distances. Rare cases of hermaphroditism have nevertheless been reported (Anzani et al., 1990; Levadoux, 1956). In contrast to the wild grapevine, the domesticated form of *V. vinifera* is hermaphroditic and self-compatible. Based on this reproductive difference, the two taxa were separated into two subspecies: *V. vinifera* ssp. *sylvestris* (Gmelin) Hegi and its domesticated relative *V. vinifera* ssp. *vinifera*.

The latter have been propagated vegetatively for centuries (Mylesa et al., 2011), leading to somatic mutations that have actively contributed to the increase in the number of grape varieties. Interspecific cultivars as well as PIWI are also hermaphrodites. Rootstocks on their side are mainly dioecious.

The *Vitis* taxa all over the world are light-demanding large tendrillar lianas. They live and reproduce in gaps, upper canopies, bushes along erosive river banks, and the edges of temperate (alluvial) forests in the Northern Hemisphere. American *Vitis* have a naturally larger range of habitats than the unique Eurasian *Vitis*, with individuals situated at the extremes of environmental gradients for moisture and texture (Morano & Walker, 1995).

2.2 | The study area

The study area (48°8′0″N 16°55′0″E) covered 93 km² in lower Austria (Figure 2). The climate is temperate continental, with a mean temperature of 10°C and a mean annual rainfall of 600 mm. The Danube in Austria (350 km long) has kept an alpine hydrologic regime with the highest water levels between May and June. In addition, short episodic fluctuations throughout the year can occur. Since the 1870s, the flooded area has been reduced to a 3–7 km wide area within two dykes, leading to significant incision of the river within its floodplain. Floods have also become less erosive and less frequent, but fluctuations in the water levels are still high (7–9 m) within the dykes (Liepolt, 1965). Soils are calcareous, fine to coarse-textured fluvisols.

Along the most dynamic parts of the river network, the floodplain forests are composed of mosaics of white willow (*Salix alba* L.), black poplars (*Populus nigra* L.) and white poplars (*Populus alba* L.). On the elevated terraces, the canopy is dominated by light-demanding hardwood species such as oak (*Quercus robur* L.), ash (*Fraxinus excelsior* L.),

white poplar and elm (*Ulmus minor* Mill.), and canopy liana (*Hedera helix* L., *Clematis vitalba* L., *V. vinifera* ssp. *sylvestris*). These forests have traditionally been fragmented by numerous pathways for hunting (a total of 420 km long) and also include some permanent meadows.

Before becoming a single national park, the area included several types of protected areas. In 1978, the Lobau was designated as a protected area (Naturschutzgebiet). The Untere Lobau was included in a UNESCO Biosphere Reserve the same year. In 1979, the area called Donau–March–Thaya Auen received the status of Naturschutzgebiet. The area including Donau–March Auen and Untere Lobau became a Ramsar site in 1983. In 1996, the Donau-Auen National Park (DANP) was created. This area was designated an IUCN category II National Park in 1997, and some of its areas are included in the Natura 2000 network. With the creation of the DANP, commercial forest management was abandoned, but the former forest management is still visible in the landscape, with variations according to the owners' practices. For example, Obere, Untere Lobau, and Mannswörth were administrated by Vienna, and the rest by the federal forest company. Globally, hybrid plantations were more frequent within the dykes, while oak plantations were more frequent outside the dykes. With regard to human practices in the more distant past, the DANP was managed in different ways, with regard to both river management and forestry. In the Unterer Lobau near Vienna, the flooding periods are long and frequent, with traditional extensive forest management. In the eastern part of the DANP, from Mannsdorf Under Donau to the Slovakian border, forests were intensively managed until the creation of the National Park.

2.3 | Plant material sampling

One hundred and sixty-five *Vitis* individuals (i.e., physically separated above ground) were found in the study area. Fifteen individuals could not be found or were not reachable. Each sample location was recorded by GPS. For each sample, we collected the following data: geographic coordinates, morphological data (number of stems at the base, DBH, and height of the main stem), and ecological data: number of host trees identified by species used for ascending, situation related to the dykes (within or outside) and habitat (forest edge with meadow, forest edge with channel or forest interior in a gap).

2.4 | DNA extraction and amplification

The leaves collected from the 165 individuals were dried in silica gel. To identify hybrids, we added 21 cultivars and 19 rootstocks as an outgroup, all commonly cultivated in Austria and Europe. These included the hybrid Mgt 41b, which is a hybrid between a *V. vinifera* cultivar and *V. berlandieri*. The 19 rootstocks were from collections of the Institut für Rebenzüchtung Geilweilerhof (Germany) and from the Agroscope Viticulture Research Centre Pully (Switzerland).

Genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. Twenty-four microsatellites and five chloroplastic regions were amplified by PCR. Amplifications were carried out in 10 µl reactions containing 1x GoTaq Reaction Buffer, 0.75 mM $MgCl_2$, 5 µg BSA, 0.25 mM dNTPs, 0.25 µM of each primer, 0.5 U GoTaqG2 DNA Polymerase (Promega), and 2–5 ng of template DNA. The PCR cycling conditions consisted of an initial activation step of 4 min at 94°C, followed by 30 cycles each of 60 s at 92°C, 50 s at 52–56°C (Appendix 1), and 60 s at 72°C, with a final extension step of 10 min at 72°C. Macrogen did the genotyping. Amplified fragment lengths were assigned to allele sizes with GeneMapper software v 3.7 (Applied Biosystems). Among the 24 pairs of markers, four markers (VVMD-28, VMC-5A1, VMC-1C10, VVS2) did not amplify correctly. Five samples that did not amplify at least 15 pairs of markers were also removed from statistical analysis. All grape varieties and rootstocks amplified correctly. As a result, we retained 200 samples (i.e., 160 grapevines found in the wild; 21 cultivars; 19 rootstocks), analyzed with 20 microsatellite (nSSR) loci and five chloroplastic (cp) DNA loci.

2.5 | Genotypes

We carried out a STRUCTURE 2.3.4 analysis on the 200 individuals (Pritchard, Stephens, & Donnelly, 2000). The following options were used: 10,000 burn-in, 20,000 MCMC, admixture model and correlated allele frequencies. This method is based on the use of Markov Chain Monte Carlo (MCMC) simulations to infer the assignment of genotypes to *K* distinct clusters. The underlying algorithms attempt to minimize deviations from Hardy–Weinberg and linkage disequilibria within each cluster. In accordance with Evanno, Regnaut, and Goudet (2005), we did 10 iterations for each *K* value ($K = 1$ to $K = 6$). The most likely number of clusters (*K*) was estimated in Structure Harvester, using the maximum value of L(*K*) and calculating delta ΔK.

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 of GenAlEx 6.5. (Peakall & Smouse, 2006). We checked for private alleles within the pure wild grapevines, cultivars and rootstocks. We also used these results for the identification of hybrid/introgressed origins.

2.6 | Haplotypes

The cp DNA markers were used to determine: (1) the genetic characterization of hybrids and introgressed individuals, (2) the direction of hybridization, given that the cpDNA is inherited from the mother (Arroyo-Garcia et al., 2006; Arroyo-García et al., 2002), (3) the place where hybridization/introgression occurred (i.e., within or outside the DANP with, in the latter case, birds transporting seeds from the fields to the forest), and (4) the potential diversity of haplotypes in the wild grapevine population.

2.7 | Genetic diversity and geographic structure (on the 20 SSR)

The distribution of the population tended to be aggregated at two levels. At the first level, a western group (Untere Lobau and Obere Lobau) was separated from an eastern group by 4 km, with the latter extending up to the Slovakian border. At the second level, there

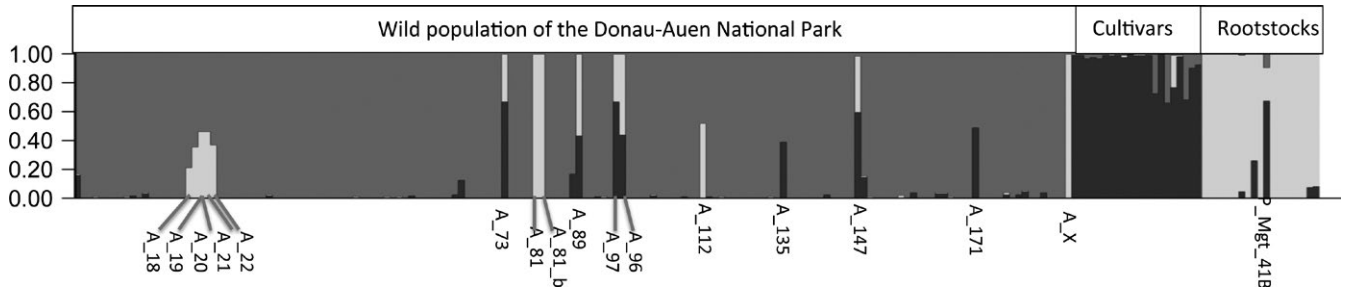

FIGURE 3 Population structure of the *Vitis* complex of the Donau-Auen National Park inferred with the Bayesian clustering algorithm implemented in STRUCTURE. Each individual is represented by a vertical bar, partitioned into *K* segments representing the proportions of ancestry of its genome in *K* = 3 clusters

were five groups (Mannswörth, Eckartsau, Orth, Untere Lobau, Obere Lobau). To examine this geographic pattern, we used the individual-based Bayesian clustering methods implemented in STRUCTURE 2.3.4. We investigated intraspecific population structure and admixture. We used an admixture model with allele frequencies correlated according to Evanno et al. (2005). Ten independent analyses were carried out for each number of clusters *K* ($1 \le K \le 26$), with 80,000 MCMC iterations after a burn-in of 20,000 steps.

2.7.1 | Focus on the true wild grapevine

To investigate the potential geographic structure, we performed complementary analysis with R ADEGENET package (Jombart, 2015; R Core Team 2013). The genetic diversity was assessed with GenAlEx 6.5 (Na, Ne, Ho, He). Clones were detected using GenAlEx and were confirmed in the raw data set.

2.7.2 | Focus on the hybrids

First, we identified the clones in the hybrid population and calculated the respective distances between them. Second, we removed them in order to analyze the full and half sibship assignments, as well as parent assignments of the hybrids/introgressed individuals. The analysis was performed in Colony 2.0 (2008; updated 2014 http://www.zsl.org/science/software/colony). We considered the hybrids as offspring, and the true wild grapevines, cultivars and rootstocks as putative parents. The following parameters were used: Mating system I: female polygamy/male polygamy, Mating system II: without in-breeding/without clones; Species: dioecious/ diploid, Length of run: medium; Analysis method: full likelihood (FL), and Sibship size scaling: no prior. For the other parameters, we used the default values.

2.8 | Morphology versus habitats

To investigate the influence of the flooding process on *Vitis* morphology, we compared statistically variations in DBH, height and number of stems between individuals within and outside dykes. As the data were not normally distributed, nonparametric tests (Mann–Whitney) were used. The same tests were used for investigating the relationships between ecological characteristics (flooding, habitat) on *Vitis* morphology.

3 | RESULTS

3.1 | Picture of the *Vitis* complex

The structure analysis (Figure 3) performed on the 200 grape samples suggested that two groups could be retained among the 160 individuals collected in the wild: one containing all *V. vinifera* subspecies and the other regrouping hybrid rootstocks. However, we retained *K* = 3, separating the true wild grapevines (ssp. *sylvestris*) from cultivars (ssp. *vinifera*) and hybrid rootstocks. In the rootstock clade (in green), 41 B Millardet et de Grasset (41 B MGt) showed alleles of *V. vinifera*, which is normal as it was issued from a crossing between *V. vinifera* and *V. berlandieri*. In summary, of the 160 *Vitis* individuals collected in the wild from the DANP and analyzed, 144 *Vitis* were genetically different and 16 were clones. Among the 144 *Vitis* individuals, 132 were true wild grapevines and 12 were hybrids/introgressed individuals. Among the 12 hybrids, we found the following taxa: one rootstock × rootstock, five true wild grapevine × rootstock, three cultivar × rootstock, and one true wild grapevine × cultivar × rootstock. Clones were found in true wild grapevines (12) and crossings of rootstock × rootstock (1), true wild grapevine × rootstock (1), and cultivar × rootstock (2) (Figure 4).

3.1.1 | Haplotypes

We identified a total of five haplotypes distributed in both wild grapevines and hybrids (see Sections 2.4 and 3.1). 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 as well as some rare true wild grapevines; and H5, which regrouped all the American rootstocks of various origins.

3.1.2 | Host trees

Vitis climbed on a total of 330 trees or shrubs belonging to 24 species in the DANP, the most frequently being *Cornus sanguinea* L. (20%), *Populus alba* L. (15%), and *Acer campestre* L. (14.7%). A single *Vitis* individual may use one to five different hosts.

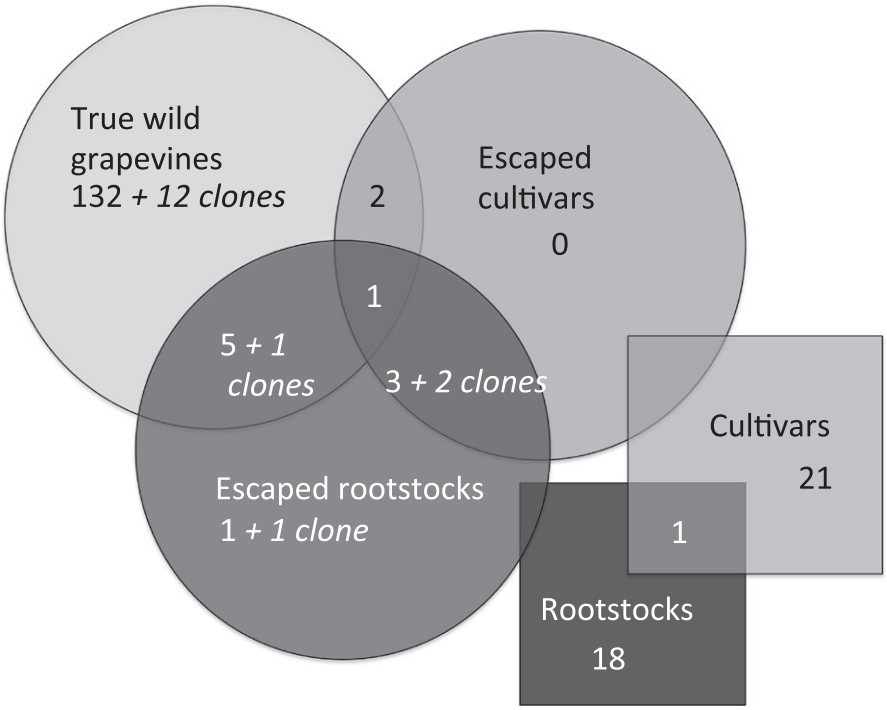

FIGURE 4 Distribution of the 160 studied individuals of the Donau-Auen National Park (DANP) within the categories of True wild grapevines, escaped cultivars and escaped rootstocks. The circles contain the numbers of individuals and clones in the three categories. The squares contain the numbers of cultivars/varieties and rootstocks added to the study

3.2 | Focus on the true wild grapevine

3.2.1 | Geographic structure

The analyses in STRUCTURE and ADEGENET revealed no geographic structure among the 132 true grapevines, despite the wide distribution of individuals. Concerning the global genetic diversity, all markers were polymorphic, with the number of alleles ranging from two to 12 according to the markers. The Shannon's Information Index was 0.8. The heterozygosity values ranged from 0.03 to 0.80. The mean heterozygosity value was 0.418, which was identical to the expected heterozygosity (0.418). (Table 1). Both results indicated a random mating population, with free gene exchanges.

3.2.2 | Clones

Twelve clones were found close to each other, within 2–36 m. Ten true wild grapevines produced clones. Eight of them were by pairs, and two of them by threes.

3.2.3 | Private alleles

Of 144 individuals (132 true wild and 12 clones) of the wild grapevine, we found 16 private alleles distributed on nine markers. In contrast, although the cultivars and rootstocks had reduced numbers of individuals, they had many more private alleles (respectively, 30 and 72) distributed on 15 and 18 markers of 20 (Table 2).

3.2.4 | Haplotypes

The following haplotypes were found in the 132 individuals: H1, common in the wild populations of western Europe, was found in 128 individuals; H2, common in the wild populations of eastern Europe, was found in three individuals; and H4, commonly found in cultivars such as Chasselas and Cabernet Sauvignon, was also present in some of the true wild grapevines.

TABLE 1 Summary of genetic diversity in the true wild grapevine population (144 individuals)

	Na	Ne	Ho	He	I
Mean	5.55	1.998	0.418	0.418	0.8
SE	0.555	0.213	0.047	0.046	0.089

Ho and He, observed and expected heterozygosities, respectively. I, Shannon's Information Index; Na, number of alleles; Ne, effective number of alleles.

TABLE 2 Number of private alleles in the wild grapevines, cultivars, and rootstock

	Wild grapevines *N* = 144	Cultivars *N* = 21	Rootstocks *N* = 18
Nb alleles	16	30	72
Nb markers	9	15	18

N, total number of individuals.

3.2.5 | Morphology versus habitat

Most individuals were found on the left side of the Danube in the study area. Eighty-six individuals grew outside the dykes against sixty within dykes. Taking into consideration only the habitats, 42% of the true wild grapevines lived at the edges of forest/meadow, 33.3% at the edges forest/channels, and 23.9% in forest gaps. The

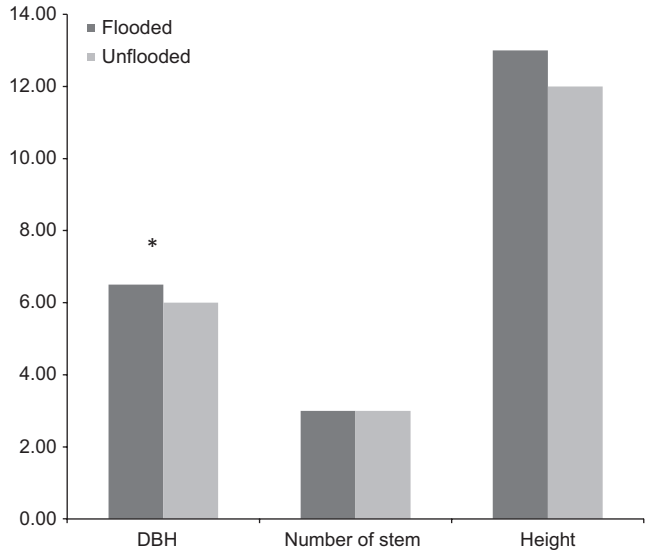

FIGURE 5 Comparison of ecological variables (DBH, total height, number of stems) in relation with the flooding process

Mann–Whitney test indicated that DBH varied significantly with the occurrence of flooding, with higher trunk diameters in flooding areas (Figure 5). The number of stems per individual depended on the habitat, with a higher number of stems in gaps and the edges of forests with channels (the mean number of stems was 4.8 and 5, respectively) than in the edges of forests with meadows (mean number of stems was 3). The total height of the grapevine was significantly higher in the gaps compared with the edges of forests with channels or meadows ($p < .001$).

3.3 | Focus on hybrids/introgressed

3.3.1 | Orientation of crossings and parentage

Table 3 gives some of the characteristics of the hybrids. From the haplotypes, we were able to deduce the direction of hybridization. Eight had the H1 haplotype typical of the majority of *sylvestris* in western Europe; two had the H3 haplotype similar to Chardonnay and Merlot; two had the H4 haplotype similar to Chasselas, Cabernet Sauvignon, and some rare true wild grapevines; and four had the H5 haplotype regrouping all American rootstocks of various origins. We expected to find the parental origin from the genotypes. We found five types of hybrids.

True wild grapevine × rootstock

Among the six hybrids, five of them (18, 19, 20, 21, 22) were closely related (half-sibs) and distributed along 300 m of an active channel of the Danube. They shared one allele per locus. According to the results of Colony, the putative mother may have been 6 km downstream on the edge of the forest and a channel. The confidence index was nevertheless too low. No. 112 was also a crossing between an unknown mother true wild grapevine and the pollen of rootstock.

TABLE 3 List of hybrids/introgressed individuals (haplotype, parentage, sex, clones, number of private alleles from wild grapevine, cultivar, or rootstock)

Individual number	Haplotype	Parentage	Sex	Clones	Number of private alleles from *V. sylvestris*	Number of private alleles from cultivars	Number of private alleles from roostocks
A_018	H1	True wild grapevine × rootstock	Presence of grapes		2		2
A_019	H1	True wild grapevine × rootstock	Unknown		1		3
A_020	H1	True wild grapevine × rootstock	Male	20 = 21			5
A_022	H1	True wild grapevine × rootstock	Unknown		1		4
A_112	H1	True wild grapevine × rootstock	Presence of grapes		2		7
A_135	H1	True wild grapevine × (Gruener Weltiner) vinifera	Presence of grapes		1	1	
A_171	H1	True wild grapevine × (Blaufrankisch) vinifera	Unknown		1	2	
A_089	H3	Vinifera × rootstock	Presence of grapes	89 = 97		2	6
A_096	H4	Vinifera × rootstock	Presence of grapes	96 = 73_b		2	4
A_X	H5	Riparia gloire (rootstock) × rootstock × vinifera	Unknown			1	13
A_081_b	H5	Rootstock × rootstock	Unknown	81_b = 81			16
A_147	H5	Rootstock × vinifera × true wild grapevine	Presence of grapes		1	1	7

True wild grapevine × cultivar (*vinifera*)

Two individuals were issued from a crossing of a true wild grapevine and a cultivar.

No. 135 was a crossing between female 99 and the Grüner Weltiner cultivar. No. 135 was located along a road within the national park, and the mother was located 100 m downstream along a dead arm. No. 171 was a crossing between female 144 and a Blaufrankisch cultivar. The mother was located 600 m upstream along a dead arm.

Cultivar × rootstock

Three hybrids were crossings between ssp. *vinifera* as the mother and a rootstock. No. 96 was at the edge of the DANP along a cultivated area: it has Cinsaut as a mother, and an unknown rootstock pollen. No. 89 and No. 97 (a clone of 89) were crossings including Baco Noir and Riparia Gloire. These two plants were also situated at the edge of the DANP.

Rootstock × rootstock

Three rootstocks were issued from various American taxa. No. 81 and No. 81b (a clone of 81) included *Vitis riparia* in the parentage. X had *Riparia* in both parents. All were situated along the main stream.

Rootstock × cultivar × wild grapevine

No. 147 had *V. riparia* parents but also Tinturina (identical to Usellina) in the genotype. It contained private alleles of wild grapevines. It was along the main stream in an industrial area.

3.3.2 | Clones

As mentioned, we found four clones among the hybrids/introgressed (Figure 4). All types of hybrids/introgressed forms were thus able to reproduce vegetatively. The maximum distance between two clones was about 350 m.

3.3.3 | Ecology

Hybrids were found within and outside the dykes, mostly on forest edges.

More precisely, most hybrids that included the genome of the true wild grapevine (18, 19, 20 and 21) were found close to each other along a branch of the main channel of the Danube. This part is active with erosive activity from flooding. The hybrids were present on a terrace along a sandy road. They had many stems, with up to 15 stems for No. 20. They all presented dense foliage from the ground up to 20 m. No. 22 was located close to Orth an der Donau along a pathway commonly used by bikers, and not far from the vineyards at the border of the park. No. 96, 112, 135 and 171 were located along a road close to an ancient main branch of the Danube, which was still connected to the main stream when widespread flooding occurred. The hybrids including those issued from crossings between rootstocks (X, 81, 81_b, and 147) were mainly established along the main channel of the Danube. Given the low number of hybrids, no statistical analysis was performed. Hybrids/introgressed individuals were vigorous with a deep cover of foliage up to 20 m high. They had many stems at the basis, and two of them were young individuals. Ten of 16 produced flowers and fruits. They were dioecious or hermaphrodite. In spite of their vigor, these non-native taxa of grapevines had penetrated into forest gaps or massively invaded the anthropized sites of the DANP.

4 | DISCUSSION

Our study pointed out a variety of *Vitis* taxa including endangered native species and hybrids with cultivars and escaped rootstocks. The number of true wild grapevines can be interpreted as the consequence of relatively suitable ecological conditions (e.g., maintenance of flooding events, large forest cover) compared with other populations of Europe (Spain, Portugal, Italy, France), which are now reduced to a few individuals with a significant reduction in the observed heterozygosity (Andrés et al., 2012; Di Vecchi-Staraz et al., 2008; Grassi et al., 2003; Lopes, Mendonça, Rodrigues dos Santos, Eiras-Dias, & da Cămara Machado, 2009).

The relatively high genetic diversity of true wild grapevines seems to be a legacy from the beginning of the Holocene when Danubian populations received genes from two migrating populations, one originating in southern Italy and moving northward via the Alps into central Europe, the second originating in the Balkan area and migrating westward (Grassi, De Mattia, Zecca, Sala, & Labra, 2008; Taberlet, Fumagalli, Wust-Saucy, & Cosson, 1998). This legacy was kept in the genome of the population for millennia, until the 19th century.

The heterozygosity was lower than that observed in other *Vitis* populations of Europe (Arnold, Schnitzler, Parisot, & Maurin, 2009; Bodor et al., 2010; Zoghlami et al., 2013), but the genetic diversity was still quite high. Of course, the current situation is far from optimal if we consider the historical reports (see Section 1). The low survival can easily be explained by the conditions generated by embankment, which has destroyed suitable sites for the establishment of young plants, such as upper-forested terraces. A second factor that may explain both the low densities and perhaps the clustering of the current population is the past forest management, which became more intensive after river regulation, with forest managers removing the climbers. A third factor is the low regeneration potential. According to observations by the DANP staff, seedlings may be abundant in spring, but they disappear quickly over the year. The sinking of the water table has induced dryness in the top layers of the soil, making it unsuitable for the survival and development of young plants. Another consequence of the sinking water table is that the typical fluvisols currently found in the area have already started to evolve (Arnold, 2002), similar to the observed shifts in plant communities. Along rivers with altered disturbance regimes, tree communities no longer belong to the same plant community as their understorey (Roulier, 1998; Roulier, Teuscher, & Weber, 1999) and seedlings of grapevines are not part of these plant communities.

The range of lengths and diameters was found rather high among adult grapevines. The larger diameters found in forest gaps and edges between forests and channels within the dykes can be explained by

good conditions of light, nutrient, and moisture. These individuals invested their efforts in a single stem in order to reach the canopy rapidly, in particular when gaps are small and surrounded by tall trees. Single stems are also the result of growth without any trauma such as breakage following host fall.

The gene pool of the naturalized grapevines found in the DANP shows high genetic diversity due to genetic admixture among different taxa. The detailed pedigree reconstruction of the hybrids/introgressed *Vitis* allowed us to prove that the hybridization pattern is thus symmetric in nature. In viticulture, artificial bidirectional interspecific crossing has been successful and the development of these crossings is ensured by human care. Yet it has never been demonstrated that this could spontaneously occur. Our study also showed that hybrids involving rootstock genes were established preferentially along ancient main branches of the Danube, which are sometimes quite active, or along the main stream. Another interesting result is that hybrids and introgressed individuals were not so abundant in this area and did not succeed in penetrating the forest interior. Perhaps the competitivity of native plant species in the understorey, shown through architectural and phytosociological studies (Schnitzler, 1994), has prevented their establishment or, like the native grapevines, they cannot integrate the changing plant environment. There are certainly additional causes, such as strict governance regarding the cleaning of vineyard peripheries. This would reduce the feral propagule pressure.

Based on our results and the literature, we can conclude that the current population of wild grapevine of the DANP is one of the last bastions of the former vast metapopulation that extended throughout Europe. This area has maintained enough suitable habitats to preserve true wild grapevines from attacks by American pests and diseases, thanks to the accessibility of groundwater to roots and the maintenance of flooding, the preservation of a large forest cover, and the strict protection of the species. These results are of great importance for conservation biology. However, as dynamic floods seem to have gone forever from large river plains, the establishment of native offspring is probably impossible. If a re-wilding strategy is considered in the DANP (i.e., re-creation of erosive zones along the main river and adjacent channels), one should take into account that hybrids may take advantage to this new situation. This is, however, the only chance for wild grapevine populations to regenerate. Whatever the case, re-wilding actions must not only address protection of one specific subspecies, even endangered, but they must also consider the interest of the global ecosystem functioning. We thus hope for the return of erosive floods in a not too distant future.

ACKNOWLEDGMENTS

The authors would like to thank Christian Fraissl, Constanze Rak Karoline Zsak and Teresa Knoll for their support on the field part of the project and constructive comments on the paper. The authors thank the Institut für Rebenzüchtung Geilweilerhof (Germany) and the Agroscope Viticulture Research Centre Pully (Switzerland) for the selection of cultivars and rootstocks. The Wüthrich and Matthey-Dupraz Funds financed part of this research. The authors also thank the reviewers for their useful comments, and Catherine Stott for the correction of the manuscript.

DATA ACCESSIBILITY

Material can be obtained at the DANP in Orth and der Donau. Other data are archived at the University of Lausanne.

AUTHOR CONTRIBUTIONS

Dr. Claire Arnold directed the study, did part of the fieldwork, and wrote part of the article. Mag. Olivier Bachmann collected the samples in the collections and the Donau-Auen National Park, and he did the laboratory analysis and the statistics. Prof. Dr. Annik Schnitzler, went to the field, did the ecological part of the study, and wrote part of the article.

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How to cite this article: Arnold C, Bachmann O, Schnitzler A. Insights into the *Vitis* complex in the Danube floodplain (Austria). *Ecol Evol*. 2017;00:1–11. https://doi.org/10.1002/ece3.3187

APPENDIX 1

TABLE A1 List of 24 nSSR and 5 cpDNA primers, references, and annealing temperatures. (In gray and italic primers that did not amplify correctly)

Primer	Reference	Cycles
VVMD 5	Bowers, Dangl, Vignani, & Meredith, 1996	94°C-4 min; 30 cycles (92°C-60 sec, 54°C-50 sec, 72°C-60 sec) 72°C-10 min
VVMD 7	Bowers et al., 1996	94°C-4 min; 30 cycles (92°C-60 sec, 52°C-50 sec, 72°C-60 sec) 72°C-10 min
VVMD 8	Bowers et al., 1996	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VVMD 17	Bowers, Dangl, & Meredith, 1999	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VVMD 24	Bowers et al., 1999	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VVMD 25	Bowers et al., 1999	94°C-4 min; 30 cycles (92°C-60 sec, 53°C-50 sec, 72°C-60 sec) 72°C-10 min
VVMD 26	Bowers et al., 1999	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VVMD 27	Bowers et al., 1999	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VVMD 28	*Bowers et al., 1999*	*94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min*
VVMD 31	Bowers et al., 1999	94°C-4 min; 30 cycles (92°C-60 sec, 53°C-50 sec, 72°C-60 sec) 72°C-10 min
VVMD 32	Bowers et al., 1999	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VVMD 36	Bowers et al., 1999	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VrZAG 62	Sefc et al. 1999	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VrZAG 79	Sefc et al. 1999	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VMC 1C10	*Vitis Microsatellite Consortium*	*94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min*
VMC 2A5	*Vitis* Microsatellite Consortium	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VMC 2B3	*Vitis* Microsatellite Consortium	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VMC 2C3	*Vitis* Microsatellite Consortium	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VMC 2H4	*Vitis* Microsatellite Consortium	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VMC 4G6	*Vitis* Microsatellite Consortium	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VMC 5A1	*Vitis Microsatellite Consortium*	*94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min*
VMC 5C5	*Vitis* Microsatellite Consortium	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VMC 5H2	*Vitis* Microsatellite Consortium	94°C-4 min; 30 cycles (92°C-60 sec, 56°C-50 sec, 72°C-60 sec) 72°C-10 min
VVS 2	*Thomas & Scott 1993*	*94°C-4 min; 30 cycles (92°C-60 sec, 54°C-50 sec, 72°C-60 sec) 72°C-10 min*
VndhF1	Bachmann & Arnold in prep	94°C-4 min; 30 cycles (92°C-20 sec, 51°C-20 sec, 72°C-60 sec) 72°C-10 min
VndhF2	Bachmann & Arnold in prep	94°C-4 min; 30 cycles (92°C-20 sec, 51°C-20 sec, 72°C-60 sec) 72°C-10 min
VtrnK-1	Bachmann & Arnold in prep	94°C-4 min; 30 cycles (92°C-20 sec, 53°C-20 sec, 72°C-60 sec) 72°C-10 min
VtrnK-2	Bachmann & Arnold in prep	94°C-4 min; 30 cycles (92°C-20 sec, 49°C-20 sec, 72°C-60 sec) 72°C-10 min
VtrnC	Bachmann & Arnold in prep	94°C-4 min; 35 cycles (92°C-20 sec, 51°C-20 sec, 72°C-60 sec) 72°C-10 min

3. Samples

The team of the Donau-Auen National Park sent us 74 samples in April and 17 additional one in June 2017. A total of 91 samples were thus analysed. We also collected leaves from grape varieties from the Research Center of Pully as standards. Among them, we finally included one sample from the previous study (AU172) as well as two grape varieties: Chasselas, Merlot 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.

In the first set of samples, packs were numbered from 1 to 14, however we noticed that there were no pack 4. Sample names are not standardised and some were, sometimes, difficult to read. In the second set of samples numbers were sometimes identical. We thus renumbered each samples from 1 to 91 (Table 1 and Pictures Annexe 4). Pictures will allow you to reattribute the samples to the right collection in case of misspelling. In Pack 8 number 00121 M31 contained dried berries containing each 2-4 seeds. We could not consider these seeds. Indeed each seed has a different combination of DNA of the mother and the father. We should have extracted the embryo of each seed and thus kill the embryo. The result would not meet the aim of the current project.

Package	Sample Number	Info on Bag1	Info on Bag2	Info on Bag3
Pack 1	DA01	0038	97	
Pack 1	DA02	0030	294	
Pack 1	DA03	00031	295	
Pack 1	DA04	00029	296	
Pack 1	DA05	00028	297	
Pack 1	DA06	00032	310	
Pack 1	DA07	0034	311	
Pack 1	DA08	00033	312	
Pack 1	DA09	00035	313	
Pack 1	DA10		314	
Pack 1	DA11		315	
Pack 2	DA12	00122	M73	
Pack 2	DA13	00123	M94	
Pack 2	DA14	00124	M64	
Pack 2	DA15	00086	M93	
Pack 2	DA16	00124	M69	TRAUBE
Pack 3	DA17	00022	207	
Pack 3	DA18	Kleine	302.2	23.8.216
Pack 3	DA19	00024	309	
Pack 3	DA20	00025	89	
Pack 3	DA21	00026	81	
Pack 3	DA22	00027	102	
Pack 5	DA23	0010	20	
Pack 5	DA24	00011	18	
Pack 5	DA25	00013	304	
Pack 5	DA26	Verjung ung	2	
Pack 6	DA27	00015	30	17.08.16
Pack 6	DA28	93A		
Pack 6	DA29	00016	303	
Pack 6	DA30	00017	277	
Pack 6	DA31	00018	305	
Pack 6	DA32	00019	93	
Pack 6	DA33	00020	307	
Pack 7	DA34	00047	44	
Pack 7	DA35	00049	Gazleitung West Forster Wiese	
Pack 7	DA36	00053	64	Ulme
Pack 7	DA37	00054	65	

Package	Sample Number	Info on Bag1	Info on Bag2	Info on Bag3
Pack 10	DA46	00041	20.09.16	
Pack 10	DA47	00042	Steinerwehr	
Pack 10	DA48	00043	Stemerwekgrabenquer	
Pack 10	DA49	00044	Nr.14 5	
Pack 10	DA50	00046	Nr.M7 FA; FA	
Pack 11	DA51	00085	M61	
Pack 11	DA52	00088	M57	
Pack 11	DA53	00090	M83	
Pack 11	DA54	00121	M31	
Pack 12	DA55	00000	192	22.09.16
Pack 12	DA56	00001	299	
Pack 12	DA57	00003	300	
Pack 12	DA58	00005	22	
Pack 12	DA59	00006	182	
Pack 12	DA60	00008	270	11.08.16
Pack 13	DA61	00045	Nr. M4 Düne Vitis	20.09.16
Pack 13	DA62	00048	M10	ESCHE
Pack 13	DA63	00050	M14	ESCHE
Pack 13	DA64	00051	M23	feldahorn
Pack 13	DA65	00052	M63	Silberpa ppel
Pack 13	DA66	00055	M66	
Pack 14	DA67	00056	Nr.M	03.10.16
Pack 14	DA68	00057	M41	
Pack 14	DA69	00058	M40	
Pack 14	DA70	00061	Nr.M	
Pack 14	DA71	00060	Nr.M.18	
Pack 14	DA72	00059	M19	
Pack 14	DA73	00062	M26	
Pack 8	DA74	00080	M35	
Shipment 2	DA75	298		
Shipment 2	DA76	330		
Shipment 2	DA77	337		
Shipment 2	DA78	337	H1= VITIS	
Shipment 2	DA79	337	VITIS	
Shipment 2	DA80	339		
Shipment 2	DA81	346		
Shipment 2	DA82	348		

Package	Sample Number	Info on Bag1	Info on Bag2	Info on Bag3
Pack 8	DA38	00091	M30	
Pack 8	DA39	00081	M47	
Pack 9	DA40	00082	M49	
Pack 9	DA41	00083	M53	
Pack 9	DA42	00084	M54	
Pack 9	DA43	00087	M60	
Pack 9	DA44	00089	M67	
Pack 9	DA45	00120	M97	

Package	Sample Number	Info on Bag1	Info on Bag2	Info on Bag3
Shipment 2	DA83	349		
Shipment 2	DA84	351		
Shipment 2	DA85	353	VITIS PROBE 001	
Shipment 2	DA86	354	VITIS PROBE 003	
Shipment 2	DA87	355		
Shipment 2	DA88	357		
Shipment 2	DA89	358		
Shipment 2	DA90	359	HS=VITIS	
Shipment 2	DA91	360		

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

4. DNA Extraction

The total DNA of leaf samples was extracted in early July 2017 after the delivery of all 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).

5. Genotyping

5.1. 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 sufficiently 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 12 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.

Among the 12 nSSR primers, one did not amplify correctly for most samples and was thus removed from the statistics. VMC 5C5 did not amplify on 25.8% of the samples but was still sufficiently informative and variable to be retained. 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	2.3 %
VVMD 5	Bowers et al. 1996	15	0.8 %
VVMD 7	Bowers et al. 1996	17	4.7 %
VVMD 24	*Bowers et al. 1999*		
VVMD 25	Bowers et al. 1999	11	3.1 %
VVMD 27	Bowers et al. 1999	17	0 %
VVMD 31	Bowers et al. 1999	11	2.3 %
VVMD 32	Bowers et al. 1999	16	1.6 %
VrZAG 62	Sefc et al. 1999	13	0 %
VrZAG 79	Sefc et al. 1999	14	0 %
VMC 2H4	Vitis Microsatellite Consortium	24	0.8 %
VMC 5C5	Vitis Microsatellite Consortium	9	25.8 %

Table 2. List of 12 nSSR primers, references, number of alleles per locus and percentage of missing data (non amplified samples). (In grey and italic VVMD24 did not amplify correctly).

5.2. SSR Data

Raw data were standardised. In addition to the 91 samples, we added one sample from the previous study (AU 172) as well as 21 Cultivars of *Vitis vinifera* origin and 15 Rootstocks of American *Vitis* origin. In total, the dataset is composed of 128 samples.

5.3. Detection of suspected clones within the dataset

For this purpose we first investigated the raw data (Annexe 5), sorting data by size.

A UPGMA clustering based on the Genetic distance matrix was done (Annexe 6). A PCoA (Principal component analysis) was performed in GenAlEx 6.5 (Peakall & Smouse, 2006). This allows also to confirm the clonality.

In table 3 we regrouped individuals, which share exactly the same genotype for at least 10 of the 11 primers. Some samples did not amplify for one (locus) primer but we have at east 7 groups of very closely related individuals.

Suspected Clones			Remark
DA06	DA09	DA02	Identical on 10 primers VMC5C5 did not work for DA06 and DA09
DA27	DA55	DA74	Identical on 10 primers VMC5C5 did not work for DA27 and DA55
DA28	DA32		Identical on all primers
DA 77	DA83	DA85	Identical on all primers
DA89	DA90		Identical on 10 primers VMC5C5 did not work for DA90
DA56	DA75		Identical on 10 primers VVMD7 did not work for DA56
DA78	DA79	DA82	Identical on 10 primers VVMD7 did not work for DA78

Table 3. Groups of suspected clones. In red the 11 clones.

5.4. Clustering

The UPGMA cluster allows to identify the closely related individuals, globally 3 main groups are present (Annexe 6). In green we find a group made of 18 individuals where rootstocks are present; in red, a group (86 individuals) composed of supposed wild grapevines. Finally the blue group is made of 24 individuals and clustering mainly cultivars of *V. vinifera*.

We can already see here that some individuals found in the Park do share alleles with cultivars or rootstocks.

5.4.1. Green Group (Rootstocks)

Focus on the Green group of rootstocks (Fig. 2):

Within the first 7 samples we find rootstocks of *V. riparia* (Group 1), within the 3 next we find rootstocks with *V. rupestris* origin (Group 2). Within Group 3, we have rootstocks with *V. berlandieri* origin. Fercal is different because it has alleles from *V. berlanderi, V. vinifera* and *V. longii* (http://plantgrape.plantnet-project.org/fr/porte-greffe/Fercal).

Rootstocks from Group 2 and 3 involve *V. riparia* and *V. berlandieri* in their parents.

The 4 individuals from the Park are in Group 1.

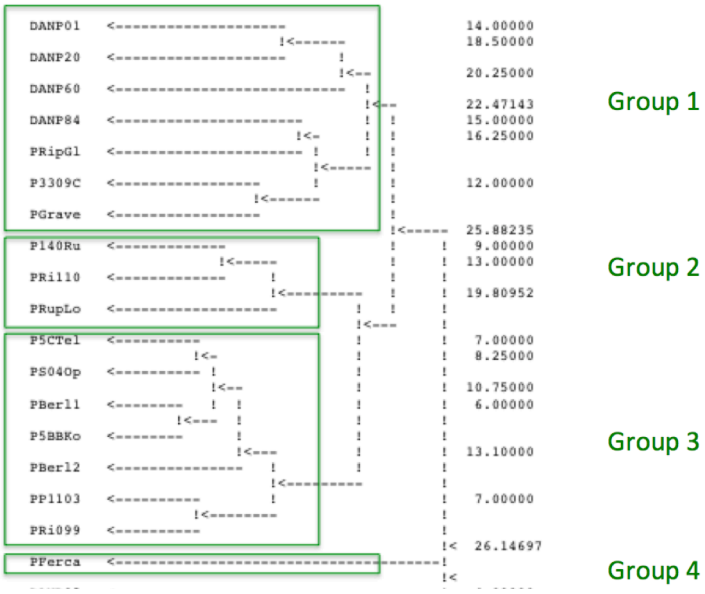


Fig. 2. Zoom on the part of the UPGMA regrouping rootstocks. Four groups are highlighted.

5.4.2. Red Group (wild)

As mentioned, this red group is formed mainly by "wild" grapevines.

In this group, a set of 12 individuals is clearly different from the other individuals collected in the park: DA02, DA03, DA06, DA09, DA08, DA07, DA04, DA05, DA21, DA27, DA55, and DA74. They are highlighted in violet in the cluster (Annexe 6).

The following couple of individuals share one allele per locus: DA11 – DA25, DA61 - DA63, DA38 – DA43 – DA51, DA23 – DA24, DA46 – DA72, DA31 – DA35.

We do not have information on the location of these individuals within the DANP, thus we can't know if this interpretation makes sense or not. We can say that they could be related, additional locus could be studied to confirm their parentage.

5.4.3. Blue Group (vinifera cultivars)

This group is relatively well defined. With this analysis, two individuals from the park do cluster with cultivars, DA58 with Pinot noir and DA76 with BlauFrankisch. DA58 do share some alleles with Pinot noir but can't be a direct hybrid of Pinot, the same occurs with DA76 and BlauFrankisch. We can't say that they are directly parents.

5.5. 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=0.775) and rootstocks (He=0.810) (Table 4). The diversity is lower (He=0.632) 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. The observed heterozygosity (Ho) is lower than the expected heterozygosity (He). This is usually a sign of inbreeding and senescence of a population.

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, 26 private alleles are found, in the set of cultivars used within this study there were 14 private alleles and within the set of rootstocks 27. For instance in Locus VVS2, alleles 123 is only present in the PG group while 153 is only present in the samples of the DANP.

Pop		Na	Ne	I	Ho	He	F	No. Private Alleles
DNP	**Mean**	10.273	3.133	1.427	0.555	0.632	0.141	26
	SE	0.945	0.424	0.112	0.058	0.040	0.052	
CP	**Mean**	8.091	4.699	1.732	0.816	0.775	-0.053	14
	SE	0.563	0.342	0.070	0.029	0.017	0.032	
PG	**Mean**	8.455	5.508	1.872	0.852	0.810	-0.047	27
	SE	0.434	0.362	0.056	0.068	0.013	0.081	

Table 4. Summary of genetic diversity in the 3 groups (128 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.

5.6. Structure Analysis

From the structure analysis (Fig. 3) performed on the 128 grape samples, we retained K=3, separating the true wild grapevines (ssp. sylvestris) from cultivars (ssp. vinifera) and hybrid rootstocks. In the rootstock clade (in green), Fercal showed alleles of *V. vinifera*, which is normal as it has a *V.vinifera* in its genealogy.

Among the 92 samples from the DANP, 24 individuals show a different pattern.

18 were already found in the previous cluster analysis (Annexe 6): DA01, DA02, DA03, DA04, DA05, DA06, DA07, DA08, DA09, DA20, DA21, DA27, DA55, DA58, DA60, DA74, DA76, and DA84.

The structure method is based on ancestry models and private alleles. Individuals are assigned probabilistically to populations or jointly to two or more populations if their genotypes indicate them to be admixed. It allows a much more precise analysis. Thus, six additional individuals revealed different pattern: DA13, DA14, DA23, DA24, DA19 and DA67 (in purple in Fig. 3).

A summary of these results is given in Table 5.

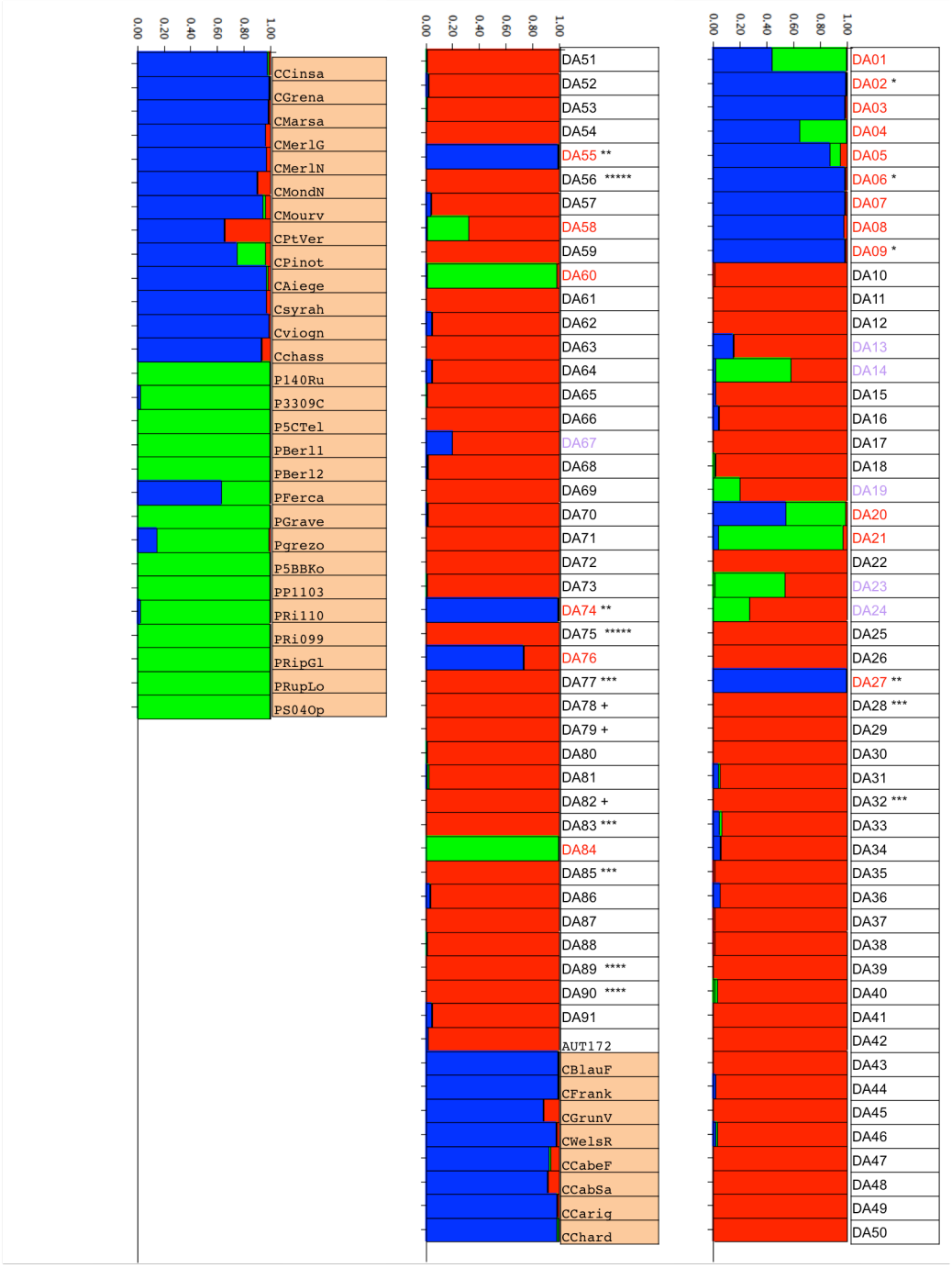


Fig. 3. Population structure of the *Vitis* complex of the Donau Auen National Park inferred with the Bayesian clustering algorithm implemented in STRUCTURE. Each individual is represented by a vertical bar, partitioned into *K* segments representing the proportions of ancestry of its genome in *K=3*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

Clone 1	Clone 2	Clone 3	Haplotype	Putative Crossing
DA01			H3	Cultivar x Rootstock
DA02	DA06	DA09	H3	Cultivar x Cultivar
DA03			H3	Cultivar x Cultivar
DA04			No amplification	Cultivar x Rootstock
DA05			H1, H2 or H5	Cultivar x Rootstock
DA07			H1, H2 or H5	Cultivar x Cultivar
DA08			H1, H2 or H5	Cultivar x Cultivar
DA13			H1	Wild x Cultivar
DA14			H1	Wild x Rootstock
DA19			H3	Wild x Rootstock
DA20			H3	Cultivar x Rootstock
DA21			H1, H2 or **H5**	Rootstock x Rootstock
DA23			H1	Wild x Cultivar
DA24			H1	Wild x Cultivar
DA27	DA55	DA74	H1	Cultivar
DA58			H1	Wild x Rootstock
DA60			No amplification	Rootstock x Rootstock
DA67			No amplification	Wild x Cultivar
DA76			H1	Wild x Cultivar
DA84			H5	Rootstock x Rootstock

Table 5. Clones, haplotypes and putative origin of the crossing.

At K=3 some of these samples get clustered with *Vitis* Cultivars, however they are not closely related to the set of cultivars used in this study. We thus examined K=4 (Fig. 4) and we noticed that this group got separated from the cultivar clade except for DA76.

Austria is located at the convergence of several migration roads of species after the Glaciations. They can actually belong either to other grape cultivars or we can imagine that they could be issued from wild grapevines originated from the East.

A closer look at these samples should be given to the location and the ecology of these samples within the DANP. One thing is that they present a higher rate of crossing with rootstocks. Which makes us thing that they are indeed cultivars not taken into account in our set of cultivars within this study.

In summary, within the 92 Vitis individuals collected in the DANP and analysed, 81 *Vitis* were genetically different and 11 were clones. Among the 81 *Vitis* individuals, 61 were true wild grapevines and 20 were hybrids/introgressed individuals (Table 5) either between true wild grapevine × rootstock, true wild grapevine × cultivar, cultivar × cultivar, cultivar × rootstock or rootstock × rootstock.

We do find all possible crossings in this dataset.

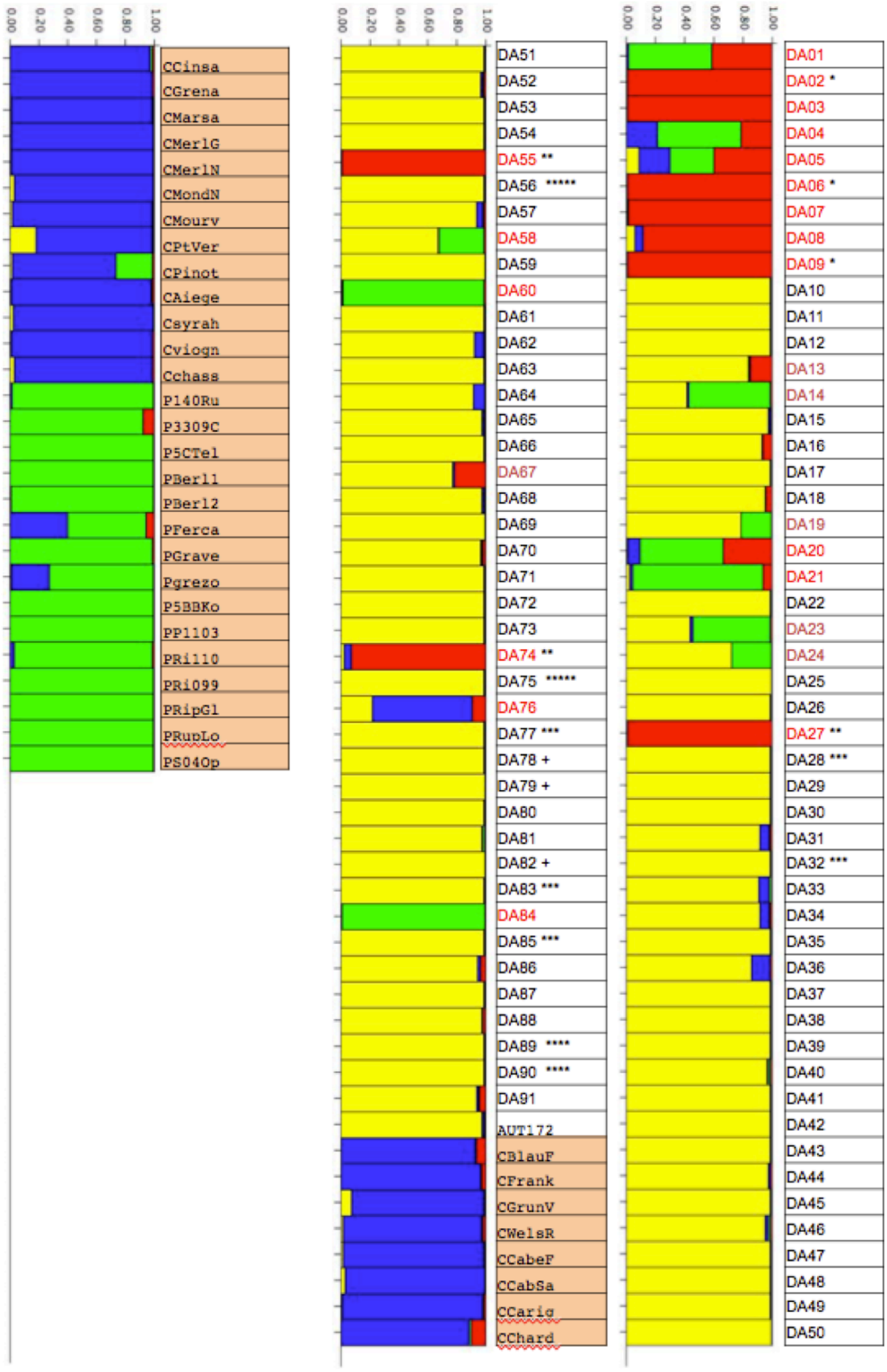


Fig. 4. Population structure of the *Vitis* complex of the Donau Auen National Park inferred with the Bayesian clustering algorithm implemented in STRUCTURE. Each individual is represented by a vertical bar, partitioned into *K* segments representing the proportions of ancestry of its genome in *K=4* clusters. Clones are identified by * and +.

6. Chloroplast

6.1. 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.
We identified a total of five haplotypes distributed in both wild grapevines and hybrids (Table 6). 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 as well as some rare true wild grapevines; and H5, which regrouped all the American rootstocks of various origins.

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

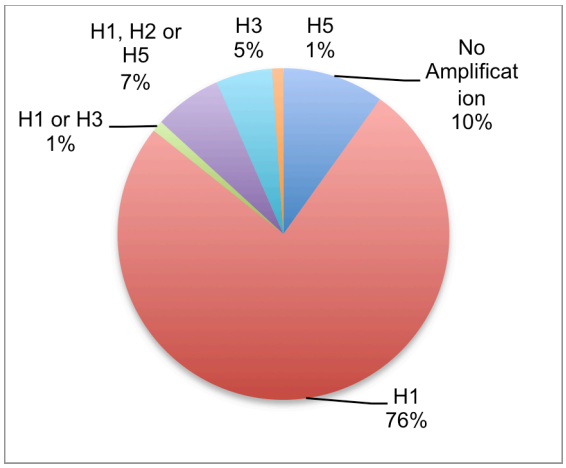
Table 6. Chloroplastic regions and length of the amplified fragments (in bp) related to the five haplotypes generally identified in wild *Vitis* in Europe.

6.2. CP Methods

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

6.3. CP Results

90% of samples did amplify (Annexe 7) at all locus or at least sufficiently to allow an identification to the *V.vinifera* or the Rootstock group.
69 out of 91 samples are belonging to haplotype (H1). Most of the suspicious samples did not amplify well enough to clearly identify the mother inheritance (Fig 5).



But in some cases it is clear and we can thus estimate the direction of the crossing (see table 5).

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

Fig. 5. Percentage of haplotypes within the DANP

7. Discussion and conclusion

In article Arnold et al. 2017, page 13 and 14 of this document, you will find the main points relevant to this current complementary study.

In addition we can add the following points:

A substantial number of propagules regularly reach the park. They are issued from vineyards and gardens surrounding the DANP. The Danube carries also vine shoots, berries and spread them within the park. Humans, animals and insects spread berries and pollen. This phenomenon is occurring since ages and will always be present and if not increase. However we do notice that proportionately to the propagule pressure, the success in their establishment within the park is reduced for *Vitis* species. Only a small number of these propagules succeed in forming adult individuals. However among the young plants, we do notice that hybrids/introgressed individuals do have more success than true wild grapevines.

1. In this set of 91 samples the number of hybrids or introgressed individuals is higher than in the previous study. This is quite normal. The previous study was based on individuals recorded since 1957. For most individuals, the "wild" identity was at least based on morphology and in principle suspected rootstocks were not even considered. We do not know if the new individuals were collected within forests, what was their environment and size. Young plants at the border of the park, along pathways or in erosive parts of the river bank do have a greater probability of beeing crossings involving cultivars or rootstocks.
2. Clones are rare but are present in the three groups: wild, cultivar rootstock and their crossings. Crossings are occurring in each direction for instance from wild grapevines (pollen donor) or to wild grapevines (pollen receptor).
3. The genetic diversity is low even if the calculation in this report includes the hybrids and introgressed individuals, which contribute to increase the He and Ho values. But this reflects the real current diversity of grapevines individuals within the DANP. The current population integrates a complex of *Vitis* species, which is more effective for individuals integrating or combining rootstock, cultivar and wild genes than for individuals of "pure true wild genotype". The disequilibrium between Ho and He is indicating a probable inbreeding process.

As we already mentioned, a better flooding dynamic should be welcome for the *Vitis* species within the park, but will for sure also lead to an increasing number of *Vitis* crossings in the park. Of course any solution should take into account the presence and benefit for other species present in the park. Crossings may represent a simple and effective way for wild grapevines to adapt to current ecological conditions and at least conserve a niche in the DANP environment.

8. References

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