Strategies and methods for conserving and exploring the intra -varietal genetic diversity of ancient grapevine varieties

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Abstract. The antiquity and diversity of traditional grapevine varieties guarantee a strong historical and natural character and add high economic value to viticulture and wine. A high level of intra-varietal diversity of quantitative traits was naturally created and accumulated over centuries. Nowadays, this diversity allows one to carry out selection within a variety and adapt it to the most diverse environmental, agricultural, and market contexts. However, an unusually intense process of genetic erosion of intra-varietal diversity broke out in the 1980s and threatens to destroy within a few decades the diversity created over centuries and millennia. Therefore, the definition of strategies and methods for conserving and exploring intra-varietal genetic variability is crucial for the sustainability of viticulture rich in history and traditions. Those strategies are presented in this work.

1 Introduction

Plant varieties obtained through plant breeding techniques are widely used in modern agriculture, with remarkable results in terms of increased yields, quality, and adaptation to biotic and abiotic factors. Even before Mendel [1] discovered the theoretical foundations of genetics, plants were artificially crossed to obtain more efficient cultivars for agriculture [2-4]. Since the 1920s, Fisher [5] and others made great advances in the knowledge of the genetics of continuous variation traits (Quantitative Genetics), as are practically all the traits of plants with economic interest, which resulted in new and powerful plant breeding technologies increasingly applied in agriculture. Since the beginning of this century, other new molecular technologies of gene transfer and gene editing have appeared, with enormous potential for the construction of new genotypes that are better adapted to all the demands of agriculture and consumers.

However, viticulture is far from obeying this pattern of evolution regarding cultivated varieties observed in the generality of other agricultural species. For winemaking purposes, what is cultivated almost exclusively in the most important winegrowing countries are ancient varieties of the species *Vitis vinifera* domesticated centuries or millennia ago by ancestral vinegrowers and multiplied until today by vegetative propagation. The few exceptions to this rule are the cases of rootstocks, interspecific hybrids that appeared from the end of the 19th century onwards, which constitute the root part of the plants and do not have a direct relation with the quality of the production, and the "direct producer" hybrids (from approximately the same period) whose wines do not reach the quality standard of *V. vinifera* and which today have a residual presence in viticulture. There are also some varieties from intra-specific artificial crossbreeding but mainly designed to table grapes.

The explanation for this particular situation is not simple, but some of the reasons are more or less obvious: first of all, the ancient varieties are very numerous (more than 6000 already described in the Vitis International Variety Catalogue (VIVC) [6] and have very diverse characteristics. Therefore, this base of inter-varietal diversity has been sufficient to ensure the adaptation of the species to the majority of the objectives and changes in the viticultural context over centuries and millennia. But even when this is not fully verified (e.g. disease resistance), the introduction in the viticulture of new varieties obtained using recent plant breeding technologies and with an interspecific genetic basis has other weaknesses. The first is that wine quality is an extremely complex concept, closely associated with the *Vitis vinifera* species, and very difficult to achieve with grapes from interspecific hybrids. On the other hand, wine has a very special origin and natural history, since it accompanies human beings since the time of domestication and the birth of agriculture, in personal behavior, in social relationships, and in artistic and religious manifestations, i.e., it carries countless traditions, which give it a unique noble status and add to its high value. However, the cultivation of recent varieties obtained by interspecific crossing techniques or genetic transfer tends to give a more technological character and overshadows this natural and historical mark of wine, contributing to a decrease in its value.

However, these limitations regarding the applicability of certain modern genetic improvement techniques to grapevine varieties do not apply to other more classical techniques with still largely unexploited potential. A typical case is that of direct selection (according to various methodologies) applied to the intra-varietal diversity of ancient varieties, a type of diversity that began to be perceived in the 1940s [7-9] and that can be exploited today through efficient methodologies [10-14].

Traditionally, intra-varietal diversity has been used in many countries through clonal selection, mainly since the 1950s, to improve the yield, quality, and other traits of traditional varieties, with results that are generally considered relevant for the progress of viticulture. However, the clonal selection has also two less positive consequences: on the one hand, the genetic homogenization resulting from selection makes the behavior of the varieties unstable concerning the

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environment (Genotype×Environment (G×E) interaction), that is, more irregular behavior in different locations, years, and cultural contexts; on the other hand, the systematic cultivation of one or a few clones of each variety, leads to the loss of intra-varietal diversity (genetic erosion), making it unsuitable for future selections focused on viticultural objectives different from the current ones.

The question of halting the erosion of intra-varietal diversity of ancient grapevine varieties has been a concern in Portugal and has been resolved through different complementary strategies. Those strategies are presented in this work. The overall objective is to secure the sustainable use of ancient grapevine varieties by future generations and to deploy a system to obtain more resilient vegetative propagation material that will encourage producers to develop new types of wines, creating new market opportunities and strengthening their competitiveness and growth.

2 Methods

Long-term planning procedures for conserving and exploiting intra-varietal genetic variability discussed in this work can be summarized as follows:

(1) prospecting genetic resources in old vineyards and conserving *ex-situ* large representative samples of the intra-varietal diversity of varieties;
(2) selecting with outstanding and statistically predictable genetic gains of yield and quality, through the "polyclonal selection" methodology;
(3) encouraging the usage of varieties that are not under selection yet by making available to winegrowers mixtures of conserved genotypes, instead of single environmentally unstable genotypes (sensitive to G×E interaction);
(4) promoting the organization of a national network of private old vineyards with the purpose of conserving existing diversity and creating new intra-varietal diversity.

(1) Prospecting genetic resources in old vineyards and conserving *ex-situ*

To recover genetic resources in old vineyards, the solution is to prospect and conserve *ex-situ* a representative sample of the intra-varietal diversity and then make inferences regarding the entire variety.

How do obtain a representative sample of an ancient grapevine variety? The answer is described in detail in several works [10, 11, 15]. The key rules can be summarized as follows: (1) to prospect plants in old vineyards that were planted before selection and nursery activities because only those vineyards preserve the diversity that was created in the past; (2) the total number of plants should come from the largest possible number of vineyards, and only a few plants from each vineyard should be sampled (5 or fewer); (3) within each vineyard, the plants should be separated and must be marked casually (except in cases of serious diseases of a systemic type); (4) according to simulation studies, the minimum sample size needed to represent the variety in a growing region is approximately 70 genotypes. As varieties are usually grown in several regions, samples with 400 or more genotypes for each variety are frequently prospected and conserved.

(2) Select using polyclonal selection methodology

Polyclonal selection methodology is described in [10, 11, 13] and in Resolution OIV-VITI 564B-2019 [12]. It is a combined strategy between conservation and selection: (1) a representative sample of the intra-varietal diversity is conserved in the field trial for performing polyclonal selection; (2) the selection performed (a mixture of 7-20 genotypes) is based on sound quantitative genetic and statistical tools [16-18], allowing selection with high predictable genetic gains for the target traits; (3) the selected material obtained maintains a heterogeneous nature and shows low sensitivity to G×E interaction; (4) the selection performed uses diversity that is evaluated and is conserved, therefore, the data can be used to apply other selection criteria adaptable to new contexts.

The theory of mixed models [19-21] has been applied for the analysis of intra-varietal diversity of yield and other quantitative traits in grapevine field trials, and to perform the polyclonal selection. Examples of mixed models that have been fitted to data from those field trials are described in [22-26]. Typically, the genotypic effects are considered to be random; the effects of the experimental design included are usually assumed as random (for example, complete block effects, in the case of randomized complete block design; resolvable replicate effects, effects of rows and columns nested within complete replicates, in the case of resolvable row-column designs). In the simplest models, random errors and all random effects are assumed to be independent and identically distributed normal random variables. All random effects are assumed to be mutually independent. The residual maximum likelihood (REML) estimation method is used for the estimation of the covariance parameters. Several key indicators are obtained: broad-sense heritability, empirical best linear unbiased predictors (EBLUPs) of genotypic effects and respective prediction error variances, and genetic gain of selection. In practice, in the context of animal and plant breeding (including in grapevine), linear mixed models can be fitted using usually the ASReml-R package [27] within R [28], or Proc Mixed of SAS [29].

(3) Encouraging the usage of varieties that are not under selection yet

For a country, it is not feasible to have all autochthonous varieties under polyclonal and clonal selection. However, the intra-varietal diversity of all of these varieties should be preserved and used when necessary. Therefore, another consequence of the first strategy (prospection and *ex-situ* conservation of the intra-varietal of all autochthonous varieties) is to make available propagation material containing intra-varietal diversity.

(4) Promoting the organization of a national network of private old vineyards

The above-mentioned strategies for stopping genetic erosion relate exclusively to the diversity of a variety created naturally in the past but they tell us nothing about the new diversity that may be created in future years of vineyard variety growth. However, it is easy to conclude that without the introduction of new viticultural techniques related to diversity, the centuries-long evolutionary history of traditional varieties and intra-varietal diversity will end here since: (1) the diversity created in each year of vegetative growth is simply destroyed by pruning the plants; (2) new vineyards are planted with markedly homogeneous material from previous generations. Techniques for continuing the annual accumulation of diversity are under development, as it will be seen below.

3 Results and Discussion

(1) Prospecting genetic resources in old vineyards and conserving *ex-situ*

A unified approach for conserving and exploiting genetic resources of ancient grapevine varieties has been implemented in Portugal since 1978, by an organized national network of around 150 technicians from all over the country. Currently, it is reinforced and supported by the Portuguese Association for Grapevine Diversity (PORVID). This work comprises a network of field trials planted all over the country and an Experimental Centre for the Conservation of Grapevine Diversity of the Portuguese Association for Grapevine Diversity (PORVID), which is a farm dedicated to the conservation of the intra-varietal diversity of all autochthonous Portuguese varieties. At present, more than 30,000 genotypes of at least 218 identified varieties are conserved [14], and the goal of conserving 50,000 is set to be reached within 4 years.

To reinforce the need of prospecting genotypes in old vineyards in the main growing regions of the variety to obtain a representative sample of the intra-varietal diversity, some examples of the work carried out in Portugal are provided in Table 1. For example, for widely grown Portuguese autochthonous varieties, such as Arinto, Fernão Pires, Síria, and Trincadeira, diversity was prospected in 4-5 wine demarked regions and those varieties are now conserved in *ex-situ* collections with more than 300 accessions per variety. For varieties that are also grown in other countries, such as Aragonez, Bastardo, Jaen, and Tinta Caiada, the strategy was to extend the prospection in regions of those countries (in the examples provided, Spain and France).

(2) Select using polyclonal selection methodology

In Portugal, polyclonal selection has been applied to 64 varieties. The strategy is to have 100 varieties under selection within the next 10 years.

Polyclonal propagating material has been produced in Portugal since 1985, however, all the material produced has been traded under the lowest category, standard material. In 2021, Portugal established national legislation [30] with the procedures to the official recognition of voluntary certification of grapevine polyclonal propagating material.

The predicted genetic gains of the polyclonal selected material for important traits have been described in several works [10, 11, 13, 14, 31]. For example, with the polyclonal selection in Portuguese grapevine varieties, predicted genetic gains from 5.9% to 46.0% for yield, 2.8 to 13.2% for soluble solids, 2.0% to 28.3% for acidity, and 2.0% to 16.4% for anthocyanins have been obtained [11, 31]. In Portugal, the use of these selected materials is generating yield gains corresponding to over 10,000,000 €/year, as well as quality gains and several other intangible results.

Table 1. Examples of genotypes prospected in the main growing regions of the variety and the number of genotypes conserved (in the field and pots) by the Portuguese Association for Grapevine Diversity (PORVID).

Variety	Prospected regions	No. genotypes conserved
Aragonez/ Tempranillo	Alentejo, Douro, Dão (Portugal); Rioja, Valdepeñas (Spain)	383
Arinto	Vinhos Verdes, Bairrada, Lafões, Lisboa (Portugal)	566
Bastardo/ Trousseau	Dão, Beira Interior, Douro, Trás-os-Montes (Portugal); Jura (France)	493
Fernão Pires	Bairrada, Dão, Lisboa, Tejo (Portugal)	467
Jaen/ Mencia	Dão (Portugal); Bierzo (Spain)	462
Síria	Algarve, Alentejo, Dão, Douro, Beira-Interior (Portugal)	499
Tinta Caiada/ Parraleta	Alentejo, Douro (Portugal); Somontano (Spain)	306
Touriga Nacional	Dão, Douro (Portugal)	465
Trincadeira	Alentejo, Beira-Interior, Dão, Douro, Lisboa, Tejo (Portugal)	543

(3) Encouraging the usage of varieties that are not under selection yet

As a consequence of the large-scale work of prospection and conservation, propagation material with intra-varietal diversity is available for varieties that are not under selection yet. In Portugal, material of this type for 51 varieties is made available by PORVID (Table 2 shows only some examples of these varieties). Making available mixtures of genotypes representative of the intra-varietal diversity (instead of one or only very few ones from an ampelographic collection) will allow the planting of more resilient commercial vineyards (higher level of intra-varietal diversity and lower sensitivity to GE interaction) and the production of new types of wine.

Table 2. Examples of varieties that are not under selection yet in Portugal but for which there is available vegetative material that contains intra-varietal diversity.

Variety	**No. Genotypes in the mixture**
Marufo	103
Diagalves	100
Branda	98
Manteúdo	82
Pilongo	72
Pintosa	71
Tália	65
Malvasia-Rei	47
Tinta-Carvalha	33
Tamarez	30

(4) Promoting the organization of a national network of private old vineyards for the purpose of conserving existing diversity and creating new intra-varietal diversity

As we have been describing, the erosion of intra-varietal diversity is a highly sensitive problem in current viticulture that we are solving through genetic conservation, methodologically based and applied on a large scale to autochthonous Portuguese varieties. But it is a conservation that only contemplates the diversity created and accumulated in the past, over numerous generations of vegetative multiplication, and which can still be found today in some old vineyards, prior to the generalization of narrow genetic band selection processes, "ready" grafts, and certification systems. The same does not happen in modern vineyards, since they tend to be planted with more or less homogeneous materials resulting from selection and certification processes. And, even if the natural variation mechanisms work in these vines as they do in the old ones, they will not generate abundant diversity during their short lifespan (of the order of 20-40 years) and, whatever the extent of diversity created, it will be lost with the grubbing-up of vines (not transferred to new vines). Erosion has been mitigated by *ex-situ* conservation, which is more or less efficient depending on the country, but without replacing the process of accumulation between generations and growth in diversity.

This means that viticulture is currently going through a historic moment of profound change towards the impoverishment of intra-varietal diversity: going against the direction of the natural evolution of varieties over millennia, making them more unstable in face of the environment (G×E interaction) and preventing future generations from adapting them (through selection) to future changes. In the context of our experimental work on the conservation and use of diversity, there is an approach that is already underway and is subject to further development, which consists of substantially extending the useful life of vines with a high diversity content, through appropriate techniques for fault replacement, total replanting of vines with "genetic copies" of previous vines, and others. In the medium term, the expansion of such system is plausible and desirable, creating a national network of old vineyards with high diversity. Another process being planned is to grow plants to obtain maximum linear elongation of the main cordon each year and over several years (based on intercalary rooting of the main stem) and maximize the effects of accumulation of mutations.

4 Conclusions

In summary, our experimental strategy regarding intra-varietal diversity is based on adherence to the natural processes of its creation, accumulation, and conservation and on sound quantitative genetic-statistical methods to perform selection for relevant traits (yield, must quality traits, and tolerance to biotic and abiotic stresses). That is, a viticultural strategy that respects nature and is adaptable to future changes.

The selected polyclonal materials and mixtures of clones of non-selected varieties will encourage producers to produce new types of wines, creating new market opportunities, and strengthening their competitiveness and growth.

Acknowledgments

The research was also supported through funding of the projects "Conservation and selection of ancient grapevine varieties" (PDR2020-784-042704) and "Save the intra-varietal diversity of autochthonous grapevine varieties (PRR-C005-i03-|-000016).

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