Mechanisms and Candidate Genes for Seed and Fruit Set in Grapevine

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Mechanisms and candidate genes for seed and fruit set in grapevine

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Abstract

Background: Grapevine reproductive development has direct implications on yield. It also impacts on berry and wine quality by affecting traits like cluster compactness, bunch and berry size, berry skin to pulp ratio or seedlessness. Seasonal fluctuations in yield, fruit composition and wine attributes, which are largely driven by climatic factors, are major challenges for worldwide table grape and wine industry. Accordingly, a better understanding of reproductive processes such as gamete development, fertilization, seed and fruit set is of paramount relevance for managing yield and quality. With the aim of providing new insights into this field, we searched for clones with contrasting seed content in two germplasm collections.

Results: We identified eight variant pairs that seemingly differ only in seed-related characteristics while showing identical genotype when tested with the GrapeReSeq_Illumina_20K_SNP_chip and several microsatellites. We performed multi-year observations on fruit and seed set deriving from different pollination treatments, with special emphasis on the pair composed by Sangiovese and its seedless variant locally named Corinto Nero. The pollen of Corinto Nero failed to germinate *in vitro* and gave poor berry set when used to pollinate other varieties. Most berries from both open- and cross-pollinated Corinto Nero inflorescences did not contain seeds. The genetic analysis of seedlings derived from occasional Corinto Nero normal seeds revealed that the few Corinto Nero functional gametes are mostly unreduced. A number of genes potentially involved in sporogenesis and gametogenesis showed contrasting expression between Corinto Nero and Sangiovese and five missense single nucleotide polymorphisms were identified from transcriptomic data. The above findings suggest that the seedless phenotype of Corinto Nero is driven by pollen and/or embryo sac defects, and both events likely arise from meiotic anomalies. Finally, three genotypes, including Sangiovese and Corinto Nero, were unexpectedly found to develop fruits without pollen contribution and occasionally showed normal-like seeds.

Conclusions: Our collective results suggest that parthenocarpy and stenospermocarpy are not restricted to Black Corinth (alias Korinthiaki) and Sultanina-derived cultivars. The single nucleotide polymorphisms identified between Sangiovese and its parthenocarpic variant Corinto Nero are suitable for testing as traceability markers for propagated material and as functional candidates for the seedless phenotype.

Key words: *Vitis vinifera*, seedlessness, somatic variation, reproductive development, flower, berry, fertilization, parthenocarpy, stenospermocarpy, single-nucleotide polymorphism

Background

Fruit set is defined as the transition of a quiescent ovary to a rapidly growing young fruit [1]. The decision of whether or not to set fruit generally depends on the successful completion of pollination

while fruit further growth is determined by fertilization, which initiates seed development [2, 3]. In the typical double fertilization of angiosperms, two sperm nuclei fuse with the egg cell and the binucleate central cell of the female gametophyte inside the ovule, leading to the formation of the zygote and the primary endosperm, respectively. Seed coats originate from the maternal integument cell layers of the ovule. As the seed(s) develops, the walls of the ovary thicken and form the fruit. The coordination between these events relies on the signals originating from seed(s). In the absence of pollination and fertilization, the ovary ceases cell division and abscises. An exception is parthenocarpic species for which an uncoupling of fruit and seed development occurs being the ovary able to develop in the absence of fertilization.

Seedless fruit set and its advantages for productivity and quality

Parthenocarpy has always been attractive to growers, because it may circumvent the environmental constraints on pollination and fertilization and ensure yield stability. Moreover, seedless fruits are favourable to both food processing industry and consumers, being easier to deal with and to eat/digest. Accordingly, the wide occurrence of parthenocarpy in fruit crops is likely the effect of a selective pressure for seedlessness during their domestication and breeding. However, parthenocarpic genotypes are also found in wild species or in non-fruit crops, suggesting adaptive reasons for empty fruit formation in higher plants [4, 5].

Seedlessness is one of the most prized quality traits also in grapevine (*Vitis vinifera*), as demonstrated by the increasing world demand for seedless table grapes [6]. Besides increasing fruit appeal for fresh consumption, the absence of seeds might have additional favourable effects on the quality of grapes, musts and wines. First, seedlessness might contribute to lower cluster density. A loose cluster is a desired trait in grapevine breeding, as demonstrated by the extensive studying of bunch compactness [7, 8]. This architecture leads to a considerably enhanced resilience to infections that decrease yield and compromise chemical and sensory properties of grapes and derived products [9, 10]. In addition, a loose cluster architecture contributes to harmonized ripening periods among berries. Second, a high

seedless berry portion in total berry weight has been found to positively affect important wine characteristics, like color, taste and aroma, favouring the full oenological potential of the vineyard [11, 12]. Finally, the development of parthenocarpic grapes could be crucial to ensure a stable production, which is a major concern for grape growers and winemakers, especially in view of climate change [13, 14]. The main source of yield variability is bud fertility (number of inflorescences per bud), but flower number or size and fruit set rate contribute as well. Among the factors compromising successful fruit set, extreme temperatures (heat and cold) and rainy conditions can impair pollen grain development and functionality and can reduce ovule fertility. Micronutrients, like Zinc and Boron, also affect grapevine reproductive performance [15-20]. Parthenocarpic plants can circumvent the problem of low fruit set making fruit development possible under environmental conditions adverse for pollination and/or fertilization; thus, parthenocarpy has the potential to modulate seasonal fluctuations in yield.

Seedlessness types in grapevine

Vitis vinifera varieties are commonly differentiated as being either seeded or seedless. Normal seeds have a thin outer integument and a sclerified inner integument and contain an embryo and endosperm. When placed in water, these seeds do not float. Two kinds of seedlessness are reported in grapevine: parthenocarpy and stenospermocarpy [21, 22]. Parthenocarpy is the only method by which truly seedless berries are produced. Sometimes, the stimulus of pollination is needed for berry set. In stenospermocarpy, in contrast, fertilization takes place but embryo and/or endosperm abort. In spite of this interruption in seed development, the ovary wall continues to grow to a certain point. The earlier breakdown occurs, the smaller and more rudimental seed traces are present in the mature berry. Parthenocarpy is mainly observed in a group of cultivars whose prominent representative is ‘Black Corinth’ (alias Korinthiaki). The vast majority of their berries completely lack seeds, are very small and spherical; their use is chiefly to make raisin. Molecular analysis has elucidated that parthenocarpic Corinth type cultivars, including Black Corinth, White Corinth (with a pink variant

named Red Corinth), Cape Currant and Corinto bianco, are not genetically related [23, 24]. In line with this, different reproductive defects have been observed in the above varieties, concerning ovules, embryo sacs and pollen [21, 22, 25-27]. Stenospermocarpy is characteristic of an ancient oriental cultivar known as 'Kishmish' (Sultanina or Thompson seedless in the western countries). This variety shares the name Kishmish (or similar) with others often derived from it, and with different genotypes usually of oriental origin [28, 29]. Sultanina has been the major source of seedlessness in table grape breeding programs around the world [23, 30]. Stenospermocarpic berries contain partially developed seeds or seed traces that can go unnoticed, for which reason they are generally considered seedless for commercial purposes; their size, although small, is compatible with requirements for fresh fruit consumption and can be increased by hormone sprays. Sultanina stenospermocarpy has been associated with defects in seed coat development; in particular, endotesta growth and lignification do not occur [31]. Correlated abnormalities in precursor inner ovule integument development have been observed at the time of anthesis [25, 26].

The genetic determinism of seedlessness in grapevine

Parthenocarpy has been recently related with impaired meiosis that terminates in the lack of a mature embryo sac and in pollen sterility in Corinto Bianco, a seedless variant of Pedro Ximenez. Fourteen single-nucleotide polymorphisms (SNPs) distinguishing these two lines were identified, among which seven specific to Corinto Bianco were proposed as candidate parthenocarpy-responsible mutations. They include a putative deleterious substitution in a HAL2-like 3′-phosphoadenosine-5′-phosphate phosphatase [27].

To our knowledge, no other study has been undertaken to unveil the molecular bases of parthenocarpic phenotype in different cultivars, where independent somatic mutations affecting sexual reproduction are expected. It is conceivable that the fertilization-independent fruit growth in these lines is triggered by a genetically determined de-regulation of the hormonal balance, as reported

for other crops. In fact, an increased hormonal level in the ovary can substitute for pollination and prompt fruit growth [3].

On the other hand, the genetic architecture of Sultanina stenospermocarpy has been extensively investigated. In 1996, [32] proposed that three independent recessive genes, which are regulated by a major dominant inhibitor locus named *SDI* (*Seed Development Inhibitor*, according to [33]), control seed development. Different QTL (quantitative trait locus) studies located *SDI* on linkage group (LG) 18, explaining up to 70% of the phenotypic variance in seed content [34-40]. Based on genetic linkage and putative homology, the seed morphogenesis regulator gene *AGAMOUS-LIKE 11* (*VvAGL11*) was proposed as the *SDI* candidate gene [37, 38]. Recent integrative genetics and genomics approaches revealed a missense polymorphism (a SNP at position chr18:26,889,437 resulting in an Arg197Leu substitution) in *VvAGL11* as the functional mutation leading to seed abortion in all Sultanina-related seedless table grape varieties. The concurrent post-zygotic variation identified for this polymorphism and seedlessness phenotype in seeded somatic variants of Sultanina supports a causal effect. A working model has been proposed in which the Arg197Leu mutation disrupts the function of multimeric complexes containing VvAGL11 proteins. In turn, this prevents proper seed coat differentiation and finally leads to endosperm degeneration and embryo development arrest [31, 41].

In the last two decades, a number of other genes have been proposed to play a role in stenospermocarpic ovule/seed abortion or in normal seed development [42-55]. Nonetheless, the differential expression detected for these genes in the comparison of seeded and seedless whole fruits might be a consequence (instead of a cause) of the seedless syndrome (with the concurrent lower proportion of seed-related tissues) if these transcripts accumulate specifically in seeds [41].

Additional candidate genes were identified based on the association between structural variations and seedlessness [56, 57].

Reasons and tools for investigating new seedlessness sources

Only the Sultanina mutation is currently used for commercial purposes (fresh consumption or raisin). However, most of the Sultanina-related table grape cultivars require the spray application of gibberellic acid (GA) at pre-veraison to increase berry size for enhanced market appeal (with the potential drawback of exacerbating berry drop, as described by [58]). In fact in grapevine, as in other species, fruit weight is positively correlated with seed content that is seed number and weight [59 with references herein, 60-62]. This relationship has been attributed to hormones produced in the seeds and transported to the pericarp [63, 64]. In particular, auxins, gibberellins and cytokinins control the first steps of berry development by promoting cell division [61, 65, 66]. Nevertheless, a number of genetic studies (mainly QTL analyses) indicated that seed traits and berry size might be partly dissociated [35-37, 39, 67, 68]. On this basis, it is conceivable that new seedless genotypes that do not require the application of GA sprays may be identified [69]. The discovery of new sources of seedlessness with a genetic determination different from the *SDI* mutation may be beneficial from the table grape consumer's perspective as well. In fact, when employed in breeding programs for Sultanina-derived stenospermocarpy, the SNP at position chr18:26,889,437 is able to discern only those individuals that surely are not seedless, while it does not discriminate between the different seedless classes (C1-C3, as defined by [32]), which are not equally desirable.

Exploiting seedless grapes might also be interesting in the oenological sector, for example to produce specific style wines. In particular, the absence of seeds affects the berry skin to pulp ratio (altering the proportion of skin-available compounds) and increases the quality of ready to drink wines by reducing the astringency conferred by tannins from immature seeds.

Finally, understanding seed and fruit set is both a biological and an agronomic challenge. The clarification of the mechanisms that regulate the reproductive system is crucial since yield stability of a crop, including grapevine, depends on genetic factors controlling seed and fruit development. Identifying these factors might provide useful tools (cultural practices, treatments, molecular markers) for managing yield and for breeding new cultivars or selecting clones resilient to climatic change conditions (e.g. genotypes able to set fruit in the absence of pollination/ fertilization).

In perennial plant species, where mutants are difficult to generate and to screen, natural somatic variants represent a unique resource to understand the genetic control of target traits, because they result from the effect of single mutation or epimutation events in a given genetic background [70-73]. Somatic variants affecting primary berry features like color, seedlessness, or aroma have been identified and exploited throughout the history of viticulture [74]. Others variants for vegetative or reproductive development have been maintained as curiosities and in some cases they proved useful to investigate gene biological function. Among the reproductive traits affected by somatic variation, there are inflorescence differentiation, inflorescence size and branching and flower development. Somatic variants affecting berry development and ripening are also known [70 with references herein, 75-78]. The mechanisms causing somatic variation in grapevine may include changes in disease (e.g. virus load), epigenetic differences, genetic alterations, or various combinations of these effects [70].

The present study was undertaken to provide new insights into the regulation of seed and fruit formation in grapevine. To this purpose, a deep phenotypic and molecular characterization of eight pairs of somatic variants with contrasting seed content identified in germplasm collections was performed. Sangiovese and its seedless variant called Corinto Nero were studied in more detail.

Results

Genotyping variant pairs

SSRs (Simple Sequence Repeats)

The accessions belonging to each pair (Table 1) shared the same microsatellite profile (Additional file1: Table S1 and Additional file 2: Figure S1), confirming that they are somatic variants. Liseiret/Aspirant proved to be identical to Heunisch Weiss, synonym Gouais Blanc [79]. Corinthe Noir and Termarone/Termarina Rosa were identical to Black Corinth/Korinthiaki [24] and to Verano/Termarina [80], respectively. The last genetic profile was also identical to Mammolo/Sciaccarello from [81]. Besides this core sample set, other genotypes were added for

different purposes in the study (see Methods). These additional accessions were also checked by markers for their true to type status.

SNPs (Single Nucleotide Polymorphism)

After SNP data set filtering, pairwise analysis revealed identical SNP profile at all the passed loci of the GrapeReSeq_Illumina_20K_SNP_chip for Sangiovese/Corinto Nero, Termarone/Termarina Rosa, Chasselas Blanc/Chasselas apyrène and Pedro Ximenez/Corinto Bianco. Potentially different SNPs between the other somatic variants were not confirmed by Sanger sequencing of PCR products (Additional file1: Table S2).

Phenotyping variant pairs upon open-pollination

The Shapiro-Wilk test revealed that most of the investigated traits did not follow a normal distribution. The only exceptions were fruit set and bunch length. In a number of cases (e.g. mean flower number per inflorescence, bunch width, bunch length/width ratio and seed number per seeded berry), the deviation from normality was only contributed by seedless variants (Additional file 3: Figure S2A).

The differential behavior of seedless and seeded variants was stable across seasons/locations for most traits (Additional file 3: Figure S3).

Flower number and fruit set rate

Flower number: Before applying the VitisFlower app to the estimation of flower number, its reliability was tested by comparing in the lab the estimated value with the manual count for six Chasselas Rose inflorescences of different size. The correlation was high ($R^2 = 0.90$), in line with the results of the developers (a similar value was also obtained in 2019 when photographing and emasculating Sangiovese and Corinto Nero inflorescences in the field).

The average number of flowers per inflorescence varied from a minimum of 266 (Chasselas Rose) to a maximum of 818 (Corinto Bianco). Significant ($P < 0.05$) differences were found between several

seedless and seeded variants (Figure 1A and Additional file 1: Table S3). In particular, the seedless variants Corinto Nero, Chasselas apyrène and Corinto Bianco showed a significantly greater number of flowers per inflorescence with respect to their seeded counterparts. Although we could not perform any statistical comparison due to the missing seeded reference (Dastatchine did not produce enough inflorescences), it was evident that additional seedless accessions (Sultanina and Corinthe Noir) exhibited a high flower number. This general behavior was inverted for Aspirant that had a significantly lower number of flowers than Liseiret.

We also noticed that the flowers of seedless Moscato Bianco have the so-called "star" conformation with petals freely opening from the top of the calyptra instead of abscising from the base and being subsequently shed fused together as a "cap" (Figure 2A-B). Stamens are short and anthers remain stuck to the calyptra.

Fruit set rate: Both parametric and non-parametric tests indicated that there were no significant differences between fruit set rate measured at fruit set stage and at harvest. The only exception was Corinthe Noir that had a lower fruit set at harvest (34%, while at the earlier stage it was 68%), since most berries were dried and part of them had already fallen.

In the whole set of accessions under study, the mean fruit set rate (as estimated at harvest) ranged from a minimum of 8.9% (Termarina Rosa) to a maximum of 57% (Aspirant). All seedless variants (except for Aspirant) showed lower fruit set rates than their seeded counterparts, with statistically significant differences observed for all pairs but Corinto Nero/Sangiovese and Termarina Nera/Sangiovese (Figure 1B and Additional file 1: Table S3). Nonetheless, differences in fruit set rate between Corinto Nero and Sangiovese were significant in self-pollination conditions at IPSP (data not shown). Fruit set could not be figured for Corinto Bianco because all inflorescences dried after flowering.

Bunch, berry and seed features

Bunch traits: The average bunch density was lowest in Sultanina (2, according to the OIV 204 descriptor) and highest in Sangiovese and Dastatchine (6). The majority of seedless variants had looser bunches compared to their seeded counterparts. The most evident exception was Termarina Rosa (Additional file 1: Table S4).

As a rule, clusters from seedless variants were significantly lighter than clusters from the corresponding seeded lines (Figure 1C). In most cases, they were also shorter and narrower, with a greater length/width ratio (Figure 1D-F). Some examples of clusters in variant pairs are shown in Figure 3. Similarly to what observed for flower number per inflorescence, berry number per bunch did not show a clear pattern related to seed content and it appeared instead to be genotype-dependent. In particular, the seedless variants Corinto Nero and Corinto Bianco exhibited a significantly greater number of berries per cluster with respect to their seeded counterparts. This trend was inverted for the seedless Moscato Bianco that had a significantly lower number of berries than its wild-type (Figure 1G).

Berry and seed traits: Berries from all seedless accessions proved to be significantly lighter compared to berries from the corresponding seeded clones (Figure 1H). In the set of IPSP accessions (where both berry length and width were measured), berries from seedless lines were shorter and narrower than berries from seeded lines and, as a general trend, they had a more rounded shape (Figure 4). Among the seedless accessions, the lowest berry weight was registered in parthenocarpic Corinto Bianco and Corinthe Noir while stenospermocarpic Sultanina had the heaviest berries. As expected, all the seedless clones had a significantly lower percentage of seeded berries (with fully developed seeds, as indicated by the arrow in Figure 5A) compared to their wild-type counterparts (Figure 1I). In particular, Aspirant, Moscato Bianco mutant, Termarina Rosa, Sultanina and Corinthe Noir proved to be absolutely devoid of normal seeds (however, a few Corinthe Noir and Moscato Bianco mutant seeded berries, which were also bigger than normal, were noticed in 2019, as shown in the section "Inspection of seeds and traces at veraison"). For other seedless lines, the proportion of seeded berries ranged from 1% (in Corinto Bianco) to 45.6% (in Termarina Nera) (Additional file 1: Table S3). For

Corinto Nero the average percentage of seeded berries was 9.3%, which is consistent with the values previously calculated from a greater number of berries (5%, 3.1% and 4.3% of seeded berries out of 2133, 1539, 1456 total berries collected in 2008, 2009 and 2010 respectively). It can be easily noticed that the two seedless variants of Sangiovese, Corinto Nero and Termarina Nera, show a rather different phenotype with a higher percentage of medium sized berries and seeded berries in the last one especially when subjected to open pollination (Figure 6C). The seeded berries contained in the seedless accessions displayed a comparable size to that of berries from their seeded counterparts (data not shown).

Seeded berries from seedless accessions contained one apparently normal seed on average (Figure 1J). In particular, all seeded berries from Chasselas apyrène and Corinto Bianco showed one seed, while a few seeded berries from Corinto Nero and Termarina Nera had a second seed. However, the majority of these seeds are not expected to be viable, as suggested by empty seed rate (data not shown). For example, this rate (as estimated by floatability) proved to be more than 11-fold higher in seeded berries from Corinto Nero (72.3%) compared to Sangiovese (6.3%) (Table 2). Seeded berries from seeded accessions accommodated from one to two normal seeds on average. Among seeded lines, the minimum and the maximum number of seeds were observed in Dastatchine and in Liseiret/Sangiovese, respectively (Figure 1J). The majority of fully developed seeds were found in large berries (class A), as shown in Figure 6 and in Additional file 4: Figure S4. The mean seed fresh weight was not significantly different between Corinto Nero and Sangiovese seeded berries, while Termarina Nera seeded berries contained significantly heavier seeds. Among the seeded varieties, Liseiret and Moscato Bianco showed the lightest seeds, whereas Dastatchine had the heaviest ones, which suggests a negative relationship between seed number and weight in these genotypes (Figure 1K).

In addition or as an alternative to normally developed seeds, various rudimental seeds and seed traces were found in most cases (Figure 5). For the Sangiovese/Corinto Nero case study, we also quantified the proportion of seeded berries, berries with only traces and totally seedless berries in 2018, as shown

in Additional file 5: Figure S5. Upon open-pollination, all Sangiovese berries contained at least one apparently normal seed, while the majority of Corinto Nero berries were totally seedless, a smaller percentage contained traces and only 2.5% accommodated a seed.

The phenotypic characterization of all the accessions in open-pollination conditions confirmed the existence of a significant correlation ($R = 0.79$ with Spearman's rs test, $P < 0.05$) between mean berry weight and mean seed number per berry. Mean berry weight proved to be significantly correlated ($R = 0.67$ with Spearman's rs test, $P < 0.05$) also with mean seed weight only in the pool of seeded accessions. For example, Dastatchine (and Pedro Ximenez to a lesser extent) had both the heaviest seeds and the heaviest berries. When considering seedless variants (those berries with at least one seed) this correlation was lost instead (data not shown).

Significant ($P < 0.05$) correlations were also found between bunch density and a number of traits evaluated in this work or in other studies as derived or combined traits. In particular, when considering all the accessions in the same analysis, bunch compactness proved to be positively correlated with fruit set rate, bunch weight, length and width (as well as the ratio between bunch weight and size), berry weight, percentage of seeded berries and number of seeds per berry. Most of the genotypes had a similar relationship between bunch compactness and the above traits, with the only exception of Termarone (alias Sciaccarello), Termarina Rosa wild-type. When performing a separate analysis for each genotype, an additional positive correlation was found between bunch compactness and berry number (as well as the ratio between berry number and bunch length), which can justify the use of berry number as an indicator of bunch compactness (Additional file 1: Table S5).

Inspection of seeds and traces of reproductive structures at veraison

Two different berry size categories (small and large) were observed for all the seedless accessions but Sultanina, and only in Liseiret among the seeded ones (Table 3). However, it is important to remark that almost all collected berries were small in the seedless genotypes with two berry size categories.

Large berries of all these seedless variants, but Aspirant and Termarina Rosa, contained seeds. The floatation test suggested that the seeds of Corinto Nero and Moscato Bianco mutant were vital, whereas the majority of those of Chasselas apyrène were not (Table 3). When potentially viable seeds were dissected, a well-developed endosperm was usually observed, while the embryo was not. This is probably due to the type of section performed, thus the presence of an embryo cannot be excluded. Details about the structure of the seeds are available in Additional file 6: Figures S6-10.

Aspirant biggest berries accommodated only traces of reproductive structures, but initiation of seed components could be generally observed in a more advanced stage of development than in smaller berries (Additional file 6: Figure S6). In the case of Termarina Rosa, berries of both size categories showed similar traces instead (Additional file 6: Figure S9A-C). Unlike the other seedless variants, berry size differences in Aspirant and Termarina Rosa are probably due to a phenological lag between berries sampled from different parts of the bunches or from different bunches. By the time of harvest, all the berries would have likely reached a homogenous size. In fact, this was also observed for Aspirant seeded counterpart (Liseiret), whose small and large mature berries presented well-developed seeds.

Significant differences were found in seed length and width in the seeded/seedless pairs analyzed, that are Sangiovese/Corinto Nero and Moscato Bianco/Moscato Bianco mutant (Additional file 1: Table S6). It is noteworthy that Corinto Nero seeds were on average larger and wider than those of all the other accessions. Detailed description of the seeds extracted from each of these genotypes is shown in Additional file 6: Figures S8, S9 and S11.

We assumed that, in case traces of reproductive structures were observed in seedless berries of the reference cultivars for parthenocarpy (Corinthe Noir) and stenospermocarpy (Sultanina), they are likely remnants of unfertilized ovules and seed traces, respectively. Soft traces were found in the analyzed berries of these two genotypes (Additional file 6: Figure S10). However, significant differences in length and width of the traces were detected (Additional file 1: Table S7). In particular, traces of Corinthe Noir proved to be much smaller compared to the great majority of traces of

Sultanina (Figure 7A). As regards the other seedless variants that were analyzed, Corinto Nero and Termarina Rosa traces clustered together with Corinthe Noir ones, whereas Chasselas apyrène and Aspirant traces mainly laid within the size range of Sultanina (Figure 7B). In fact, significant differences both in trace length and width were found between accessions grouped in the Corinthe Noir cluster (Corinthe Noir, Corinto Nero and Termarina Rosa) and those of the Sultanina's size range (Sultanina, Chasselas apyrène and Aspirant), but not between accessions within each group (Figure 7, Additional file 1: Table S7). Based on these results and on the observations at the stereomicroscope (Additional file 6: Figures S6-10), we hypothesize that most of Corinto Nero and Termarina Rosa traces are likely unfertilized ovules, while those found in the seedless berries of Chasselas apyrène and Aspirant are probably seed traces.

When analyzed at six stages from flowering to pepper-corn sized berries, the ovules of the Sangiovese seedless variant essentially remained within the same range of length and width, which further confirms the above hypothesis that they are unfertilized ovules. Oppositely, the ovules of Sangiovese wild-type increased in size with the progress of the phenological stages, that is to say, they are likely fertilized ovules evolving to become a seed (Figure 7C and Additional file 7: Figure S12).

Investigation of the mechanisms possibly responsible for the seedless phenotype

Evaluation of sanitary status

ELISA test and PCR detected the presence of some viruses in the analyzed accessions, but their distribution does not support a specific role of these pathogens in the seedless phenotype (Additional file 1: Table S8).

Evaluation of male gamete (pollen) functionality

Pollen viability and germination

The *in vitro* viability and germination of Corinto Nero pollen grains proved to be null or close to zero in three seasons. Conversely, Sangiovese pollen viability and germination rates were on average 20

and 40%, respectively. The behavior of Corinto Nero pollen closely resembles that of Corinto Bianco, for which we observed no viability and germination ability, while the pollen grains of its seeded counterpart (Pedro Ximenez) showed high germinability instead. Oppositely, both Chasselas apyrène and Sultanina had functional pollen (Figure 8A-B). High viability and germination were registered also for Corinthe Noir pollen in two seasons (with average values of 79% and 44%, data not shown).

Pollination treatments

A) Self- vs open-pollination: The Shapiro-Wilk test revealed that most of the investigated traits in self-pollination conditions did not follow a normal distribution. The only exceptions were fruit set and bunch length. When seeded and seedless accessions were analyzed separately, it emerged that the deviation from normality in the distribution of some traits (mean bunch weight, bunch width and berry number per bunch) was only contributed by seedless accessions. Oppositely, the deviation from normality in the distribution of mean seed weight was only due to seeded accessions (Additional file 3: Figure S2B).

Fruit set rate: Additional file 1: Table S9 reports fruit set rate values calculated at harvest except for Corinthe Noir, for which we considered the value obtained at harvest not reliable due to dried and fallen berries. If fruit set rate as estimated at fruit set stage is taken instead, this cultivar presented the highest rate among all accessions in both pollination conditions tested. Among the seeded varieties fruit set rate ranged from 31% (Moscato Bianco and Termarone) to 56% (Sangiovese) in open-pollination conditions and from 20% (Termarone) to 46% (Chasselas Rose) in self-pollination conditions. In seedless accessions, excluding Corinthe Noir, it ranged from 11% (Moscato Bianco mutant) to 57% (Aspirant) and from 6% (Sultanina) to 56% (Termarina Nera) in open- and self-pollination, respectively. It is noteworthy that coulure was observed in Sultanina upon self-pollination. As a general rule, most accessions presented higher rates in open- than in self-pollination, except for Termarina Nera, Moscato Bianco mutant and Chasselas both apyrène and Rose (Additional file 8: Figure S13A). However, no significant differences were observed between open- and self-

pollination for any of the accessions for which a statistical comparison was feasible, except for Corinto Nero located at IPSP (Additional file 1: Table S9). Comparison of seeded/seedless pairs was only possible for Sangiovese/Corinto Nero and for Chasselas Rose/Chasselas apyrène for which differences in fruit set rate in self-pollination conditions were statistically significant according to both parametric and non-parametric tests (data not shown).

Bunch traits: Bunch size was significantly affected by the pollination treatment in Sangiovese and even more in Corinto Nero. In particular, upon open-pollination Corinto Nero showed significantly heavier, longer and wider clusters, with a greater number of berries compared to self-pollination. The bunch features of other varieties (e.g. Pedro Ximenez) did not appear to be influenced by the pollen source. Oppositely, Corinthe Noir showed significantly bigger and denser bunches upon self-pollination with respect to open-pollination (Additional file 1: Table S9 and Additional file 8: Figure S13B-F).

Berry and seed traits: The average weight of Sangiovese berries was significantly greater upon open-pollination compared to self-pollination. A similar difference was observed in Termarina Nera, Moscato Bianco, Termarone and, to a lesser extent, also in Corinto Nero. The percentage of seeded berries was differently influenced by the pollination type in Sangiovese and its seedless variants. While Sangiovese open- and self-pollinated bunches exhibited the same percentage of seeded berries, the fraction of Corinto Nero berries with normal seeds was significantly higher after open-pollination. With the lowest p-value for the difference between pollination treatments, this trait proved to be the most sensitive to the pollen source (Additional file 1: Table S9 and Additional file 8: Figure S13G-H).

B) Pollination of Nebbiolo/Trebbiano Toscano with Corinto Nero pollen to test the *in vivo* performance: Berry set was poor when Nebbiolo was cross-pollinated with Corinto Nero pollen compared with fruit set rate of the self-pollinated inflorescences of Nebbiolo. Similar results were obtained when Trebbiano Toscano was cross-pollinated with Corinto Nero pollen. Almost all seeds obtained from Nebbiolo and Trebbiano Toscano cross-pollination with Corinto Nero pollen did not

germinate. Detailed information about the features of the clusters and berries obtained in each condition is shown in Table 4.

C) Emasculation of some pairs and additional varieties: this experiment was originally done to evaluate the parthenocarpic potential of Corinto Nero, given that this accession was found to set fruit in self-pollination conditions in spite of having non-functional pollen, and was then extended to other accessions. While the emasculated and covered inflorescences from most of the treated genotypes dried, Sangiovese, Corinto Nero and Gamay proved to set fruit after anther (and, when tested, also stigma) removal. This ability was confirmed in different seasons and locations but Sangiovese lost its ability to set fruit when emasculation/destigmation was performed at the earliest stage (E-L 15), whereas Gamay was apparently not influenced (Table 5). In 2019 the fruit set rate calculated for Sangiovese and Corinto Nero after emasculation was 42% and 21%, respectively (compared to 66% and 50% upon open-pollination) (data not shown).

Sangiovese clusters derived from emasculated inflorescences showed only a few large berries (class A) with seeds (from 1.9% to 8.2% when pooling berries from all clusters). Most berries were significantly smaller (classes B and mainly C) compared to the control and contained traces of reproductive structures instead. These traces included very small remnants as well as notable rudimental or incomplete seeds. Corinto Nero clusters derived from emasculated inflorescences resembled control bunches: very few large berries that harbored seeds were developed (from 0.4% to 7.6%), whilst the majority of berries were small (class C) and contained tiny traces. Gamay clusters and berries formed after emasculation were smaller with respect to the control. Only a few berries (0.6% in 2015) showed normal seeds, whereas most berries accommodated rudimental or incomplete seeds (Figures 6C and 9A-B, Additional file 8: Figure S14).

All the seedlings derived from occasional normal seeds extracted from emasculated bunches, that are four plants from Gamay, three from Sangiovese and one putative from Nebbiolo (two examples are shown in Figure 9C), had a microsatellite profile that was fully compatible with self-pollination.

Interestingly a Gamay seedling deriving from emasculation was completely homozygous (Additional file 1: Table S10). Some of the seedlings had variegated leaves with green and albino sections.

Evaluation of female gamete (embryo sac) functionality

The four emasculated inflorescences of Corinto Nero that were manually pollinated with Nebbiolo pollen set fruit (Additional file 8: Figure S15). However, most berries were of medium or small size (97.3%) and did not contain seeds (95.5%); the few recovered seeds failed to germinate.

Exploration of potential causes of gamete non-functionality

In 2016, 629 seeds were extracted from the Corinto Nero seeded berries occasionally obtained by open pollination. About 28% (against 95% in Sangiovese) were kept for sowing, as they were potentially viable (non-floating). The percentage of *in vitro* germination was similar in Sangiovese (54%) and Corinto Nero (51.5%) (Table 2). A total of 67 Corinto Nero seedlings were analyzed for ploidy level and genotyped at unlinked microsatellite loci (Additional file 1: Table S11). According to flow cytometry analysis, these plants had different ploidy levels. In particular, 42 plants were 4C (probable tetraploid), 14 were 3C (probable triploid), eight were 2C (probable diploid) and three were 6C (probable hexaploid). The Corinto Nero offsprings showed three different genotypes: 48 individuals (72%) displayed the same genotype as Sangiovese/Corinto Nero plants (Corinto Nero-like, type 1 according to [27]); 14 (21%) had the same genotype as Sangiovese/Corinto Nero plus additional exogenous alleles in several loci (Corinto Nero-like + exogenous alleles, type 2); five plants (7%) exhibited loss of Sangiovese/Corinto Nero heterozygosity in one or more microsatellite loci as well as additional exogenous alleles in several loci (Corinto Nero segregant + exogenous alleles, type 3). No plant had a profile consistent with being derived from regular self-fertilization (type 4).

Overlapping of ploidy and microsatellite data revealed that 42 out of 48 type 1 offspring were 4C, suggesting that they were generated by fertilization of a diploid Corinto Nero female gamete by a diploid Corinto Nero male gamete or, as an alternative, they derived from a tetraploid Corinto Nero

egg cell. Of the six remaining Corinto Nero-like genotypes, two were 2C (probable apomixis), one was 3C (possible fertilization of a diploid Corinto Nero egg by a haploid Corinto Nero sperm nucleus or vice versa) and three were 6C (possible fusion of a diploid and a tetraploid gamete). Thirteen out of 14 type 2 plants were 3C, indicating the fertilization of a diploid egg cell by a haploid non-Corinto Nero sperm cell, while one was 2C, which needs to be better understood. Finally, all five type 3 plants were 2C, which is consistent with the fertilization of a haploid egg by a haploid non-Corinto Nero sperm cell. While no Corinto Nero self-crossed offspring plants were identified, the above genotypes suggest that only in a few cases (at most 6) regular Corinto Nero haploid female gametes might have been formed through meiotic reduction.

Pollen morphometric data, which were collected in view of the generally accepted correlation between pollen grain size and ploidy level, highlighted the great size variability of Corinto Nero pollen, due to heterogeneous and extreme values (15-36 µm, Figure 8C) that are not usually observed in grape cultivars [82, 83]. About half of Corinto Nero pollen grains showed diameters lower than 22 µm and, similarly to Corinto Bianco pollen grains, they were on average smaller compared to those from other varieties, including Sangiovese. Moreover, several Corinto Nero pollen grains were collapsed and/or damaged.

Investigation of the genetic basis of the seedless phenotype

VvAGL11

Genotyping with the CAPS-26.88 marker confirmed that the Sultanina accession used in this study had the point variation (G>T) causing the stenospermocarpy-associated Arg197Leu substitution in the *VvAGL11* gene. All the other accessions were homozygous for the seeded allele (G/G), with the only exception of Aspirant. This accession was genotyped several times for the SNP position, corroborating the G/T genotype. Such polymorphism differentiated Aspirant from its seeded counterpart, Liseiret (Additional file 1: Table S12).

Significant differences in *VvAGL11* expression levels were observed between Sultanina and Dastatchine and between Aspirant and Liseiret at the analyzed stages (Additional file 9: Figure S16).

Genes with validated SNPs between Sangiovese and Corinto Nero

A total of 71,557 SNPs and 37,121 INDELs satisfied the initial filtering criteria. From this list, it was required for any position to be considered a candidate SNP, to be present in at least two libraries and to be different between Corinto Nero and Sangiovese. This approach identified 1670 SNPs. When combined with variant effect prediction and functional gene annotation, 99 missense SNPs were selected for Sanger sequencing. Of these, five were confirmed to be true polymorphisms (Table 6 and Additional file 1: Table S13). All but one were retrieved in additional plants of Sangiovese (clones R10 and VCR4) and Corinto Nero (four accessions from Sicily). The only exception was the 4148 C>T variant on chromosome 6, which was uniquely found in the Corinto Nero accession from Calabria, the one deeply investigated in this study (data not shown).

The same sequences were obtained using either DNA isolated from root/berry pulp or skin tissues of Corinto Nero (data not shown).

Differentially expressed genes between Sangiovese and Corinto Nero

Among the genes that were found to be differentially expressed between Sangiovese and Corinto Nero (according to Additional file 7: Tables S5 and Additional file 10: Table S7 in [84]), we identified genes that control sporogenesis, gametogenesis, pollen-pistil interaction and seed development as potentially linked to the Corinto Nero seedless phenotype (Additional file 1: Table S14).

Discussion

This study represents an integrative approach towards clarifying the mechanisms that underpin seed and fruit development in grapevine.

The members of each pair of variants have been phenotyped in the same vineyard and over multiple growing seasons in order to minimize the effect of environmental conditions and viticultural practices

on their reproductive development [17, 85]. For example, micronutrient (in particular Zinc and Boron) deficiency might originate parthenocarpic fruit set [20, 86]. Moreover, a great degree of berry transcriptomic plasticity is documented for some genotypes like Sangiovese [87].

The investigated variants and their seedlessness type

The seedless phenotype has spontaneously arisen in several grapevine cultivars as a result of somatic mutation; however, Sultanina has been the only source of seedlessness in table grape breeding so far. A high level of somatic variation could have a genetic basis (e.g., a more unstable genetic background) or simply reflect a longer history of cultivation or a larger extension of growth (that means a higher number of vegetative propagation cycles). All the genotypes investigated in this study are ancient cultivars that are known for having many clonal variants.

Sangiovese has a long-standing documented history, as demonstrated by its first mention in 1590 in Soderini's treatise "La coltivazione delle viti". At present it is the most important cultivar in Italy, where it covers a large amount of acreage (71,558 ha, according to [88]) and is the basis for the production of internationally known wines, such as Chianti, Brunello di Montalcino and Vino Nobile di Montepulciano. This variety is also grown in Argentina, California, France and few other countries, but to a much lesser extent. Sangiovese is characterized by great phenotypic heterogeneity (especially in the composition of berry metabolites) and is the cultivar with the highest number of registered clones (128) in the Italian National Catalogue of Grapevine Cultivars [89]. Despite its large presence and long history in the Tyrrenian sea basin, several genetic studies (the first being [90]) demonstrated that Sangiovese has clear and dated relationships with ancient Southern Italian varieties and strongly suggested that it has been cultivated in Southern Italy for a long time. Consistent with this view, the Sangiovese seedless somatic variant evaluated in our study, wrongly named Corinto Nero, was originally identified in the Librandi collection in Calabria, South Italy [91]. Other accessions of Corinto Nero with Sangiovese DNA profile were also recovered from Aeolian Islands in Sicily. In the past these grapes were mostly addressed to the production of "Passolina" raisins (synonym of

Black Corinth, according to [92]), while nowadays they are exclusively employed, in the maximum proportion of 5%, to darken the PDO wine Malvasia delle Lipari [93]. Further seedless variants of Sangiovese were collected in other Italian regions. One of them, called Termarina Nera from Emilia Romagna was included in this study. Another accession is grown in Campania (Southern Italy) under the name of Acinella (that means small berry) (Antonella Monaco, personal communication), as well as another form found in Piedmont. The appellation "Corinto Nero" for this seedless variant of Sangiovese refers to a phenotype resembling Korinthiaki (syn. Black Corinth in California/Corinthe Noir in France), the well-known parthenocarpic cultivar from Greece. In line with the name assigned to this Sangiovese's variant phenotype, our collective findings suggest that the mechanism underlying Sangiovese/Corinto Nero seedlessness is parthenocarpy as well. Similarly to what observed in Korinthiaki/Black Corinth [25, 26] and in Corinto Bianco [27], both parthenocarpic according to the cited studies, Sangiovese/Corinto Nero seedless phenotype is not uniformly expressed, as revealed by the occasional appearance of individual berries with normal size and seeds in several clusters.

Moscato Bianco (syn. Muscat à Petits Grains Blancs) is considered one of the founders of the Muscat family [94]. In the *V*IVC database [95, 96], it has 327 synonyms. A red parthenocarpic variant of Muscat à Petits Grains Blancs was previously reported by [23] under the name of "Cape Currant" (the appellation "Currant" evolved from "Corinth", according to [92]). The complete absence of seeds, seed rudiments and remains of unfertilized ovules suggests that the mutant Moscato Bianco described in our study is parthenocarpic as well, probably of the vegetative type [97]. Moreover, its seedless phenotype is concomitant with the presence of "star" flowers. This conformation was earlier observed in numerous varieties and it was associated to male sterility, aberrant ovules with incomplete integuments (equated with ovules from White and Red Corinth described by [26]), poor fruit set and parthenocarpic berry development [98, 99]. Our mutant Moscato Bianco shows also an altered vegetative growth (hairless leaves with a wider petiolar sinus and smoother teeth), as similarly reported for star Chardonnay [98].

Gouais Blanc (syn. Heunisch Weiss; Liseiret in the present study), the genitor of hundreds traditional grape cultivars [100], has been cultivated since ancient times in nearly all the temperate European grape growing countries [101] due to its high crop and resistance to cold. These factors presumably induced extensive intra-varietal diversity. Indeed, considerable morphologic variability of Heunisch Weiss clones has been described. Moreover, a stenospermocarpic variant was identified at the JKI Institute for Grapevine Breeding Geilweilerhof, wrongly mentioned by the historic German ampelographers as "Aspirant" [79]. The accession analyzed in the present work corresponds to this variant. Our findings support its stenospermocarpic behavior. Based on the discovery of this bud mutation and of seedless mutants in two Heunisch Weiss offsprings (Chardonnay and Iordan), a certain genetic disposition to seedlessness has been attributed to Heunisch Weiss [79]. Nonetheless, while Aspirant showed the point variation causing the Sultanina stenospermocarpy-associated Arg197Leu substitution in the *VvAGL11* gene, Iordan seedless was homozygous for the seeded allele (Additional file 1: Table S12).

The group of Chasselas comprises different synonyms and sports, including a seedless form named "Chasselas Apyrene" [30]. Based on the presence of seed traces comparable to those of Sultanina (Figure 7B), we endorse the hypothesis of stenospermocarpy for the accession of Chasselas apyrène analyzed in the present work. Unlike Sultanina, however, it does not carry the causative SNP in the *VvAGL11* gene. These findings suggest an alternative mechanism for Chasselas apyrène seedlessness. Sultanina is known to be subject to somatic variation. Mutants having smaller berries than the normal stenospermocarpic variety with no abortive seeds ("parthenocarpic" Sultanina) or mutants having larger and more round berries with greater seed traces (Sultanina "Gigas") have been observed [102. 103]. The stenospermocarpic variety used nowadays is the result of intense human selection. Seeded somatic variants have been additionally reported and are known as "Sultanine Monococco" [23, 70, 104] or "Thompson seeded" [42, 43]. Dastatchine has been mainly described as a female putative ancestor/offspring of Sultanina [23] but also as an accession of Sultanine Monococco [105]. The

Dastatchine accession analyzed here corresponds to Sultanine Monococco, i.e. the seeded variant of Sultanina, not its genitor or progeny.

As concerns the other genotypes investigated in the present work, Corinto Bianco was previously reported as a parthenocarpic variant of the ancient seeded cultivar Pedro Ximenez [24], while Termarina proved to be a parthenocarpic variant of the seeded cultivar Sciaccarello (syn. Termarone). Termarina has been grown in north-central Italy since at least 1600 [80]. The Termarina Rosa accession analyzed here shows the same microsatellite profile as the Termarina described by [80]. Based on the small berry size (like in Corinth grapes) and on the presence of very tiny traces (similar in size to those of Corinthe Noir, so likely ovule traces), we hypothesize that our Termarina Rosa is parthenocarpic as well. Unlike what reported by [80], we never observed the occurrence of normally sized and seeded berries.

Finally, we included in our analysis Corinthe Noir (syn. Korinthiaki/Black Corinth) as a reference for parthenocarpy. Remarkably, in several seasons we observed that this accession underwent bunch desiccation involving most berries.

With the exception of Corinto Bianco and Sultanina, that were extensively characterized in previous studies [27, 41], the mechanisms underlying seedlessness in the other variants have not been investigated so far, being analyzed here for the first time.

The use of molecular markers to differentiate somatic variants

Contrasting results have been reported so far about the ability of microsatellites to distinguish between clones (for example, [106, 107] and references therein).

In the present study, somatic variants from the same cultivar could not be differentiated by microsatellites (Additional file 1: Table S1), which points to the need for a different molecular approach. For this reason, each pair (or triplet) of clones was genotyped with the GrapeReseq 20K SNP array, which is the largest available SNP set implemented in a high-throughput genotyping technology for grapevine. This tool has been successfully applied to studies of genetic diversity,

relationships and structure as well as to QTL and association mapping (e.g. [40, 57, 108-111]). Nevertheless, the SNP-array holding 18K SNP loci, reduced to about 16K good quality loci, was insufficient to discriminate among the clones analyzed here (Additional file 1: Table S2). This result is in line with previous findings concerning biotypes of the same cultivar [112, 113] and indicates that such number of SNPs hardly covers the genome regions harboring target phenotypic traits. Therefore, other methods are necessary, for which reason we recently started the resequencing of Corinto Nero genome.

Nature of the reproductive structure traces observed in the seedless genotypes

Veraison is the onset of ripening and represents the transition from berry growth to berry ripening. Grapevine seeds at this phenological stage have reached their full pear shape and size and, from a structural point of view, they are completely developed, although further changes in color, lignification and composition will occur during berry ripening [114]. In addition, it is easier to extract and analyze traces (ovule remnants or rudimental seeds) at veraison than at maturity, reason why seeds and traces were inspected at this stage.

Fertilization is the key step differing between parthenocarpy and stenospermocarpy seedlessness: it does not occurr in the former, while in the latter seeds abort at some point after fertilization. Many parthenocarpic grapes contain very small residuals that correspond to aborted ovules, which are much smaller than stenospermocarpic seed traces [21]. In fact, in most Corinthe Noir seedless berries we observed very small and tiny remnants of undeveloped ovules with a significantly reduced size compared to the aborted seeds of Sultanina (Figure 7A). Hence, to understand the mechanism underlying seedlessness, it is essential knowing the nature of the traces found in seedless berries: unfertilized ovules or aborted seeds. In seeded cultivars, just after fertilization there is a relatively delayed development of the embryo (proembryo) and a slow formation of the endosperm while, on the other hand, a rapid development of nucellus and of ovule maternal integuments (which start differentiation into the seed coat) take place. When the integuments and nucellus reach their

maximum size and differentiation, the embryo and the endosperm start a fast development. The seed coat contains several layers of strongly sclerified cells [21, 22, 114]. Although histological analysis should be performed for confirmation, pieces of evidence that fertilization had occurred were the presence of structures such as sclerenchyma and/or endosperm, a big degenerated nucellus, and a clearly defined pear shape of seed traces extracted from Aspirant and Chasselas apyrène seedless berries. Therefore, these traces were considered remnants of seeds aborted in earlier or later stages of development (Additional file 6: Figures S6 and S7). Conversely, none of these structures or characteristic seed shape could be seen in the examined traces from seedless berries of Corinto Nero and Termarina Rosa (Additional file 6: Figures S8 and S9). In fact, these traces resulted to be similar in size and consistency to the undeveloped ovules of Corinthe Noir (Figure 7B). Hence, we hypothesize they are unfertilized ovules too. In Corinto Nero, this hypothesis is further supported by the monitoring of Sangiovese and Corinto Nero ovule size increase at six stages from flowering to pepper-corn (Figure 7C).

The effect of seed content on bunch/berry features

Fruit set: In general fruit set rate proved to be compromised in seedless compared to seeded variants, with the only exception of Aspirant (Figure 1B and Additional file 1: Table S3). This reduction is consistent with the observed positive correlation between fruit set rate and seed number per berry [115] or number of seeded berries [116]. Genotype, vine nutrition, cultural practices and weather conditions being equal, the main factors affecting fruit set are expected to be flower density, flower fertility and pollination efficiency (this last is not relevant if vegetative parthenocarpy occurs) [117-119]. As regards flower density, all the seedless variants but Aspirant and star-flower Moscato Bianco had, indeed, a higher estimated number of flowers compared to their seeded counterparts (Figure 1A and Additional file 1: Table S3). In the case of mutant Moscato Bianco the poor fruit set was probably related to the star conformation of flowers (having a potential detrimental effect on fertility, [98, 99]). Based on the frequent decrease of fruit set rate in self-pollination compared to open-pollination

conditions (Additional file 1: Table S9 and Additional file 8: Figure S13A), one might envisage that restricted pollination efficiency plays at least a partial role in lowering fruit set rate of some seedless accessions, like Corinto Nero (the observed fruit set reduction in self- compared to open-pollination was twice as much as in Sangiovese). This is consistent with the very low viability and germination of Corinto Nero pollen reported in Figure 8A-B. However, instead of depending from a limited pollen functionality, the lower fruit set in self- compared to open-pollination conditions might simply reflect a cross-pollination preference [115, 120, 121], a microclimate negatively affecting pollination efficiency or a phenological lag among flowers within the paper bag. This might hold for Sultanina, which showed the highest decrease of fruit set rate in self- compared to open-pollination conditions (Additional file 1: Table S9) in spite of the highly viable pollen (Figure 8B and results obtained by [115]).

Bunches and berries: The average bunch weight and size were significantly lower for the seedless accessions than for their equivalent seeded cultivars (Figure 1C-E). This was mainly due to the clear predominance of lighter and smaller berries in the seedless lines (Figures 1H and 4). Indeed, mean berry weight proved to be positively correlated with seed number ($R^2 = 0.79$) and seed weight ($R^2 = 0.67$ in the set of seeded accessions). These findings are in agreement with previous reports [60-62, 115]. The most likely explanation is that seed content influences berry growth (especially affecting cell division) through hormonal mechanisms, more seeds or larger seeds producing more hormones than fewer or smaller ones [2, 122]. In the seedless variants for which both diameters were measured, the decreased berry weight was associated to an evident spherical shape (Figure 4A). This could be due to pleiotropic effects on fruit size and shape. It is noteworthy that [73] documented a negative correlation between fertility index and berry traits, in particular berry shape index (length/diameter ratio).

Interestingly, seed content affected also cluster density, as demonstrated by the positive correlation of bunch compactness with the percentage of seeded berries, number of seeds per berry and berry weight (Additional file 1: Table S5). This is in agreement with the findings of [116].

The occurrence of berry set after emasculation

Whilst unpollinated and unfertilized flowers usually abscise, Sangiovese, Corinto Nero and Gamay emasculated and bagged inflorescences were repeatedly observed to set fruit (Table 5). In all three genotypes, only a few normal-sized berries contained seeds, whereas the majority of berries were small and accommodated traces instead (Figures 6C and 9A-B, Additional file 8: Figure S14). This phenomenon is not reported as a characteristic grapevine feature [120] and establishing the underlying biological mechanism is especially interesting.

Emasculation was performed when flowers were still closed; therefore, cross-pollination mediated by wind or by insects has to be excluded, as confirmed by the self-pollination compatible microsatellite profile of the few seedlings derived from germinated viable seeds (Additional file 1: Table S10). The segregation of SSR alleles, along with the paucity of fertile seeds, is also against the involvement of apomixis, which is asexual reproduction through seed [123].

Cleistogamy (self-pollination without calyptra fall) or bud-pollination (self-pollination taking place before the flower opens) might be possibly engaged. The occurrence of these phenomena has been hypothesized in some cultivars, while not appearing in others [124]. For example, [125] reported that at the time of opening, anthers in all flowers of Müller-Thurgau and Pinot Noir had already dehisced. About 16-18% of the flowers of Pinot Noir and 60-63% of Müller-Thurgau proved to be pollinated before opening and growth of pollen tubes had already started. [126] observed that at 2 weeks before anthesis Cabernet Sauvignon anther membranes were degraded and mature pollen grains had been released, while the cap was still attached to the flower. At this stage, an early seed structure had begun to develop. Given the assured seed set by cleistogamy and bud-pollination and the viability of Sangiovese (this work) and Gamay pollen [127], these methods of self-pollination triggered before emasculation might eventually have played a role in fruit set following emasculation, especially for the few normal-sized seeded berries. Nevertheless, we consider this hypothesis unlikely because at the time of flower emasculation anthers were still green and had not dehisced yet.

Likewise, we cannot exclude that, while castrating, some anthers bursted and allowed the pollen to escape, as already reported by [128] and [129]. If some pollen by this time was already mature, it might have retained its vitality until the pistils became receptive, especially in flowers emasculated just before blooming.

In any case, we believe that the prevalent mechanism underlying berry formation (mainly small and seedless) after inflorescence emasculation, not only in the seedless genotype (Corinto Nero) but also in Sangiovese and Gamay, should have been parthenocarpy. This is also proved by the occurrence of inflorescences setting fruits after removal of both anthers and stigma during emasculation (Table 5). Indeed, grapevine has a characteristic facultative parthenocarpy, of both the vegetative (not requiring pollination) and the stimulative (requiring pollination) types, a phenomenon that intensifies when proper pollination is prevented by emasculation or by adverse environmental conditions [2, 85, 130, 131]. Previous reports of parthenocarpic fruits produced by emasculating and bagging the flower clusters are available for White Corinth, Black Monucca, Himrod seedless, Sultanina, Red Globe, Campbell Early and Muscat of Alexandria. In particular, this last variety was observed to produce some berries without seeds, some berries with empty seeds, some berries with seeds that had an endosperm and some berries with seeds that contained an embryo [2, 130, 132, 133]. This parthenocarpic potential might be an intrinsic property of grapevine (not restricted to specific genotypes) that becomes only expressed in the absence of fertilization upon certain conditions. In the case of emasculation, whether or not these special conditions exist, the successful outcome of this process might be considerably affected by the timing of emasculation (as shown for Sangiovese, Table 5). In the present study, it is noteworthy that the accessions setting fruit after emasculation (Sangiovese, Corinto Nero, and Gamay) are all early-flowering varieties. It is conceivable that in these genotypes developmental processes had progressed enough to result in ovary growth into fruit after removal of suppression signals from stamens or after perception of other signals in response to the damage of reproductive structures. Conversely, it is likely that in late-flowering cultivars (Grenache, Nebbiolo, Trebbiano Toscano, etc) emasculation was done too early in terms of

reproductive organ development. Indeed, the stage of inflorescence development as determined by the E-L scale does not necessarily reflect the stages of development for the fertile organs (gametes), especially at key steps such as meiosis. In particular, the duration of reproductive organ development between meiosis and bloom is cultivar-dependent [134, 135]. An effect of developmental timing on fruit set is also supported by the observation that berries derived from flowers that open first have less probability to abscise than the flowers that open later within the same cluster, because of polar auxin transport [136]. However, we cannot exclude that the individual genotype plays a role in this phenomenon, which could be enhanced in certain cultivars. For example, a study evaluating the reproductive performance of ten grapevine varieties [131] showed that Sangiovese is characterized by high bunch weight, high fruit set, high number of seeded and seedless berries, low proportion of live green ovaries relative to the total number of flowers, low coulure index (proportion of flowers that do not develop into either a berry or a live green ovary). Similarly, another study assessing the reproductive performance of 120 varieties [116] classified Sangiovese and Gamay into a group characterized by higher fruit set rates and lower coulure values, lower number of flowers and an intermediate number of seeded berries with respect to the other classes. The above features come out in favour of an intrinsic predisposition of these two cultivars to set fruit. Similarly to what we observed for Sangiovese, Corinto Nero and Gamay, some degree of background parthenocarpy following emasculation and coincident elimination of inhibitory signals from floral whorls surrounding the carpel was also seen in Arabidopsis ecotypes, several tomato lines and sweet pepper genotypes [137, 138].

The mechanisms possible responsible for the seedless phenotype

Sanitary status: The sanitary status of cultivar clones can be a source of phenotypic variation associated with changes of gene expression [139, 140]. Some previous studies, e.g. [11], reported an effect of virus infection on seed content, however our results (Additional file 1: Table S8) do not support a specific role of these pathogens in the seedless phenotype of the analyzed varieties.

Aberrations in reproductive development: With the only exception of star-flower Moscato Bianco (Figure 2B), we did not observe any macroscopic evident alteration in flower structure at anthesis.

The reasons of seedlessness could be related to abnormalities in ovule formation before flowering, low level of pollen fertility, insufficient pollination and fertilization at flowering, embryo/endosperm abortion after fertilization. Parthenocarpy has been found associated with alterations in early ovule development (defective integument growth and irregular meiosis reducing the production of viable female gametes) in tomato [141, 142], Arabidopsis [143, 144] and *Capsicum annuum* [138]. A connection between parthenocarpy and ovule defects exists also in grapevine; ovule development anomalies can occur before megasporogenesis (in White and Red Corinth according to [26]), at the end of megasporogenesis (in Corinto Bianco to [27]) or during megagametogenesis (in Black Corinth to [26]). Besides being a rule in parthenocarpic genotypes, even in normally seeded cultivars a high percentage of abnormal embryo sacs occurs. In fact, the fertilization of just one of the four ovules is sufficient to ensure the development of the pistil into a fruit. However, this phenomenon is more frequent in stenospermocarpic varieties, like Sultanina [21, 22, 25-27, 133]. In the present work, the majority of berries derived from the cross Corinto Nero x Nebbiolo or from open-pollination of Corinto Nero had no seeds. This indicates that the availability of viable pollen (from Nebbiolo or other cultivars in open pollination) is not sufficient to promote normal seed development in Corinto Nero and that female defects contribute to impeding this process. We hypothesize that, at the time of anthesis, Corinto Nero embryo sacs are missing or in various stages of degeneration, rarely able to function in fertilization. In fact, the ploidy level of Corinto Nero seedlings evidenced anomalies during meiosis in megasporogenesis. Therefore, Corinto Nero seedlessness is likely due to the lack of functional female gametes coupled with an alternative fertilization-independent process of fruit development.

An association has been additionally observed between parthenocarpy and male sterility in mutants and transgenic lines of tomato and apple, largely involving genes that control floral organ identity and development [141, 145-150]. Consistently, a relationship between seed set and pollen viability

or germination has been documented in grapevine, with low pollen fertility resulting in a low level of seed setting, due to an increased probability of pollination failure [27, 118, 151]. The *in vitro* tests performed in the present study revealed that Sangiovese pollen is viable and able to germinate, even if at lowest levels in the range of variation reported for grapevine cultivars [115, 119, 127 and references herehin, 152]. Oppositely, its seedless variant Corinto Nero showed negligible pollen viability and germination, as the parthenocarpic Corinto Bianco (Figure 8A-B and [27]). The very low presence of viable pollen grains in Corinto Nero might explain the only occasional formation of seeded berries after self-fertilization in case of rarely available functional ovules (Additional file 1: Table S9 and Additional file 8: Figure S13H).

The non-functionality of Corinto Nero pollen was also supported by the *in vivo* pollination experiments (comparison of self- and open-pollination, cross-pollination of Nebbiolo and Trebbiano Toscano). There are a few factors supporting the evidence of a non-functional pollen in Corinto Nero. First, although in a number of grape cultivars open-pollination has proven to be superior than self-pollination in determining seed (xenia) and berry (metaxenia) characteristics [115, 120, 121], the pollen source apparently did not affect the percentage of seeded berries in Sangiovese while the fraction of Corinto Nero berries with normal seeds was significantly higher after open-pollination (Additional file 1: Table S9 and Additional file 8: Figure S13H). Second, berry set following pollination of Nebbiolo and Trebbiano Toscano inflorescences (two highly productive cultivars) with Corinto Nero pollen was very poor. This result was not determined by a time-shift in the reproductive development of donor and recipient cultivars, as demonstrated by testing varieties with different flowering times. Conversely to what we observed for Corinto Nero and Corinto Bianco, Corinthe Noir (mainly showing stimulative parthenocarpy, [153]), Chasselas apyrène and Sultanina (both stenospermocarpic) had functional pollen (data not shown for Corinthe Noir; Figure 8B for Chasselas apyrène and Sultanina). Consistently, Sultanina has been described as an efficient pollinizer [115]. High pollen viability was also reported for another stenospermocarpic variety, Parvana [152].

Potential causes of gamete non-functionality

Non-functional gametes may be the result of failure at different points in their development. In particular, irregularities may take place during sporogenesis, during the development of surrounding structures like tapetum and nucellus or during the final steps of gametogenesis. The uninucleate pollen grain and the chalazal megaspore generate through mitotic divisions a two- or three-celled pollen grain and a seven-celled embryo sac, respectively.

Meiosis omission or abortion involving both micro and macrosporogenesis is a likely cause of Corinto Nero sterility and impeded seed formation, as reported for Corinto Bianco [27] and to a lesser extent also for other varieties [151, 154]. Indeed, the genetic analyses of Corinto Nero seedlings (Additional file 1: Table S11) revealed that Corinto Nero infrequent functional male and female gametes are mostly unreduced gametes (as inferred from 62 out of 67 seedlings), and the major part of unreduced gametes are diploid (originating at least 58 seedlings). These diplogametes might derive from apomeiosis (suppressed or imperfect meiosis), which is the first step of gametophytic apomixis [155]. The presence of two diploid Corinto Nero-like seedlings (type 1) supports, in facts, the involvement of apomixis in these two cases. Although they are typically much more frequent events among apomicts, both the formation of unreduced gametes and the parthenogenetic development of unfertilized egg cells are widely recorded phenomena in sexual species [156]. It is conceivable that the type of apomeiosis occurring in female gametes here is diplospory (the embryo sac originates from the megaspore mother cell either directly by mitosis - mitotic diplospory - and/or after interrupted meiosis - meiotic diplospory). Although diplogametes may derive from a variety of different meiotic abnormalities, they all result from one of two basic processes depending on the mode of nuclear restitution: First Division Restitution (FDR) and Second Division Restitution (SDR), which occur during abnormal development of the first and the second meiotic divisions, respectively. FDR produces gametes containing non-sister chromatids, which retain the whole (through the omission of meiosis I in FDR sensu stricto) or a large part (through other cytological alterations, e.g.

in spindle biogenesis and polarity) of parental heterozygosity [157, 158]. SDR gametes, instead, possesses sister chromatids [159]. Therefore, to further elucidate the ontogeny of Corinto Nero female diplogametes we focused on the genetic make-up of triploid seedlings at microsatellite loci that are heterozygous in Corinto Nero, as suggested by [27]. Segregation of Corinto Nero alleles was never observed in the triploid seedlings obtained in the present work and the only type 3 Corinto Nero offsprings (segregant + exogenous alleles) were diploid. This result is consistent with the occurrence of first division nuclear restitution (FDR), but it does not exclude the involvement of apospory apomeiosis (development of the embryo sac via mitosis from a diploid somatic cell positioned adjacent to the megaspore mother cell). Cytohistological studies would be required to determine the origin of the diploid precursor cell. In addition, since in tetraploid and hexaploid Corinto Nero offsprings potential losses of heterozygosity produced by meiotic segregation events are masked by chromosome duplication, we cannot exclude that additional 2n gamete-inducing mechanisms (like second division restitution, SDR) may occur, as observed in other plants. Indeed, multiple mechanisms can lead to 2n gamete formation in the same plant [160]. As it has been hypothesized for Corinto Bianco [27], Corinto Nero might be a meiotic mutant with a recessive homozygous mutation or, more probably in a somatic variant, a dominant heterozygous mutation [72]. The formation of Corinto Nero polyploid gametes (e.g. tetraploid pollen) might be due to a block in both meiotic transitions (as in the *tam-2/osd1-1* double mutant, [161]), to defects in microspore separation (as in *qrt* mutants, [162]) or in male meiotic cytokinesis (as in *tes/stud* mutants, [163]).

The variability in Corinto Nero gametophytic ploidy level is well reflected in the wide variability in pollen size (Figure 8C), in agreement with the generally accepted correlation between pollen grain size and ploidy level [157, 159]. In particular, the bigger pollen grains might correspond to viable diploid pollen grains, as proposed in the case of Corinto Bianco [127].

Based on the above discussion, it is also tempting to speculate that the greater size of Corinto Nero occasional seeds compared to those of all other accessions that were inspected at veraison (Additional file 1: Table S6) is the result of the involvement of unreduced gametes in fertilization.

Other reasons of male gamete non-functionality may be defects in tapetum development and/or resorption and in pollen development after release from the tetrads. Tapetum layer (the most inner part of anther wall) contributes to transmittal of food to microspore mother cells, formation of callose in microspore mother cells during meiosis, differentiation of microspores after tetrad phase and formation of pollen wall. It also provides for peculiar proteins that are involved in the regulation of the pollen tube growth. Early degeneration or, conversely, persistence of tapetum have been recognized as possible causes of pollen sterility in grape [164, 165]. After tapetal cell resorption, the pollen grains undergo physiological and morphological preparation for dispersal, basically involving closure of colpi and germination aperture. Shape abnormalities and lack of furrows or germinative pores in pollen grains have been frequently associated to non-germination, which implicates a morphological sterility [166, 167]. This kind of grains resembles the pollen found in female flowers of dioecious vines [82 and references herein]. In the present work, several Corinto Nero pollen grains were found to be collapsed and it is conceivable that additional structural aberrations might be responsible for negligible viability/germination of Corinto Nero pollen grains. However, a more focused microscopic investigation would be necessary to prove it. Non-germinative abnormal pollen in turn might be a major determinant of seedless fruit development under micronutrient-sufficient conditions, as previously reported [20].

The genes possibly underlying the seedless phenotype

VvAGL11

We suggest that the stenospermocarpic seedlessness of Aspirant is linked to the Arg197Leu missense substitution in *VvAGL11*, similarly to what was found for Sultanina by [41] and confirmed in the present study (Additional file 1: Table S12). The differences in *VvAGL11* expression levels between whole seeded and seedless berries of both genotypes (Additional file 9: Figure S16) should be considered instead a consequence of the lower proportion of seed-related tissues in developing fruits, as already hypothesized for Sultanina [41]. This view is further supported by the fact that, even though

Sangiovese and Corinto Nero share the same genotype at the position chr18:26,889,437 (Additional file 1: Table S12), a significant *VvAGL11* induction from stage E-L 15 to E-L 27 was exclusively observed in Sangiovese whole berries, which can be attributed to the presence of seeds (Additional file 1: Table S14). These findings also indicate that a different mechanism (not involving *VvAGL11*) is at the origin of seedlessness in Corinto Nero, as well as in the remaining somatic variants (except Sultanina and Aspirant).

Genes with validated SNPs between Sangiovese and Corinto Nero

In the last years, different molecular mechanisms responsible for somatic variation have been identified, including point mutations, insertions/deletions of transposable elements and chromosomal rearrangements (for a review see [72]). Based on this knowledge, we took advantage of the transcriptomic experiment done by [84] to perform a preliminary investigation of single nucleotide polymorphisms between Sangiovese and Corinto Nero. Five SNPs were validated between Corinto Nero and Sangiovese, which have a potential involvement in intra-varietal phenotypic variation. Even in the absence of any functional role, these polymorphisms might be useful to discriminate the two lines.

Considering that both Sangiovese and Pinot Noir are seeded varieties, the most interesting genes (with possible causal SNPs) are those showing a PN40024-like genotype in Sangiovese and a variant nucleotide in Corinto Nero, that are VIT_06s0004g03800 (4148 C>T), VIT_11s0016g03590 (3340 A>G) and VIT_11s0016g05820 (949 G>A) (Table 6). The phenotypic effect might derive from gain-of-function mutations or from loss-of-function mutations resulting in haploinsufficiency [70].

VIT_06s0004g03800 codes for a nuclear factor related to kappa-b-binding protein. The product of the orthologue Arabidopsis gene is a component of INO80 chromatin-remodelling complex. The roles of SWR1(SWi2/snf2-Related 1)/INO80-complex in nuclear activities are quite diverse ranging from double-strand breaks repair to regulation of gene expression. Interestingly, some core SWR1/INO80-c subunits have been shown to act in reproductive development, e.g. female meiosis, in Arabidopsis

[168]. The 4148 C>T SNP determines a Thr1383Met change, which has potentially a significant impact on protein function due to the contrasting polarity of the two aminoacids.

VIT_11s0016g03590 codes for a transducing protein. Out of the five genes containing SNPs, it is the only one with a differential expression between Sangiovese and Corinto Nero, which is a significant up-regulation from E-L 15 to E-L 27 only in the seedless clone [84].

The product of VIT_11s0016g05820 is a component of the CCR4-NOT complex, which is one of the major cellular mRNA deadenylases and is linked to various processes including mRNA degradation, miRNA-mediated repression, translational repression and general transcription regulation [169]. Interestingly NOT1, the scaffold protein of the CCR4-NOT complex, has been recently established as an important player during male and female gametophyte development in Arabidopsis, with its disruption showing abnormal seed set [170, 171]. Notably, the variant 949 G>A was also found in a homozygous state in Chasselas apyrène (Additional file 1: Table S13), which may further support its role in the seedless phenotype.

The two remaining genes with validated SNPs are VIT_02s0025g03330 and VIT_14s0083g00910. VIT_02s0025g03330 codes for an autoinhibited H^+ ATPase (AHA). Some AHA isoforms have been suggested to play a major role in male gametophyte formation and function [172], in particular in microspore development, e.g. [173], and in pollen tube growth [174 and references herein]. Other members of the AHA family have been shown to be involved in seed coat endothelium development and in embryo viability [175, 176]. This gene falls within the confidence interval of QTLs for cluster weight and compactness, as well as rachis and shoulder length [8].

The product of VIT_14s0083g00910 is a fucosyltransferase with a potential role in pollen tube growth [177]. This gene is comprised in the confidence interval of QTLs for seed weight [35, 41], number of berries per cluster [108, 178], rachis length [179], number of nodes of the central cluster axis [178] and flowering time [180].

At four SNP positions, all the five analyzed clones of Corinto Nero shared the same allele, which hints at a common origin and propagation history. The presence of the 4148 C>T mutation in a single

Corinto Nero clone (the one from Calabria deeply investigated here) suggests instead that this mutation is relatively recent (data not shown).

Based on the analysis of DNA extracted from different organs (layer-specific approach), a chimerical nature of the clones for the identified mutations could be excluded. This result, which contrasts with the quite common somatic chimerism reported in grapevine clones [70, 181 and references therein], can be explained by cell layer rearrangements leading to homogenization of the plant genotype [182, 183].

Differentially expressed genes (DEGs) between Sangiovese and Corinto Nero

Additional candidate genes with a potential link to the Corinto Nero seedless phenotype were identified for future validation at a deeper phenological scale (Additional file 1: Table S14). Their selection was based on the findings of the present study, the differential expression in Sangiovese and Corinto Nero [84] and the supporting evidence from the literature. They can be classified according to their involvement in the following processes [184].

Sporogenesis: This category includes genes (under sporophytic control) involved in promotion or restriction of gametophyte precursor cells and in meiosis regulation. For example, *NOZZLE/SPOROCYTELESS* (VIT_19s0014g03940) takes part in anther/ovule ontogenesis and in pollen/megaspore mother cell development in Arabidopsis [185]. Moreover, a novel role in the negative regulation of fruit set has been recently uncovered for the tomato orthologue gene *SlSPL/HYDRA* [186]. Other candidate genes are *ARGONAUTE104* (VIT_06s0009g01200), *MEIOSIS ARRESTED AT LEPTOTENE1* (VIT_11s0016g04620) and *TORNADO2* (VIT_02s0012g01410) for the establishment of sporocyte fate, *MULTIPLE SPOROCYTE* (VIT_05s0062g01100) for the inhibition of sporocyte formation, AHK, AHP, ARR components of cytokin signaling for functional megaspore specification, *ARGONAUTE9* (VIT_06s0009g01200), *RNA-DEPENDENT RNA POLYMERASE6* (VIT_04s0008g05430) and *SUPPRESSOR OF GENE SILENCING3* (VIT_07S0130G00190) for the repression of megaspore formation. Mutants for several of these genes

produce unreduced female gametophytes and exhibit apomixis-like phenotypes of the aposporous or diplosporic type [184, 187].

Additional genes have a potential involvement in the formation of Corinto Nero unreduced spores and gametes [157, 158, 188]. For example, *SWITCH1/DYAD* (VIT_00s0199g00200) codes for a protein that is required for early meiotic events. In particular, it plays an essential role in sister chromatid cohesion and recombination in profase I. Mutations in the *SWITCH1/DYAD* gene may result in defects only in the female meiosis or in both meiosis [189 and references herehin], which is in line with our findings. Moreover, [190] reported that the *dyad* unreduced female gametes fully retain parental heterozygosity (apomeiosis, which is the same mechanism proposed for the formation of Corinto Nero diplogametes in the present study). SWITCH1/DYAD has been shown to interact synergistically with MMD1/DUET (VIT_14s0006g00090) to maintain normal meiotic cell cycle progression [191]. In the Arabidopsis *duet* mutant, aberrant male meiosis leads to bi- and tri-nuclear microspores that eventually abort. Other factors with a putative role in chromosome organization during the first meiotic prophase are coded by *Arabidopsis-mei2-Like* genes (VIT_10s0003g02670, VIT_12s0142g00100, VIT_17s0000g00240), *ASYNAPTIC1* (VIT_01s0010g03590), *DMC1* (VIT_05s0020g04170), *SOLO DANCER* (VIT_01s0011g06040) and *SPO11* (VIT_19s0015g00280) [157, 192, 193]. In particular, the Arabidopsis *dmc1* mutant shows strongly reduced fertility (1.5% that of wild-type plants) due to the production of little viable irregularly shaped pollen and to the lack of a functional embryo sac in most ovules. These defects can be attributed to random chromosome segregation during both male and female meiosis, which in turn results from impaired homologous centromere pairing and bivalent stabilization. Interstingly, *DMC1* has been suggested to affect diplospory [194].

OSD1 (*OMISSION OF SECOND DIVISION1*, VIT_08s0007g07790) and *CYCA1;2/TAM* (*TARDY ASYNCHRONOUS MEIOSIS*, VIT_18s0001g02060) are involved in both meiotic transitions in Arabidopsis. Single mutants for these genes generate restituted dyads (100% in male and from 30 to 85% in female, respectively) that contain 2n gametes [161, 195]. OSD1 and CYCA1;2/TAM form

with THREE-DIVISION MUTANT (VIT_10s0003g05230) a functional network that regulates meiotic cell cycle progression [196]. OSD1 interacts also with CELL DIVISION CYCLE 20 (VIT_15s0107g00320), which has a critical role in meiotic spindle assembly and chromosome segregation [197]. Other proteins regulating spindle organization, in meiosis II, are AFH14 (VIT_02s0033g01360), JASON (VIT_12s0028g00940) and AtPS1 (VIT_18s0072g00830); their lack of function causes the formation of FDR diploid pollen [198].

Tapetum differentiation/resorption and gametogenesis: A number of genes in this category play a role in pollen development. For example, *QUARTET1* (VIT_19s0015g00150) and *QUARTET3* (VIT_01s0011g01300) are necessary for pectin degradation to separate microspores from the tetrad [199]. *SERK1/SERK2* (VIT_18s0164g00070) are required for the formation of tapetum, while *DYSFUNCTIONAL TAPETUM1* (VIT_15s0046g00320), *MYB33/MYB65* (VIT_13s0067g01630) and *MYB35* (VIT_14s0066g02180) are involved in tapetum differentiation and early function, whose impairment leads to an arrest of pollen development or compromises pollen performance. *ABORTED MICROSPORES, MYB80* and *MALE STERILITY 1* are essential for late stage functions of the tapetum. In particular, *ABORTED MICROSPORES* (VIT_01s0127g00860 and VIT_03s0038g02540), and *MYB80* (VIT_19s0015g01280), together with other bHLH (e.g. *bHLH010,* VIT_15s0107g00380) and MYB family members, activate the expression of sporopollenin synthesis genes for pollen wall formation. These genes include *ABCG26* (VIT_19s0015g00960), *ACOS5* (VIT_01s0010g03720), *CYP703A2* (VIT_15s0046g00330), *CYP704B1* (VIT_01s0026g02700), *LESS ADHESIVE POLLEN 5* and *6* (VIT_03s0038g01460 and VIT_15s0021g02170), *MALE STERILITY 1* and *2* (VIT_01s0011g06390 and VIT_08s0007g07100), *Nodulin MtN3* (VIT_17s0000g08110), *TKPR1* (VIT_03s0038g04220) and *TKPR2* (VIT_01s0011g03480) [200-203]. They all show a higher expression in Sangiovese than in Corinto Nero at stages E-L 15 and E-L 27. Candidate genes for embryo sac development include genes acting during mitosis following meiosis, e.g. *PROLIFERA* (VIT_07s0005g01430), genes involved in the regulation of female gametophyte polarity, e.g. the auxin carriers *AUX1* (VIT_08s0007g02030) and

PIN1 (VIT_17s0000g02420), the auxin response factors *ARF2* (VIT_17s0000g00320) and *ARF5* (VIT_11s0065g00310), genes controlling nuclear proliferation during megagametogenesis, e.g. *CHR11* (VIT_05s0020g01780), *INDETERMINATE GAMETOPHYTE1* (VIT_00s0340g00090) and *RBR1* (VIT_04s0008g02780), as well as genes that play a role in cell specification and differentiation during megagametogenesis, e.g. *BLH1* (VIT_08s0105g00230), *CMT3* (VIT_06s0004g01080), *GAMETOPHYTIC FACTOR1* (VIT_11s0118g00720) and *WYR* (VIT_19s0015g00610) [184]. Additional transcription factors mediating female gametogenesis are *KNAT3* (VIT_04s0008g06130), *REPRODUCTIVE MERISTEM34* (VIT_03s0063g00620) and *VERDANDI* (VIT_03s0063g01440), while further candidate genes are involved in ribosome biogenesis, protein degradation, and signal transduction [204-208]. Several of the above genes affect also pollen development.

Fertilization: The candidate genes in this category are involved in pollen tube guidance, e.g. *MIK1* (VIT_07s0005g04390), in pollen tube block regulated by ethylene signaling, in polarized pollen tube growth (dependent for example on receptor-like kinases and RAC/ROP signaling), in pollen tube growth arrest, e.g. *FERONIA* and *SIRENE* (VIT_03s0038g04340 and VIT_08s0040g00010), in pollen tube discharge, e.g. *ACA9* (VIT_09s0018g02130), and in synergid cell death following pollen tube-synergid contact, e.g *GAMETOPHYTIC FACTOR2* and *SIRENE*. The above events are widely controlled by gametophytically expressed factors [184, 205, 209-211). Most of the genes implicated in pollen tube growth are more expressed in Sangiovese than in Corinto Nero between stages E-L 15 and E-L 27, in agreement with the inability of Corinto Nero pollen to germinate (Figure 8A-B).

Ovule/seed development: The majority of the genes in this category code for transcription factors. Some of them are involved in ovule development, e.g. *AINTEGUMENTA* (VIT_09s0002g01370 and VIT_18s0001g08610), *APETALA2* (VIT_07s0031g00220), *APETALA3* (VIT_18s0001g13460), *BEL1* (VIT_02s0025g00200), *CUP-SHAPED COTYLEDONS 1* and *2*, *SEEDSTICK (VvAGL11,* VIT_18s0041g01880), *SEPALLATA3* (VIT_01s0010g03900), *SHATTERPROOF* (VIT_12s0142g00360), *SHOOT MERISTEMLESS* (VIT_10s0116g00190), *SHORT INTEGUMENTS1* (VIT_15s0048g02380) and *TRANSPARENT TESTA16* (VIT_01s0011g01560 and

VIT_10s0042g00820) [212-217]. Other genes play a role in embryo and endosperm development, which are controlled by both sporophytically expressed, i.e. *SHRUNKEN ENDOSPERM* (VIT_18s0001g12840) and *SEEDSTICK* (VIT_18s0041g01880), and gametophytically expressed factors, i.e. *CONSTITUTIVE TRIPLE RESPONSE1* (VIT_08s0007g03910), *FERTILIZATION_INDEPENDENT ENDOSPERM* (VIT_19s0014g05210), *MEDEA* (VIT_07s0005g01490), *PROLIFERA* (VIT_07s0005g01430) and *RBR1* (VIT_04s0008g02780) [204, 205]. In particular, *FERTILIZATION_INDEPENDENT ENDOSPERM* and *MEDEA* encode factors that inhibit endosperm development before fertilization and are well-known targets of genomic imprinting in the female gametophyte. For this reason, Additional file 1: Table S14 includes also genes responsible for the achievement of parent-of-origin-specific expression [184, 218].

Conclusions

The present study shows that genetic diversity preserved in grape germplasm collections may be crucial for investigating the regulation of target traits. Here, independent seedless variants were characterized at the molecular and phenotypic level. Multi-year observations on seed and fruit set deriving from different pollination treatments allowed us to attribute a biological mechanism to each genotype, revealing that stenospermocarpy and parthenocarpy are not restricted to Sultanina and Corinth cultivars, respectively. The missense substitution in *VvAGL11* that is responsible for seed abortion in Sultanina-derived seedless varieties was not detected in the seedless variants evaluated in this work, with the only exception of an apparently independent Gouais Blanc mutant. For the Corinto Nero (Sangiovese seedless variant) case study, specific defects were identified in micro- and macro-gametophytes, which act in concert to promote parthenocarpy. Moreover, evidence was found in support of the intrinsic predisposition of Sangiovese and Corinto Nero to set fruit even in the absence of fertilization. Based on transcriptomic data, some hypotheses were developed on genetic functions that might be altered in Corinto Nero.

Methods

Plant material

Seven seeded *Vitis vinifera* varieties and their corresponding seedless somatic variants were selected for genetic and phenotypic characterization (Table 1). The plants are currently grown in two grape germplasm collections in northern Italy: the Grinzane Cavour collection (http://www.ipsp.cnr.it/grape-collection/?lang=en), which is located in the province of Cuneo and is maintained by CNR-IPSP (National Research Council of Italy-Institute for Sustainable Plant Protection, Torino); the FEM collection (https://www.fmach.it/eng/Farm/Crops/Corporate-bodies/Giaroni-San-Dona), which is situated in the province of Trento (experimental field "Giaroni" in San Michele all'Adige) and is managed by FEM (Fondazione Edmund Mach). The most investigated accessions in this study, Sangiovese and Corinto Nero, have been propagated and planted in two additional locations that are an experimental field in Grugliasco (Torino) and another in San Michele all'Adige. In all the vineyards, vines are grown in vertical trellis and Guyot pruned.

Corinto Nero was initially identified as a seedless somatic variant of Sangiovese collected in the region of Calabria (southern Italy, precisely in Scalea, Cosenza province) and introduced in the Librandi winery collection, as described by [91]. Another Sangiovese seedless mutant was found in Emilia Romagna (Sesso, Reggio Emilia), under the name of Termarina Nera. The main reference seeded Sangiovese was the clone R24.

The seedless variant of Moscato Bianco was discovered in a Moscato Bianco commercial vineyard in the region of Piemonte (precisely Alba, Cuneo). Aspirant-false, the seedless variant of Gouais Blanc/Heunisch Weiss, was kindly provided by the JKI Geilweilerhof, Germany.

Termarone and its seedless variant Termarina Rosa were identified by microsatellite analysis and introduced in collection from the Italian region of Emilia Romagna.

The somatic variants of the cultivars grown in the FEM collection were found by investigating the seed phenotype (number and type of seeds from 25 randomly sampled berries in 2011 and 2012) within groups of accessions with identical profile at 22 microsatellite loci and a name possibly

referring to seedlessness [219]. Three pairs of somatic variants were at that time identified: Chasselas Blanc and Chasselas apyrène, Dastatchine-false (Sultanine Monococco) and Sultanina, Pedro Ximenez and Corinto Bianco. A fourth pair was discovered that included a Sori-false accession and Corinthe Noir (the Greek Korinthiaki). However, Sori-false was then excluded from phenotypic characterization as the putative seeded form of Corinthe Noir because in the following seasons, when all bunches were examined, most of the berries were small and seedless. Both accessions proved to be subject to reiterative berry shriveling. When phenotypic data could be collected, Corinthe Noir was kept as a reference for parthenocarpy.

Additional cultivars, clones or accessions of the above cited and of other varieties were analyzed for specific goals, as detailed along the manuscript. They include: Sangiovese clones R10 and VCR 4; four accessions of Corinto Nero from Sicily (Aeolian Islands); Chasselas Rose, a seeded berry color variant of Chasselas Blanc; Sultanina Rosa, a berry color variant of seedless Sultanina; Iordan, a Gouais/Liseiret offspring and its variant Iordan seedless; lastly, Gamay, Grenache, Nebbiolo and Trebbiano Toscano for pollination treatments.

For simplicity, we often drop the term “false” for accessions wrongly labelled.

Genotyping variant pairs

For SSR and SNP genotyping, young leaves were gathered from all the accessions reported in Table 1. Total genomic DNA was extracted according to [219].

Microsatellites

Sangiovese and Corinto Nero had been previously genotyped with 58 SSRs spread across the nineteen chromosomes of the grapevine genome [84]. The remaining accessions were genotyped for 32 out of these 58 markers (Additional file 1: Table S1). PCR amplification, amplicon separation and allele size estimation were performed as described by [219]. Primers failing to amplify at 54 °C were further tested in single panel at different annealing temperatures.

SNPs

Each accession was genotyped with the commercial GrapeReSeq_Illumina_20K_SNP_chip [220, 221] containing 18071 SNPs as described in [110]. For polymorphism detection, an in house Perl script was used to carry out pairwise comparison of the filtered genotype positions for each pair of seeded and seedless accessions reported in Table 1. To this purpose, only markers successfully detected in both lines of each pair were considered.

To validate potential polymorphisms between somatic variants, PCR amplification and Sanger sequencing were performed in the same panel of clones used for the GrapeReSeq_Illumina_20K_SNP_chip hybridization. When there were several potential SNPs, a subset was selected for validation based on variant effect prediction (with the SNPeff v3.6c program by [222]) and on functional gene annotation. Primers were designed according to the 12X.2 version of the reference genome sequence using Primer3Plus [223]. The amplification was carried out in 25 µl reactions with 20-30 ng of DNA, 1x PCR buffer, 2 mM magnesium chloride, 20 µM dNTPs, 0.5 µM each primer, and 1 U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA). The reaction was then cycled with the following conditions: initial denaturation at 95 °C for 10 min; 11 cycles of touch-down PCR [95 °C for 45 sec, 55 °C for 45 sec (- 0.5 °C every cycle), 72 °C for 1min 30sec] followed by 25 cycles of 95 °C for 45 sec, 50 °C for 45 sec, 72 °C for 1min 30sec; final extension at 72 °C for 10 min. PCR products were purified using Eurosap PCR Enzymatic clean-up kit (Euroclone S.p.A, Pero MI, Italy) following the manufacturer's instructions and then sequenced by capillary electrophoresis using the same primers as in PCR. Chromatograms were aligned with MEGA6 software [224] and visually inspected with BioEdit v7.2.0 [225].

Phenotyping variant pairs

The accessions reported in Table 1 were phenotyped for flower and fruit traits upon open-pollination in one or more seasons. Developmental stages were established according to the modified Eichhorn-Lorenz scheme [226].

Flower number and fruit set rate

Fruit set rate was evaluated as the ratio of berries over flowers per bunch, which is the only valid method recognized by [116]. The number of flowers per inflorescence was estimated at stage E-L 17 (12 leaves separated; inflorescence well developed; single flowers separated) by using VitisFlower mobile application according to the developers' specifications [227, 228]. For most accessions, five to ten inflorescences were initially chosen from different plants and different positions within the plant, in order to minimize potential effects of branching level and inflorescence position along the shoot onto flower number [17, 229]. Three photographs per inflorescence were taken (from different angles) and a mean value was calculated. The number of berries set per bunch was manually counted at harvest (E-L 38) in the lab. For the accessions of the FEM collection, berries were also manually counted in the field at stage E-L 31 (berries pea-size) by marking each berry with a permanent pen. Live green ovaries were not included in the counts, as they do not fit the definition of berry [230].

Bunch, berry and seed features

Bunch, berry and seed traits were evaluated on clusters collected at technological maturity (stage E-L 38) in one or more seasons.

Bunch features included the following OIV descriptors: length (OIV202), width (OIV203), mean cluster density (OIV204), as well as bunch weight, length/width ratio and berry number. Clusters were weighted with a precision balance. Bunch length and width were measured with a ruler. The number of berries per bunch was manually counted.

Berry traits included berry size, mean berry weight and percentage of seeded berries. Berries of each bunch were classified into three size categories: A or large (berry width > 15 mm), B or medium (12 mm ≤ berry width ≤ 15 mm) and C or small (berry width < 12 mm). Berry width (OIV221) was measured with a digital caliper applied to pictures (at IPSP in 2017 and 2018) or with an *ad-hoc* aluminum sizer card from 9 to 20 mm in 1 mm steps (at FEM in 2018). At IPSP, berry length (OIV 220) was additionally surveyed. Pools of berries of the same size class were weighted with a precision balance and an average berry weight was obtained for each cluster. The percentage of seeded berries

per cluster was calculated after opening the berries with a blade and visually inspecting the presence of normally developed seeds.

Seed traits included mean seed number per seeded berry and mean seed weight. Normally developed seeds were extracted from berries of the same size category and manually counted. Fresh seed weight was measured with a precision balance after seed cleaning and drying at room temperature. An average count and weight were obtained for each cluster.

Inspection of seeds and traces at veraison

In 2019 a pool of berries from different parts of different bunches was randomly collected at veraison for each genotype, except for Chasselas Rose, Pedro Ximenez and Corinto Bianco. Within every pool, it was observed if the berries were homogenous in size or belonged to two different size categories instead. Ten berries per size category (small and large, when available) per genotype were randomly chosen for inspection at the stereomicroscope (Stemi 2000-CS, ZEISS). Traces and well-developed seeds per berry were separately counted and successively dissected for observation of their structures. Annotations were made about their appearance and consistency. The potential vitality of the well-developed seeds was tested by a floatation test in water: the sinking seeds were considered as likely viable. A digital camera (AxioCam ERc 5s, ZEISS) was attached to the stereomicroscope and simultaneously connected to a computer. AxioVision Rel. 4.8 software (ZEISS) was used to observe the samples in "live" mode and to get digital images of the dissected traces and seeds. The size range of the analyzed berries, as well as the length and width of traces and seeds, were digitally measured from the pictures.

In addition, for Sangiovese and its seedless variant Corinto Nero, pistils from different inflorescences or from a single inflorescence with flowers at different phenological stages were collected on the same day (19/06/2019). Afterwards, four intermediate stages between flowering (stage 1) and berries pepper-corn size (stage 6) were sampled. One pistil per stage was selected for each genotype for

successive dissection, extraction and examination at the stereomicroscope of the ovules/traces. Their length and width were measured using the software cited above.

Statistical analysis of phenotypic data

Statistical tests were performed using the software PAST v3.14 [231]. The normality of phenotypic data was tested with the Shapiro-Wilk test [232] by considering the whole set of accessions or the distinct groups of seeded and seedless accessions.

Both parametric (T-student and Welch in case of unequal variance) and non-parametric (Mann-Whitney and Kolmogorov-Smirnov) tests were performed to detect significant differences between somatic variants or stages for berry count. Significant differences among different genotypes were additionally tested by using the Kruskal-Wallis test (with the Dunn's post-hoc test and Bonferroni adjustment). A significance level of $P < 0.05$ was set in all cases.

Pairwise correlations between traits were assessed with the Spearman's rs test and considered for significance at the 0.05 level.

Investigation of the mechanisms possibly responsible for the seedless phenotype

Evaluation of sanitary status

In 2011 and 2012, woody material from vines was tested for the presence of the most harmful and spread grapevine viruses by applying ELISA (enzyme-linked immunosorbent assay) test and PCR as described in [233, 234].

Evaluation of male gamete (pollen) functionality

Pollen viability and germination

Pollen viability and germination were tested *in vitro* on Sangiovese/Corinto Nero and the three variant pairs Chasselas/Chasselas apyrène, Dastatchine/Sultanina, Pedro Ximenez/Corinto Bianco, as well as on Corinthe Noir cv. Inflorescences were collected at stage E-L 23 (50% caps off) of the modified Eichhorn-Lorenz scheme [226]. No selection was done for the inflorescence and shoot position, as

pollen viability has been shown to be highly uniform within the same genotype [127]. Pollen viability and germination were analyzed over three seasons (2014, 2017 and 2018). For each accession, a pooled sample composed of inflorescences from different plants was tested.

Viability: The pollen viability of freshly harvested inflorescences was determined using the 1% TTC (2,3,5-triphenyl tetrazolium chloride) test [235]. TTC, normally colorless, in the presence of dehydrogenases (viable pollen) turns into insoluble formazan and appears red. For each sample, three or four technical replicates were performed, spreading pollen grains on different glass slides. After incubation in the dark at 37 °C for one hour, pollen viability was evaluated under a microscope (Leitz Diaplan): pollen grains were considered viable if they turned red, non-viable if yellowish or unstained. Viable and non-viable pollen grains were counted in random samples of about 300 grains per slide.

Germination: In order to ensure pollen shedding from anther sacs and separation from other flower parts, inflorescences were sieved. Spontaneously released pollen grains were collected in a Petri dish and a germination medium (20% sucrose, 100 mg/L boric acid, 300 mg/L calcium nitrate) was added [236]. After 24 h of incubation at 25 °C [237], three slides were prepared for each sample and examined under a microscope (Leitz Diaplan) using the continuous sweep method and random sweep selection. The pollen grains were considered germinated when the length of the pollen tube was at least the double of the granule diameter. At least one-hundred pollen grains per slide were observed. Both parametric (T-student and Welch in case of unequal variance) and non-parametric (Mann-Whitney and Kolmogorov-Smirnov) tests were performed to detect significant differences between somatic variants.

Pollination treatments

The following pollination treatments were performed

A) Self- vs open-pollination: Fruit set rate, bunch, berry and seed traits were evaluated in self- and open-pollination conditions (SP and OP, respectively) in most seeded/seedless pairs as described

in section “Phenotyping variant pairs”. The only exceptions were Termarina Rosa, Dastatchine and Corinto Bianco due to too few or dried inflorescences in 2018. For the self-pollination group, inflorescences were enclosed within paper bags before anthesis to avoid cross-pollination and were allowed to bloom and self-pollinate. One week after berry set, the covered clusters were exposed to full sun throughout fruit development and maturation (the same holds for B and C).

B) Pollination of Nebbiolo/Trebbiano Toscano with Corinto Nero pollen: Pre-capfall inflorescences of Nebbiolo and Trebbiano Toscano (cv. early and late flowering respectively, both fully fertile and seeded) were manually decapped, emasculated using pliers with fine tips, hand-pollinated with Corinto Nero pollen, and covered with paper bags. The experiment was repeated in different seasons (2012-2014) and locations (IPSP and FEM). Self-pollinated clusters (inflorescences enclosed in bags before calyptra shed) represented positive controls. At harvest time, the number of bunches and berries was counted, berry size and seed content were visually inspected.

C) Emasculation of some pairs and additional varieties: Pre-capfall inflorescences of Sangiovese/Corinto Nero, Gouais Blanc, Chasselas/Chasselas apyrène, Pedro Ximenez/Corinto Bianco and additional genotypes (Nebbiolo, Trebbiano Toscano, Gamay, and Grenache) were manually decapped, emasculated using forceps with fine tips and covered with paper bags. The aim was to check the eventual berry set and development excluding any pollen role. This experiment was repeated in different seasons, locations and at different developmental stages. The earliest stage (stage I) corresponded to stage E-L 15, the latest one (stage II) to stage E-L 18. In some trials stigma removal was additionally performed. Undecapped self-pollinated (covered) inflorescences were used as control. At harvest, bunch- (length, weight), berry- (size, weight) and seed- (number, weight) traits were evaluated. Occasional normal seeds formed upon emasculation were placed in pots for germination. Derived seedlings were genotyped at 18 microsatellite loci to clarify their origin.

Evaluation of female gamete (embryo sac) functionality

In 2013, four inflorescences of Corinto Nero were emasculated and cross-pollinated with viable pollen of Nebbiolo with the procedure described above. Bunch, berry and seed traits were evaluated at harvest.

Exploration of potential causes of gamete non-functionality: defects in sporogenesis

In 2016, Corinto Nero and Sangiovese seeded berries, obtained upon open pollination conditions, were collected. Seeds were extracted from berries and stored at 4 °C for 2 months in order to overcome dormancy. Seed germinability was then evaluated for both accessions. *In vitro* embryo rescue was performed according to the protocol described by [27]. Young leaves were sampled from the obtained seedlings and they were divided into two batches. The first batch was used for genotyping at ten unlinked microsatellite loci (fifteen in some dubious cases). Leaves from the second batch were sent to Plant Cytometry (https://plantcytometry.com/) for ploidy level determination by flow cytometry. The ploidy level of each plant was recorded as an index relative to plants of the same species with a known ploidy level (2C), that are Corinto Nero, Sangiovese and Cabernet Sauvignon (leaves were collected from woody cuttings kept in pots with water).

In parallel, pollen grain morphology was recorded in Sangiovese/Corinto Nero (in three seasons) and in other three variant pairs (in one or two seasons) to verify possible different size of pollen grains linked to different ploidy level. Polar and equatorial axes of 50 randomly taken pollen grains were measured for each genotype in each season by examination at light microscope using an ocular micrometer.

Investigation of the genetic basis of the seedless phenotype

Candidate genes for the seedless phenotype were identified/analyzed in one or more variant pairs:

VvAGL11

All the accessions under study were genotyped with the CAPS-26.88 marker by using the primers reported in [41] for both PCR amplification and Sanger sequencing.

Based on [41) and [104], the expression of *VvAGL11* was analyzed by reverse transcription quantitative PCR (RT-qPCR) in Sultanina/Dastatchine and Aspirant/Liseiret whole berries collected at stages E-L31 (pea-size berries, only Sultanina/Dastatchine) and E-L33 (berries still hard and green), according to the procedure described by [238]. Primer sequences were taken from [41]. Three biological- and two technical replicates were analyzed for each genotype and stage. Relative transcript levels were calculated after normalization to the grapevine actin and glyceraldehyde-3-phosphate dehydrogenase genes using the ΔΔCt method. Significant differences in *VvAGL11* expression were tested between somatic variants at the same stage by using T-student test.

Genes with validated SNPs between Sangiovese and Corinto Nero

A preliminary search for single nucleotide polymorphisms (SNPs) between Sangiovese (clone R24) and Corinto Nero (from Calabria) was addressed by a two-step process. To this purpose, we took advantage of the RNA-Seq alignments used by [84] for differential expression analysis in the pairwise comparison of developmental stages in the two lines (six libraries in total, which correspond to three stages and two genotypes). In the first step, polymorphisms were sought between Sangiovese and/or Corinto Nero and the 12X.0 version of the grapevine reference genome. Variants were called with Samtools v0.1.17 [239]. An initial filtering was done with VCFtools v4.1 [240] using a window of 10 bp, a minimum read depth of five and a minimum quality of 10. Then, to identify differential single nucleotide variants between Corinto Nero and Sangiovese with a potential impact on the seed phenotype, the following approach was adopted:

A) Through VCF filtering, it was required that the alternative base was supported by at least 3 reads and the frequency of the alternative alleles was ≥ 0.75 calculated on the total number of read pairs aligned on the region;

B) An *ad hoc* Perl script was written to take consensus positions that pass the filtering criteria in at least two libraries (that correspond to two developmental stages and can be considered as replicates) of Sangiovese and Corinto Nero, respectively;

C) Putative mutations from B were annotated on *Vitis vinifera* V1 gene predictions by using the Variant Effect Predictor SNPeff v3.6c program [222];

D) An *ad hoc* Perl script was used to carry out a pairwise comparison between Sangiovese and Corinto Nero for all putative SNPs annotated as non-synonymous;

E) Ninety-nine putative SNP positions that are different in the two clones from D were further chosen for validation. This set includes all the non-synonymous SNPs supported by three libraries and a selection (based on gene function) of non-synonymous SNPs supported by two libraries out of three (due to missing or incoherent genotype from one library).

To validate the selected SNPs, PCR amplification and Sanger sequencing were initially performed on genomic DNA from young leaves of the two clones and of Pinot Noir (as a reference) by following the approach described in the section "Genotyping variant pairs-SNPs". Primer sequences are available in Additional file 1: Table S13. Individual inferred genotypes from RNA-Seq were checked for concordance with Sanger method.

Validated variants were then tested on additional clones and accessions of Sangiovese/Corinto Nero. Chimerism was also investigated by comparing the Corinto Nero genetic make-up in genomic DNA extracted from leaf/berry skin (L1 + L2-derived tissues) and in genomic DNA isolated from berry flesh/adventitious roots (L2-derived tissues) [241].

Finally, validated variants between Sangiovese/Corinto Nero were analyzed in the other wild-type/variant pairs and in Corinthe Noir. By using the tool "Sanger data analysis" of Unipro UGENE v1.32 [242] with default settings for quality filtering, amplicons were aligned against *Vitis vinifera* V1 gene predictions containing each SNP.

Differentially expressed genes (DEGs) between Sangiovese and Corinto Nero

A set of candidate genes with a potential link to the Corinto Nero seedless phenotype were selected, according to their putative role from the literature and the findings of the present study, among the

differentially expressed genes between Sangiovese and Corinto Nero in the RNA-Seq experiment described by [84].

Supplementary information

Additional file 1: Table S1. Genotyping of variant pairs with SSR markers. **Table S2.** Genotyping of variant pairs with the GrapeReSeq_Illumina_20K_SNP_chip. **Table S3.** Statistical analysis of bunch, berry and seed traits upon open-pollination. **Table S4.** Bunch compactness according to the OIV 204 descriptor. **Table S5.** Spearman correlation between bunch compactness and other traits evaluated in this work. **Table S6.** Statistical analysis of length, width and length/width ratio of the apparently normal seeds extracted from berries at veraison. **Table S7.** Statistical analysis of length, width and length/width ratio of the ovule/seed traces extracted from berries at veraison. **Table S8.** Results of the tests for the presence of viruses. **Table S9.** Statistical analysis of bunch, berry and seed traits in open- and self-pollination conditions. **Table S10.** Microsatellite profile of Sangiovese, Gamay and Nebbiolo seedlings derived from self-pollination and emasculation with inflorescence bagging. **Table S11.** Corinto Nero offspring analyzed for ploidy level and microsatellite genotyping. **Table S12.** Genotyping of variant pairs and additional accessions with the marker CAPS-26.88. **Table S13.** Single nucleotide polymorphisms and insertions/deletions identified in the five amplicons containing the RNA-Seq SNPs that distinguish Sangiovese and Corinto Nero. **Table S14.** Candidate genes for the Corinto Nero phenotype that have altered expression between Sangiovese and Corinto Nero. (xlsx)

Additional file 2: Figure S1. VMC4F3.1 profile for three Chasselas clones. (pdf)

Additional file 3: Figure S2. Trait distribution under open-pollination (A) and self-pollination (B) conditions. **Figure S3.** Trait stability in multiple seasons and locations upon open-pollination. (pdf)

Additional file 4: Figure S4. Relationship between berry size and presence of normal seeds. (pdf)

Additional file 5: Figure S5. Percentage distribution of Sangiovese and Corinto Nero berries according to seed content in two pollination conditions. (pdf)

Additional file 6: Inspection of traces and seeds extracted from berries at veraison for the seedless accessions Aspirant (**Figure S6**), Chasselas apyrène (**Figure S7**), Corinto Nero (**Figure S8**), Termarina Rosa and Moscato Bianco mutant (**Figure S9**), Corinthe Noir and Sultanina (**Figure S10**), and for the seeded cultivars Liseiret, Moscato Bianco, Sangiovese and Termarone (**Figure S11**). (pdf)

Additional file 7: Figure S12. Sangiovese and Corinto Nero pistils at six phenological stages, with details of ovules/traces for which length and width were measured. (pdf)

Additional file 8: Figure S13. Comparison of fruit set rate, bunch, berry and seed traits after open- and self-pollination. **Figure S14.** Clusters of Sangiovese, Corinto Nero and Gamay derived from self-pollination and emasculation with inflorescence bagging. **Figure S15.** Clusters obtained from Corinto Nero inflorescences after emasculation and manual pollination with Nebbiolo pollen. (pdf)

Additional file 9: Figure S16. *VvAGL11* expression estimated by RT-qPCR in Sultanina/Dastatchine-false and Aspirant/Liseiret whole berries.

Declarations

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of seeds, Michela Bernini in lab assistance, and Pierluigi Magnago in the management of seedlings deriving from emasculated bunches.

Authors' contributions

AS and MSG conceived the project, AS, IG, LC, MSG and PMS designed the experiment. AS, CCN, IG, LC, PMS, SL and SR contributed to the pollination trials and the phenotypic characterization of the accessions, LC and PMS did the statistical analysis. IG evaluated the sanitary status of the plant materials. PMS carried out the microscopic observation of seeds and seed traces. EG and FC provided the data about pollen viability and germination. IG performed the embryo rescue. AM, AS, CCN, IG, LC, MSG, PMS, PR, SL and SR took part in genotyping the materials and interpreting the results. LC, PMS and SL analyzed *VvAGL11* expression. LC carried out the selection of candidate genes. CCN and PMS drafted some sections, while LC was the major contributor in writing the manuscript. AS, CCN, EG, FC, IG, MSG and PMS revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study that are not included in this published article (and its supplementary information files) are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1. Phenotyping of variant pairs upon open-pollination. Members of the same pair (or triplet) are depicted with the same color. For each accession, a mean value was calculated from different bunches, seasons and locations. Bars correspond to standard errors. Asterisks indicate significant ($P < 0.05$) differences between seeded and seedless variant pairs, as established by one or more test(s) among T-Student test (or Welch test in the case of unequal variances), Mann-Whitney test, and Kolmogorov-Smirnov test. Different letters indicate significant differences in the whole set of accessions (Kruskal-Wallis test followed by Dunn's post hoc test with Bonferroni correction for multiple tests, $P < 0.05$). Berries with apparently normal seeds were considered as seeded, whereas

berries containing only rudimental seeds, seed traces or unfertilized ovules were classified as seedless. Abbreviations: CN = Corinto Nero, TN = Termarina Nera, SG = Sangiovese, Asp = Aspirant-false, Lis = Liseiret, Mosc mt = Moscato Bianco mutant, Mosc wt = Moscato Bianco, Ter rosa = Termarina Rosa, Term = Termarone, Ch ap = Chasselas apyrène, Ch rose = Chasselas Rose, Sult = Sultanina, Dast = Dastatchine-false, CB = Corinto Bianco, PX = Pedro Ximenez, K = Corinthe Noir (reference for parthenocarpy).

Figure 2. Comparison between Moscato Bianco wild-type (A, C) and Moscato Bianco mutant (B, D) inflorescences and leaves. The inset in (B) shows a close-up of a «star» flower.

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Figure 6. Relationship between berry size and presence of normal seeds in Sangiovese and its seedless variants. (A) Classification of berries according to size; the prevalent type of seeds or seed traces is shown at the bottom. (B) Representative berries from Corinto Nero (on the left) and Sangiovese (on the right). (C) Percentage distribution of berries according to size and seed content. The percentage of small, medium and large berries was calculated from the total number of berries per bunch, while the percentage of seeded berries was established on the total number of berries opened for seed examination (it was a representative portion of the total number of berries when this number was too big). Berries with apparently normal seeds were considered as seeded, whereas berries containing only rudimental seeds, seed traces or unfertilized ovules were classified as seedless. For each combination of accession, season and pollination treatment, from one to nine clusters were analyzed and an average value was calculated. Abbreviations: CN = Corinto Nero, TN = Termarina Nera, SG = Sangiovese in the Grinzane Cavour collection (Corinto Nero plants from two distinct parcels were analyzed in 2017); CN*, SG* = Corinto Nero, Sangiovese in the FEM collection, respectively.

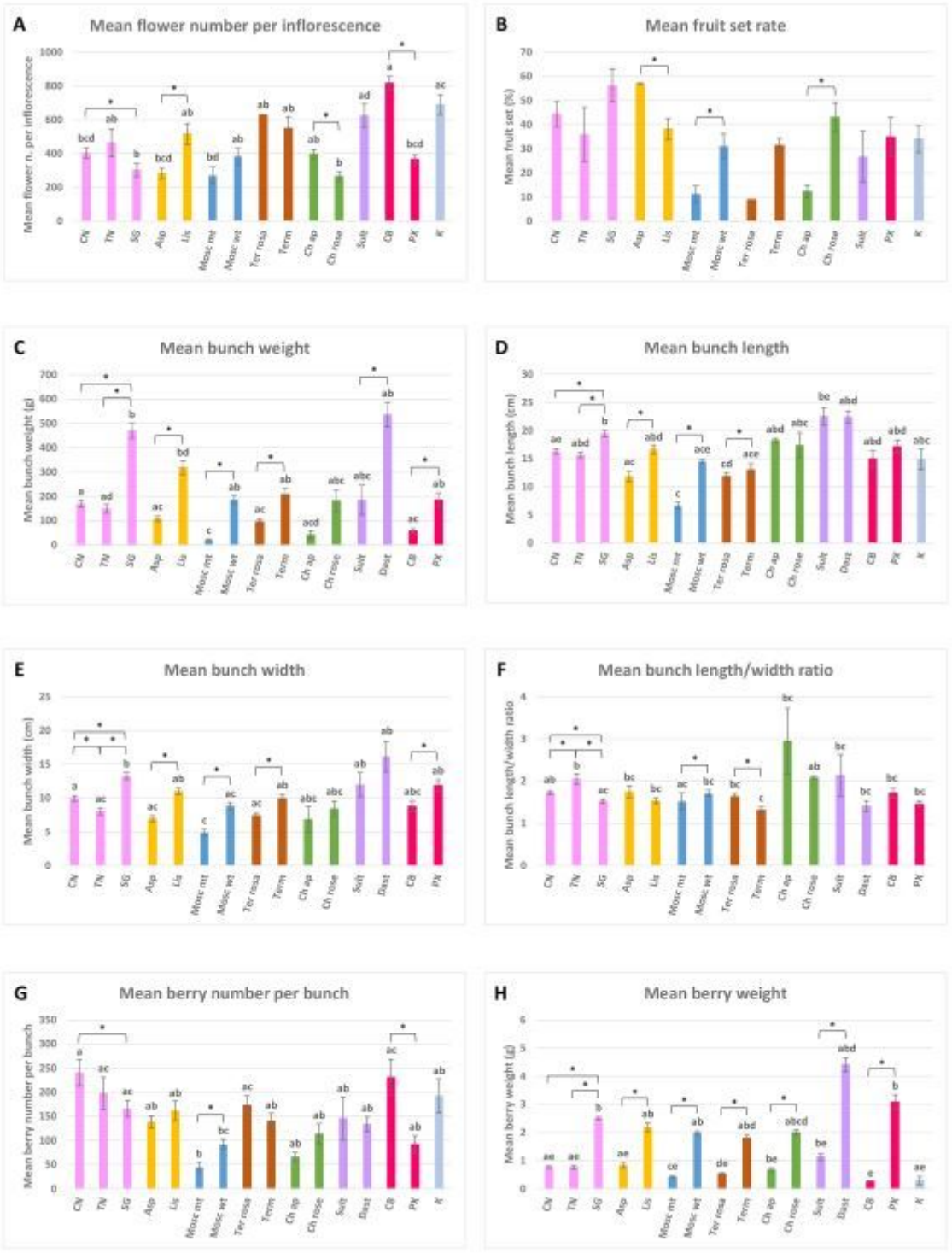
Figure 7. Scatter plots of traces' length against traces' width for the reference cultivars for parthenocarpy and stenospermocarpy, Corinthe Noir and Sultanina, respectively (A), and for the other

seedless accessions under investigation (B). Reported measures refer to traces extracted only from the smaller berries (with the exception of Sultanina having berries of a unique size). In (C) scatter plot of the length against the width of the ovules/traces of Sangiovese (SG) and Corinto Nero (CN) measured at six stages from flowering (stage 1) to pepper-corn size (stage 6), as detailed in Additional file 7: Figure S12. The intensity in the color filling the diamonds/dots increases with the stages. Ovules from stages 1 and 2 of Corinto Nero could not be measured because they were destroyed during extraction from the ovary due to their reduced size and fragility.

Figure 8. Evaluation of pollen functionality and morphology. (A) Pictures of some Sangiovese, Corinto Nero, Pedro Ximenez and Corinto Bianco pollen grains subjected to the viability (on the left) and germination (on the right) in vitro tests, as observed at the microscope (200X). (B) Mean values (± standard error) of pollen viability and germination percentage per accession; N is the number of replicates. The total number of observed pollen grains per accession ranged from a minimum of 1040 to a maximum of 4528, in relation to the available inflorescences. To detect differences between each seeded variety and its seedless variant, the non-parametric Kolmogorov-Smirnov test has been performed. (C) Box plots representing the polar and equatorial axis lengths measured on fifty randomly selected pollen grains for each genotype in each season.

Figure 9. Clusters (A), seeds and traces (B) derived from open-pollination (control), self-pollination (self) and emasculation. Abbreviations: CN = Corinto Nero, SG = Sangiovese, EMS+ST = emasculated (without stigma removal), SP = self-pollinated, stage I = stage E-L 15, stage II = stage E-L 18 of the modified Eichhorn-Lorenz scheme [226]. Red arrows indicate apparently normal seeds among several rudimental seeds. (C) Seedlings derived from occasional normal seeds extracted from emasculated bunches of Gamay (on the left) and Sangiovese (on the right). Pictures were taken 75 days after sowing.

Figures



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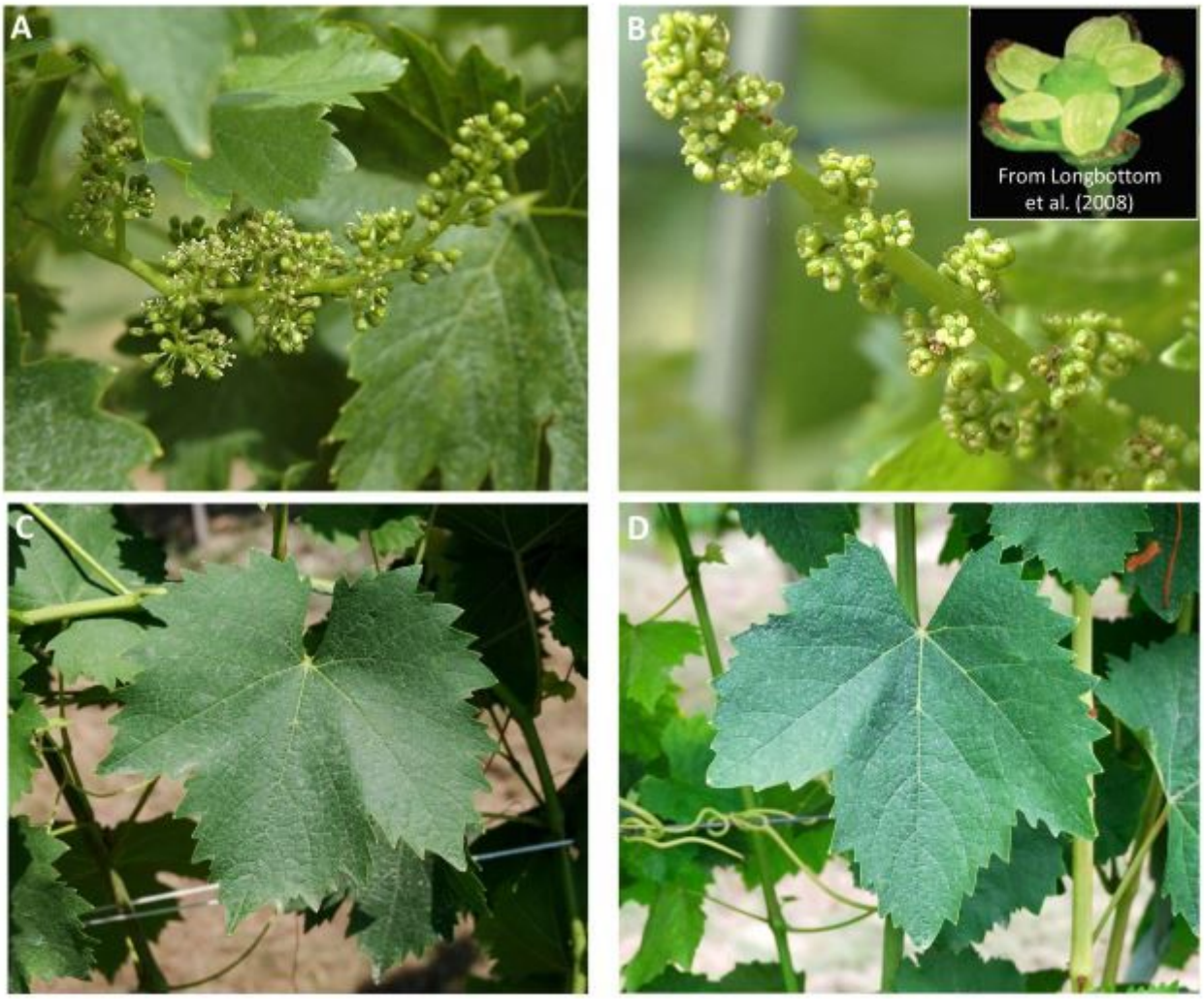

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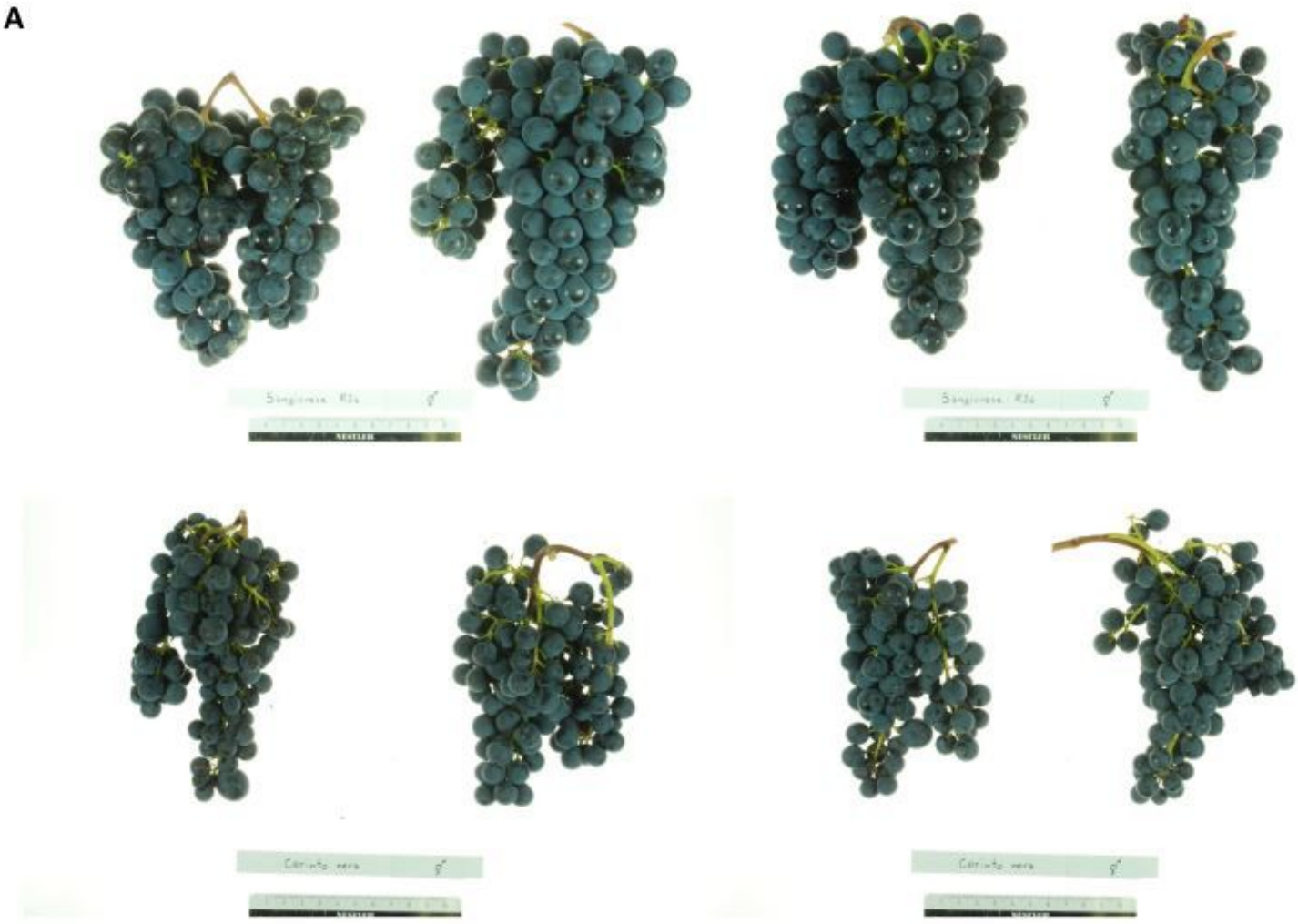

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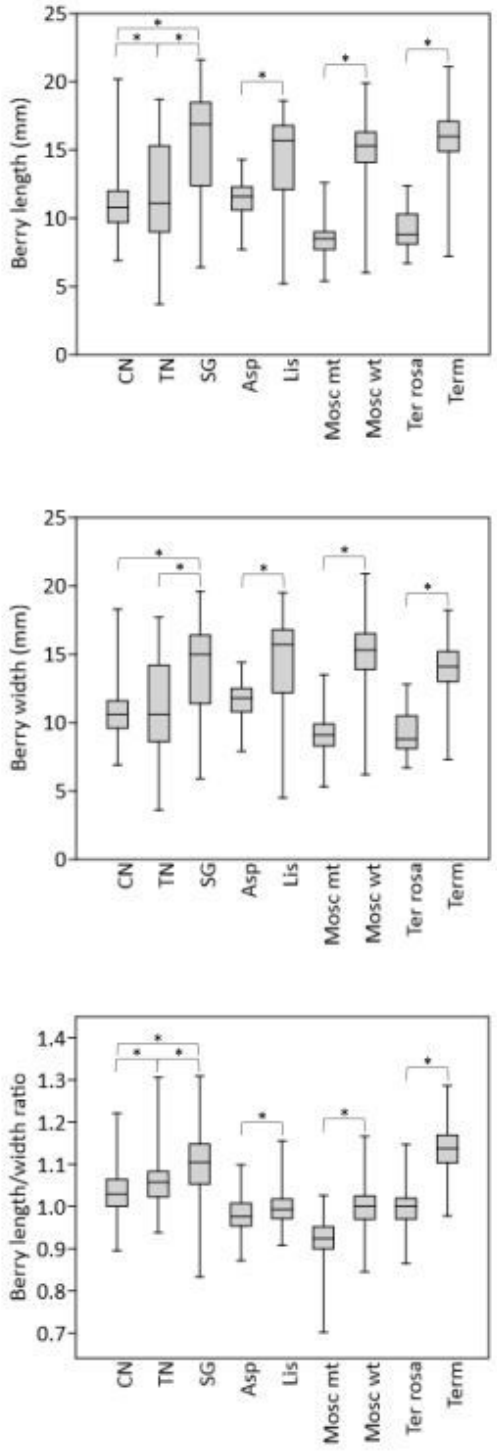

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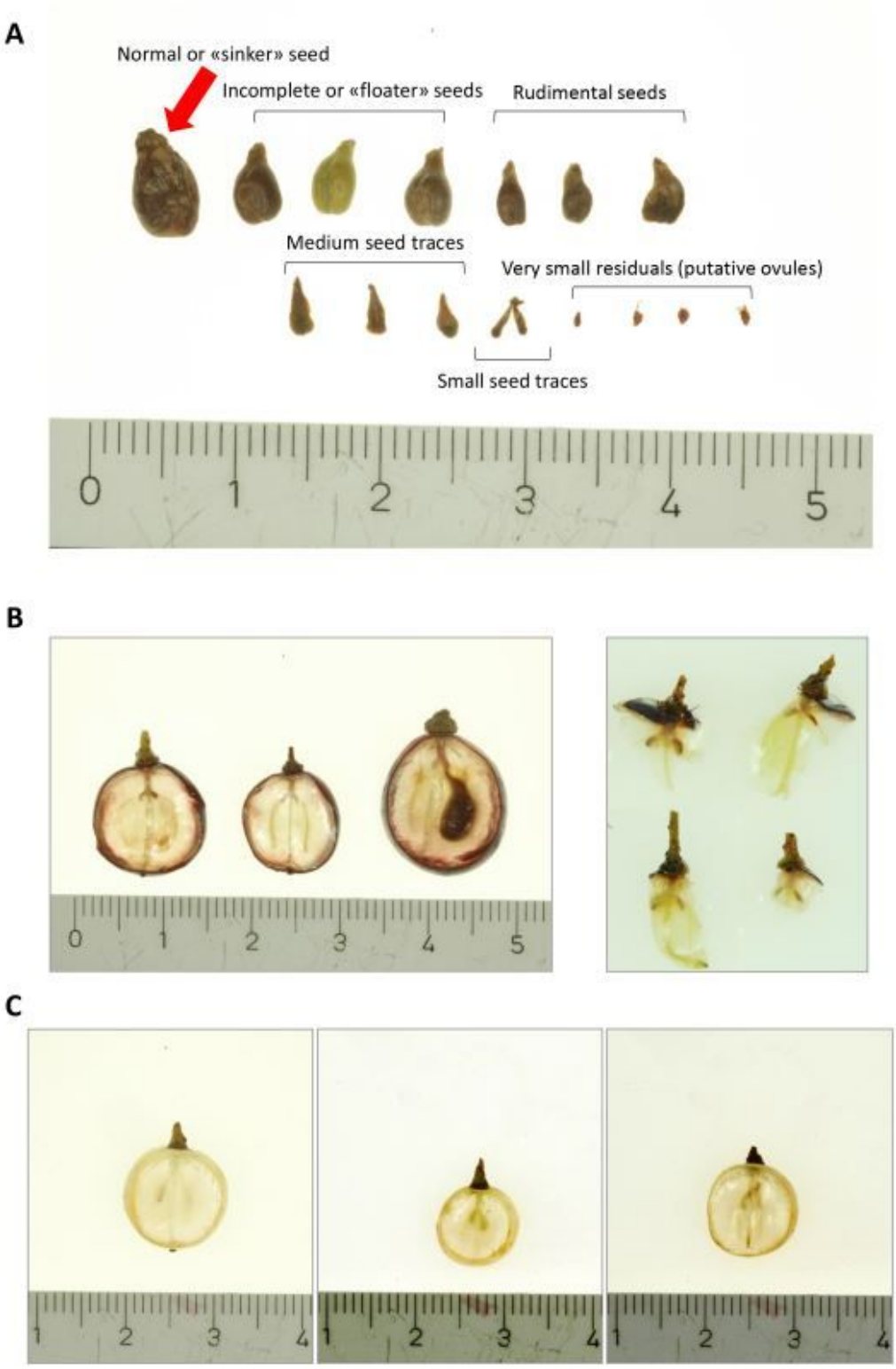

Figure 5

Seed evaluation. (A) Gradient of seed development observed in the accessions under study. Only normally developed seeds (as indicated by the arrow) were considered to estimate the percentage of seeded berries. They possess a normal testa (consisting of outer and inner integument), endosperm and embryo. The remaining structures are supposed to correspond to incomplete (“floater”) or rudimental seeds, seed traces and ovules. (B) On the left, sections of Corinto Nero berries (the rightmost berry contains a normal seed); on the right, some examples of traces extracted from the majority of Corinto Nero berries. (C) Sections of Aspirant-false berries. (D) Sections of “star-flower” Moscato Bianco berries. (E) A Chasselas apyrène berry. (F) A Sultanina berry. (G) Berries of Corinto Bianco. (H) A Corinthe Noir berry.

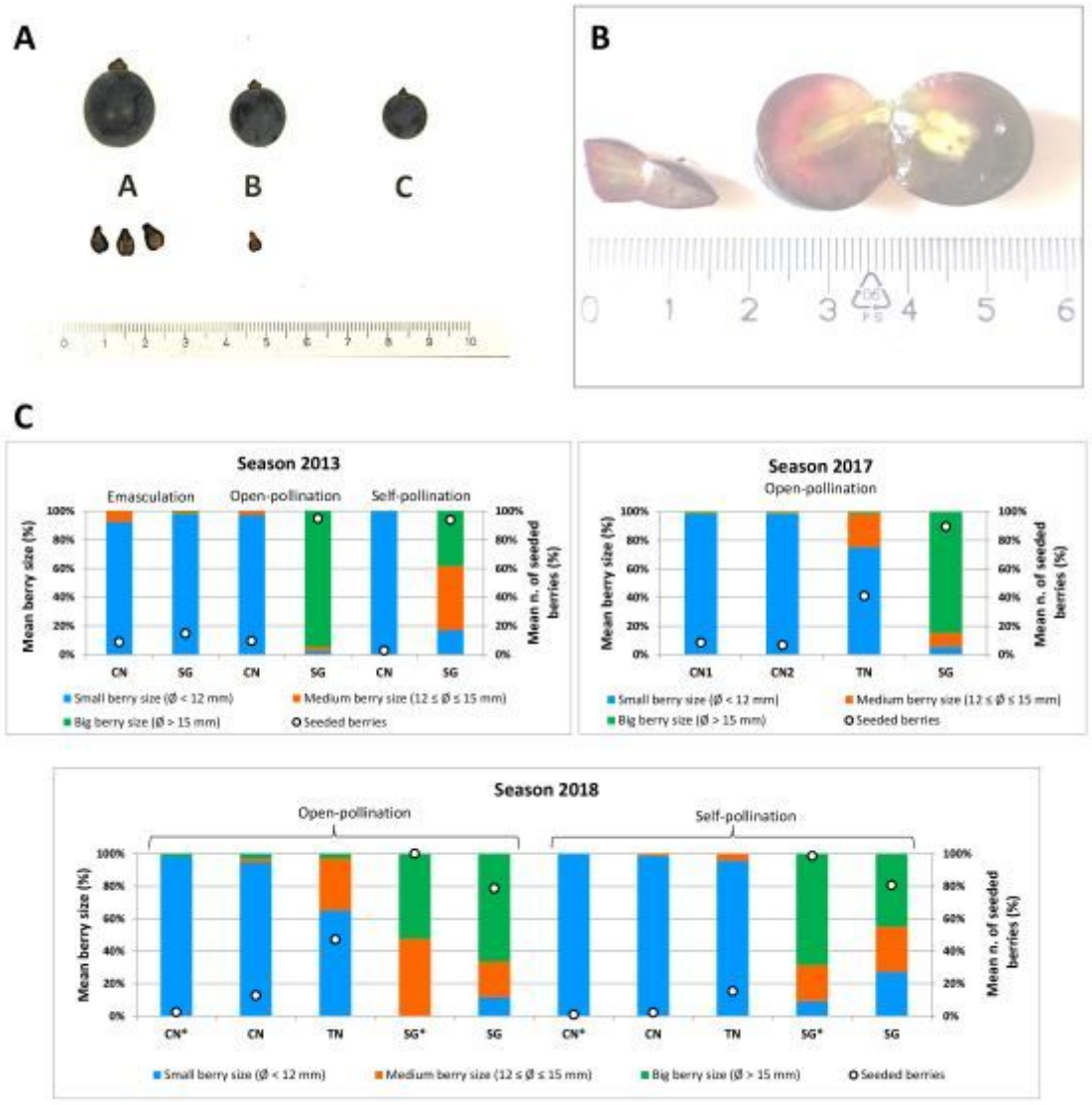

Figure 6

Relationship between berry size and presence of normal seeds in Sangiovese and its seedless variants. (A) Classification of berries according to size; the prevalent type of seeds or seed traces is shown at the bottom. (B) Representative berries from Corinto Nero (on the left) and Sangiovese (on the right). (C)

Percentage distribution of berries according to size and seed content. The percentage of small, medium and large berries was calculated from the total number of berries per bunch, while the percentage of seeded berries was established on the total number of berries opened for seed examination (it was a representative portion of the total number of berries when this number was too big). Berries with apparently normal seeds were considered as seeded, whereas berries containing only rudimental seeds, seed traces or unfertilized ovules were classified as seedless. For each combination of accession, season and pollination treatment, from one to nine clusters were analyzed and an average value was calculated. Abbreviations: CN = Corinto Nero, TN = Termarina Nera, SG = Sangiovese in the Grinzane Cavour collection (Corinto Nero plants from two distinct parcels were analyzed in 2017); CN*, SG* = Corinto Nero, Sangiovese in the FEM collection, respectively.

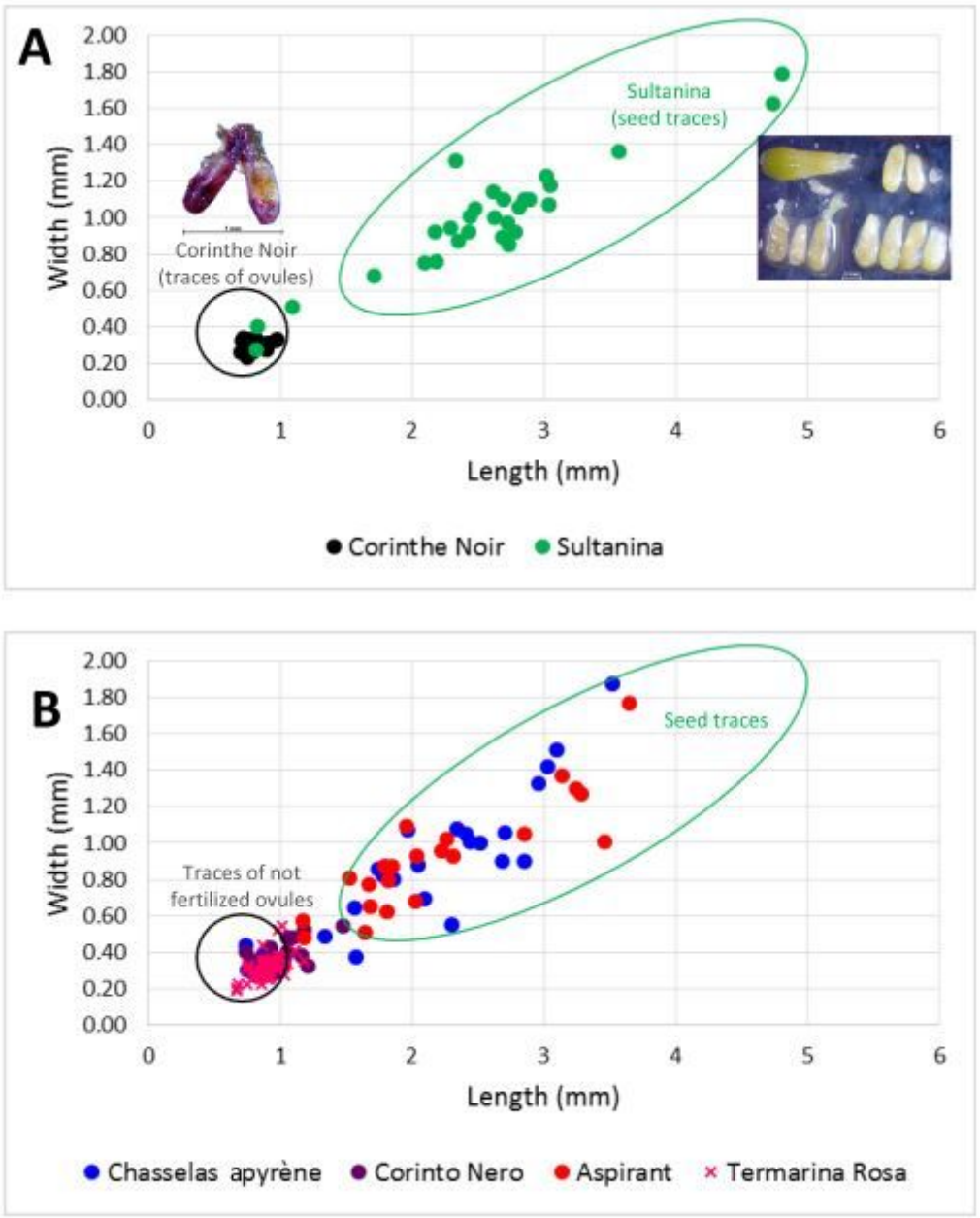

Figure 7

Scatter plots of traces' length against traces' width for the reference cultivars for parthenocarpy and stenospermocarpy, Corinthe Noir and Sultanina, respectively (A), and for the other seedless accessions under investigation (B). Reported measures refer to traces extracted only from the smaller berries (with the exception of Sultanina having berries of a unique size). In (C) scatter plot of the length against the width of the ovules/traces of Sangiovese (SG) and Corinto Nero (CN) measured at six stages from flowering (stage 1) to pepper-corn size (stage 6), as detailed in Additional file 7: Figure S12. The intensity in the color filling the diamonds/dots increases with the stages. Ovules from stages 1 and 2 of Corinto Nero could not be measured because they were destroyed during extraction from the ovary due to their reduced size and fragility.

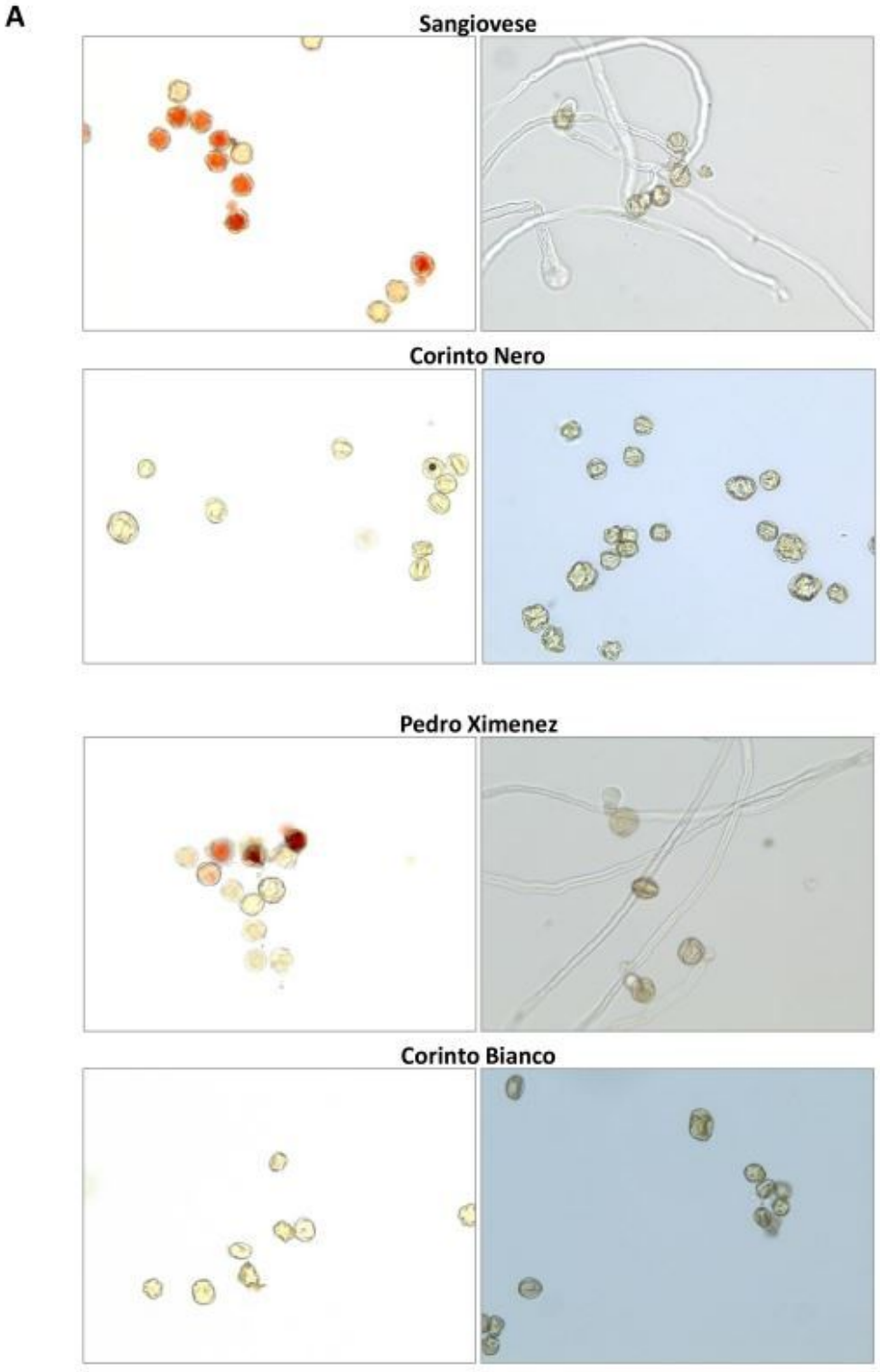

Figure 8

Evaluation of pollen functionality and morphology. (A) Pictures of some Sangiovese, Corinto Nero, Pedro Ximenez and Corinto Bianco pollen grains subjected to the viability (on the left) and germination (on the right) in vitro tests, as observed at the microscope (200X). (B) Mean values (± standard error) of pollen viability and germination percentage per accession; N is the number of replicates. The total number of observed pollen grains per accession ranged from a minimum of 1040 to a maximum of 4528, in relation

to the available inflorescences. To detect differences between each seeded variety and its seedless variant, the non-parametric Kolmogorov-Smirnov test has been performed. (C) Box plots representing the polar and equatorial axis lengths measured on fifty randomly selected pollen grains for each genotype in each season.

A

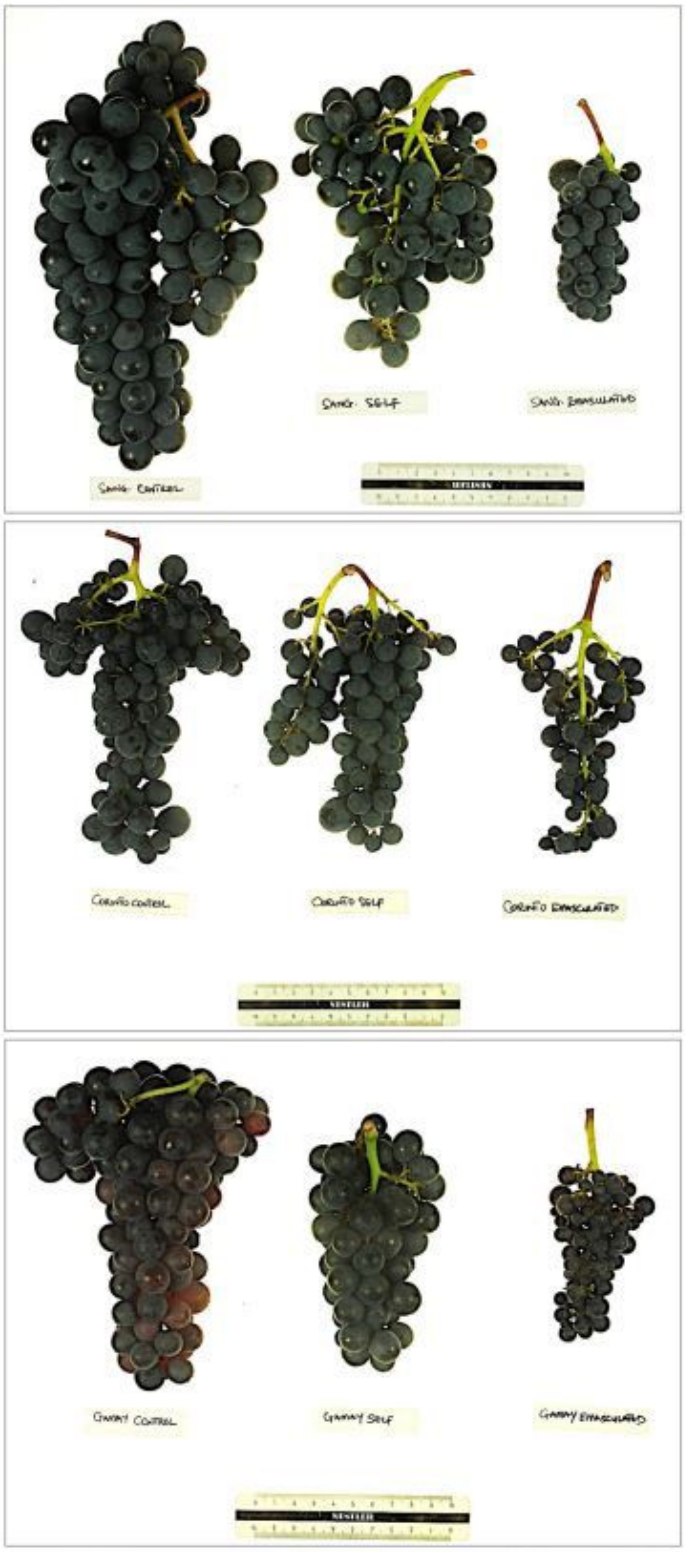

Figure 9

Clusters (A), seeds and traces (B) derived from open-pollination (control), self-pollination (self) and emasculation. Abbreviations: CN = Corinto Nero, SG = Sangiovese, EMS+ST = emasculated (without stigma removal), SP = self-pollinated, stage I = stage E-L 15, stage II = stage E-L 18 of the modified Eichhorn-Lorenz scheme [226]. Red arrows indicate apparently normal seeds among several rudimental seeds. (C) Seedlings derived from occasional normal seeds extracted from emasculated bunches of Gamay (on the left) and Sangiovese (on the right). Pictures were taken 75 days after sowing.

Supplementary Files

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