Vitis flower types: from the wild to crop plants

João L Coito [1] , **Helena G Silva** [2] , **Miguel JN Ramos** [1] , **Jorge Cunha** [3] , **José Eiras-Dias** [3] , **Sara Amâncio** [1] , **Maria M R Costa** [2] , **Margarida Rocheta** [Corresp. 1]

[1] LEAF, Linking Landscape, Environment, Agriculture and Food, Instituto Superior de Agronomia, Universidade de Lisboa, Lisboa, Portugal

[2] Biosystems and Integrative Sciences Institute (BioISI), Plant Functional Biology Centre, University of Minho, Campus de Gualtar, Braga, Portugal

[3] Instituto Nacional de Investigação Agrária e Veterinária, Quinta d'Almoinha, Dois Portos, Portugal

Corresponding Author: Margarida Rocheta
Email address: rocheta@isa.ulisboa.pt

Vitis vinifera can be divided into two subspecies, *Vitis vinifera* subsp. *vinifera*, one of the most important agricultural crops in the world, and its wild ancestor, *Vitis vinifera* subsp. *sylvestris*. Three flower types can be observed: hermaphrodite and female (on some varities) in *vinifera*, and male or female flowers in *sylvestris*. It is assumed that the different flower types in the wild ancestor arose through specific floral patterns of organ abortion.

A considerable amount of data about the diversity of sexual systems in grapevines has been collected over the past century. Several grapevine breeding studies led to the hypothesis that dioecy in *vinifera* is derived from a hermaphrodite ancestor and could be controlled by either, one or two linked genetic determinants following Mendelian inherence. More recently, experiments using molecular approaches suggested that these *loci* were located in a specific region of the chromosome 2 of *vinifera*.

Based on the works published so far, its seems evident that a putative sex *locus* is present in chromosome 2. However it is still not fully elucidated whether flower types are regulated by two linked *loci* or by one *locus* with three alleles. Nevertheless, several genes could contribute to sex determination in grapevine. This review presents the results from early studies, combined with the recent molecular approaches, which may contribute to the design of new experiments towards a better understanding of the sex inheritance in grapevine.

1 *Vitis* flower types: from the wild to crop plants

3 João Lucas Coito[1], Helena Gomes Silva[2], Miguel Jesus Nunes Ramos[1], Jorge Cunha[3], José Eiras-

4 Dias[3], Sara Amâncio[1], Maria Manuela Ribeiro Costa[2], Margarida Rocheta[1]*

6 [1] LEAF, Linking Landscape, Environment, Agriculture and Food, Universidade de Lisboa,

7 Instituto Superior de Agronomia, Tapada da Ajuda, 1359-017 Lisboa, Portugal

8 [2] Biosystems and Integrative Sciences Institute (BioISI), Plant Functional Biology Centre,

9 University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

10 [3] Instituto Nacional de Investigação Agrária e Veterinária, Quinta d'Almoinha, Dois Portos,

11 Portugal.

13 Corresponding Author:

14 Margarida Rocheta[1]*

15 Tapada da Ajuda, 1359-017 Lisboa, Portugal

16 Email address: rocheta@isa.ulisboa.pt

18 Abstract

19 *Vitis vinifera* can be divided into two subspecies, *Vitis vinifera* subsp. *vinifera*, one of the most

20 important agricultural crops in the world, and its wild ancestor, *Vitis vinifera* subsp. *sylvestris*.

21 Three flower types can be observed: hermaphrodite and female (on some varities) in *vinifera*,

22 and male or female flowers in *sylvestris*. It is assumed that the different flower types in the wild

23 ancestor arose through specific floral patterns of organ abortion.

24 A considerable amount of data about the diversity of sexual systems in grapevines has been

25 collected over the past century. Several grapevine breeding studies led to the hypothesis that

26 dioecy in *vinifera* is derived from a hermaphrodite ancestor and could be controlled by either,

27 one or two linked genetic determinants following Mendelian inherence. More recently,

28 experiments using molecular approaches suggested that these *loci* were located in a specific

29 region of the chromosome 2 of *vinifera*.

30 Based on the works published so far, its seems evident that a putative sex *locus* is present in

31   chromosome 2. However it is still not fully elucidated whether flower types are regulated by two

32   linked *loci* or by one *locus* with three alleles. Nevertheless, several genes could contribute to sex

33   determination in grapevine. This review presents the results from early studies, combined with

34   the recent molecular approaches, which may contribute to the design of new experiments

35   towards a better understanding of the sex inheritance in grapevine.

37   **Keywords**

38   *Vitis vinifera sylvestris*, *Vitis vinifera vinifera*, Domestication, Sex determinism, Dioecy

39

40 Introduction

41   The cultivated grapevine (*Vitis vinifera* subsp. *vinifera* L., hereafter *vinifera*) belongs to

42   Vitaceae. Among this family, species from the genus *Vitis* are found mainly in temperate zones

43   of the Northern Hemisphere distributed between North America and eastern Asia. *Vitis vinifera*

44   is the only *Vitis* species proved to have its origin in Europe. Fossil vestiges of *Vitis* grape seeds

45   from European tertiary period sediments suggest that Vitaceae is around 55 million years old

46   (Tiffney & Barghoorn 1976). The survival of *Vitis* genus was accompanied with a rise in *Vitis*

47   species and their distribution is consistent with the breaking up of *Vitis* large populations by ice

48   fronts, only surviving in refuges, the ideal conditions for speciation (Adam-Blondon et al. 2016).

49   In the case of *V. vinifera* the refuges corresponded to the regions stretching from the western

50   Himalayas to the Caucasus, where the origin of the current *vinifera* is hypothesized to have

51   occurred.

52   Evolutionary genomic analysis showed that *vinifera* diverged from *Vitis vinifera* subsp. *sylvestris*

53   (hereafter *sylvestris*) around 22000 to 30000 B.C. (Zhou et al. 2017). *Vitis vinifera subsp.*

54   *sylvestris* is considered the wild grapevine from which *vinifera* was domesticated (Zohary et al.

55   2000) in the region between the Black Sea and Iran, around 4000 years B.C. (McGovern 2004).

56   However, more recent archaeological samples from the south Caucasus, suggest that wine

57   making go as far as 8000 years (McGovern et al. 2017). Therefore, the domestication process

58   might have occurred earlier than the records show. The domestication and cultivation of *vinifera*

59   then spread to what is today the Palestine, southern Lebanon and Jordan (McGovern 2004). *Vitis*

60   *vinifera subsp. vinifera* (hereafter *vinifera*) continued its propagation and in 3000 B.C. it

61   appeared in Asia Minor, Southern Greece, Crete and Cyprus. By 2000 B.C. *vinifera* was found in

62   areas surrounding the Mediterranean Sea, including southern Italy and the Balkans. From there,

63 it expanded to the whole Mediterranean region including France, Spain and Portugal (Arroyo-
64 Garcia et al. 2006; McGovern 2004). The domestication of *vinifera* is linked with the discovery
65 of wine production, as well as, other products such as raisins, table grapes and grape vinegar,
66 making this species a major agronomic and economic crop worldwide (Hardie 2000; Royer
67 1888).

68 The last classification of the Vitaceae includes 14 genera (Soejima & Wen 2006). *Vitis* genus
69 itself has two subgenera: *Vitis* and *Muscadinia* (*V. rotundifolia*), both producing edible fruits
70 (Olien 1990). The distinction through karyological analysis showed 2n = 2x = 40 for muscadines
71 (subgenus *Muscadinia*) and 2n = 2x = 38 for bunch grapes (subgenus *Vitis*). Despite the
72 karyotype difference, crossing between *V. vinifera* and *V. rotundifolia* is viable however there is
73 a high sterility in the F1 hybrids with few or no viable seeds. This might be explained by the
74 phylogenetic proximity between the two subgenera confirmed by chloroplastic gene markers
75 (Ingrouille et al. 2002; Soejima & Wen 2006).

76 The high number of *Vitis* chromosomes (2n = 2x = 38 and 2n = 2x = 40) suggests a polyploid
77 origin of the genomes, as generally assumed for other plant species (Lewis 1979). Cytological
78 analysis performed during pollen meiosis of F1 offspring of *V. vinifera* x *V. rotundifolia* led to
79 the hypothesis of an allopolyploid origin of grapevine with three basic genomes. Two of those
80 genomes would be common to *Vitis* and *Muscadinia* (with haploid contents of n=6 and n=7,
81 respectively) and a third genome with n=6 for *Vitis* and n=7 for *Muscadinia* (Patel & Olmo
82 1955). However, not only *in situ* hybridization on *V. vinifera* chromosomes revealed a single
83 ribosomal *locus* (Haas & Alleweldt 2000), but the genetic maps did not give support to this
84 hypothesis above (Doligez et al. 2006; Lowe & Walker 2006). However, more recently the
85 hypothesis of a polyploid origin of *Vitis* found strong support with the publication of the
86 grapevine genome (Jaillon et al. 2007), describing the genome of *Vitis vinifera* as derived from a
87 hexaploid ancestor with a presumable haploid number of chromosomes n=7.

88

Survey methodology

90 The authors of this review article are familiar with grapevine genetics, flower development,
91 physiology and new generation sequencing techniques, having published several scientific
92 manuscripts regarding the subject. Scientific articles cited on this review were searched on
93 PubMed database, and Google scholar using: "grapevine", "*Vitis vinifera*", "flower

94   development", "homeotic genes", "flower sex", "plant evolution", "domestication", "grapevine

95   breeding" as keywords. Scientific articles, diverse in date of publishing, considered relevant on

96   the subject were selected.

97

The sexual systems in grapevine

99   Flowers of wild grapevines are mainly of two types: male (with suppressed pistils) (*Fig.* 1A) or

100   female (with reflexed stamens and infertile pollen) (*Fig.* 1B). However, in rare instances, female

101   flowers producing fertile pollen appear in wild plants (Valleau 1916).

102   Variations in the development and functional ability of stamens and pistils of the three grapevine

103   flower types have been reported (Stout 1921) although, these differences were considered as

104   being quantitative rather than qualitative. Clear differences were observed among hermaphrodite

105   flowers regarding filament length, anther size, pistil shape (*Fig.* 2A - C) and seed number (Stout

106   1921). In female flowers, Stout (1921) described a gradual phenotype regarding the reflexed

107   stamen development (*Fig.* 2D - H), with several grades of anther development, pollen grain

108   features and dehiscence (*Fig.* 2D - H). Pollen grains were irregular, shrivelled and an only few

109   were able to germinate (Stout 1921).

110   In accordance with a previous study (Dorsey 1912), Stout (1921) showed that in male plants, the

111   functional stamens may also vary in size (*Fig.* 2I - J) emphasising that these flowers exhibit

112   different types of rudimentary pistils with or without stigmatic surfaces (*Fig.* 3A - D). Carpels

113   and rudimentary ovules were produced with seed coats not fully developed (Dorsey 1912) and

114   none of them were able to produce fruits (Stout 1921).

115   In the middle of 20th century, Levadoux (1956) shed light on the differences between *vinifera*

116   and *sylvestris* of western Eurasia and concluded that no morphological character allowed a

117   specific differentiation between them since hermaphrodite plants could also occur in *sylvestris*.

118   Later, several authors contributed to clarify the floral and reproductive development of *vinifera*

119   and *sylvestris* in more detail (Caporali et al. 2003; Gallardo et al. 2009; Popescu et al. 2013).

120   The morphological differentiation between male and female flowers of the dioecious grapevine

121   can only be identified at a late stage of flower development, since at early stages a hermaphrodite

122   development pattern is observed (Caporali et al. 2003). Although male flower pistils are not fully

123   developed, the ovule development occurs, followed by megasporogenesis and megagametophyte

124   formation (Caporali et al. 2003). The suppression of femaleness appears to be the consequence of

125   the death of a specific layer, or layers, of nucellar cells and ovular integuments, which may

126   represent the targets of a programmed cell death or apoptosis process (Caporali et al. 2003). In

127   female flowers, the suppression of maleness appears to be the consequence of pollen sterility

128   (Caporali et al. 2003). Comparing to male flowers microspores, female microspores have been

129   shown to have an abnormal, round shape, without distinct colpi, and lack the typical thickenings

130   (Caporali et al. 2003). The anthers of female flowers produce much less pollen grains (10 – 100)

131   than the male (1,500 – 3,000) (Gallardo et al. 2009) and their size (polar axis x equatorial axis) is

132   smaller in female flowers than in male ones (Gallardo et al. 2009; Popescu et al. 2013).

133

134   **Flower types in grapevine: Mendelian approach**

135   Grapevine flower sex segregation was addressed using crosses between different *vinifera*

136   varieties in the outset of the 20th century (Hedrick & Anthony 1915). The authors self-pollinated

137   grapevines exhibiting upright stamens, reflexed stamens and performed crosses between these

138   two types of grapevines. The interpretation of these results proposed that grapevine sex

139   specification should be regulated by two determinants, F (female) and M (male) (Valleau 1916)

140   and assumed that female plants are homozygous, FF, and males are heterozygous, FM. In the

141   hermaphrodites both determinants would be linked giving rise to heterozygous individuals

142   represented by FH (of Hermaphrodite) (*Table* 1). The flower phenotypes from crosses between

143   female and the different hermaphrodite types, allowed the conclusion that the hermaphrodites

144   could be either FH or HH (Valleau 1916) (*Table* 1). Several crosses between hermaphrodite and

145   male plants revealed that MH male plants should also exist (Valleau 1916). The most

146   comprehensive work on the inheritance of flower sex in *vinifera* was carried out in 1938, and it

147   was suggested that flower sex determination in grapes was due to a digenic linked inheritance

148   (Oberle 1938). The author assumed a homozygous condition in female plants, a heterozygous

149   condition for the functionally male plants, and a heterozygous or a homozygous condition in the

150   hermaphrodites. Also, it was proposed that the action of recessive genes resulted in male sterility

151   and the action of dominant ones resulted in female sterility. Oberle (1938) named the dominant

152   allele responsible for perfect pollen development as *Sp*, the recessive allele that inhibits pollen

153   development as *sp*, the dominant allele that inhibits ovule development as *So* and the recessive

154   allele responsible for perfect ovule development as *so* (*Fig.* 4). The genetic formulation for the

155   different flower types would be then *so sp/so sp* for female, *So Sp/so sp* or *So Sp/so Sp* for male

156   and *so Sp/so sp* or *so Sp/so Sp* for hermaphrodite plants (Oberle 1938) (*Table* 1).

157   The flower phenotypes observed in crosses between male vs female, female vs hermaphrodite

158   and male vs hermaphrodite plants followed a Mendelian inheritance, and the ratios calculated

159   from the crosses performed by Oberle (1938) were similar to those obtained by Valleau (1916).

160   According to the authors, the crosses between hermaphrodites could only originate

161   hermaphrodites or hermaphrodites and females in a 3:1 ratio, depending on the hermaphrodite

162   genotype. Homozygous hermaphrodites crossed with males of any genotype originated 1:1 ratio

163   of male to hermaphrodite progeny. Heterozygous hermaphrodites crossed to heterozygous males

164   originated 2:1:1 ratio of male:hermaphrodite:female progeny. Moreover, the cross between

165   heterozygous hermaphrodites (*so sp/so Sp*) and homozygous *Sp* male (*So Sp/so Sp*) plants only

166   originated male and hermaphrodites in a 1:1 ratio. Female crossed with homozygous

167   hermaphrodites the resulting progeny was 100% hermaphrodite. However, females crossed with

168   heterozygous hermaphrodites the resulting proportion was 1:1 of female and hermaphrodites.

169   Finally the crosses between male and female plants, originated 1:1 proportion of males and

170   hermaphrodites or males and females, depending on the possible male genotypes.

171   In a later work, several self-pollination and crosses with different cultivated hermaphrodite

172   varieties were performed (Avramov et al. 1967). The authors obtained two sets of results:

173   hermaphrodite and female plants in a 3:1 ratio, and an offspring of only hermaphrodite

174   individuals. The factor for production of female flowers was designated S^f and the factor for

175   hermaphroditism S^h (*Table* 1) and it was proposed that S^f is recessive to S^h. Furthermore, no male

176   plants resulting from crosses were found. The conversion of male flowers to hermaphrodites by

177   the exogenous application of cytokinin suggested that cytokinins may bypass the genetic

178   regulatory mechanisms of the male determinant as a suppressor of ovule development (Iizuka &

179   Hashizume 1968). In 1970 Negi & Olmo (1970) identified a hermaphroditic male plant, defined

180   by the ability of producing both perfect male flowers and near perfect flowers, capable of fruit

181   production. This hermaphroditic male plant was self-pollinated and the offspring showed a ratio

182   of 3:1 for male and female plants (Negi & Olmo 1971). They selected, at random, some F1

183   plants and crossed them with a homozygous female *Vitis*. The results showed that, while some

184   crosses resulted in male and female plants, others resulted in only males plants in the F1,

185   meaning that some males would be homozygous for the male trait (Negi & Olmo 1971). These

186   results also suggested that *vinifera* sex may be inherited as a simple Mendelian factor and, based

187 on this assumption, a new model for sex inheritance was proposed, explained by a single genetic

188 factor with two allelic forms Su^F and Su^m (Negi & Olmo 1971). The allele Su^F represses the

189 development of functional female organs and the allele Su^m, in homozygous condition,

190 suppresses the development of male organs. The allelle Su^F is completely dominant over its

191 allele Su^m and the genetic formulae would be Su^F/Su^m or Su^F/Su^F for male and Su^m/Su^m for

192 female (Negi & Olmo 1971) (*Table* 1). Later, a model that involves a single genetic determinant

193 for sex with three different alleles, *M* for male, *H* for hermaphrodite and *F* for female

194 individuals, with a proposed $M > H > F$ dominance pattern (Antcliff 1980) was proposed.

195 In a study in which several hermaphrodite varieties of vinifera were self-pollinated some

196 interesting observations were found (Bronner 1981): 1) some hermaphrodite varieties were

197 heterozygous for the sex locus (resulting 3:1 ratio of hermaphrodites and females), 2) one variety

198 was homozygous (resulting in only hermaphrodite plants), and 3) the variety Muscat Ottonel

199 when self-pollinated resulted in a F1 with a surprising high number of male individuals (Bronner

200 1981). These observations showed in the two closed loci model occasionally crossing-over might

201 occur (depending on the variety in study) or that a more complex model might be needed to

202 explain *Vitis* flower type segregation. In 1983, the previous models from Valleau (1916), Oberle

203 (1938) and Antcliff (1980) were criticized because they did not not completely explain the total

204 absence asexual individuals (which could be assumed as *So sp/so sp* and *So sp/So sp*) and did not

205 explain the occasional appearance of male plants observed in crosses between hermaphrodites

206 (Carbonneau 1983). Then a more complex model was proposed adding an epistatic gene *E/e* with

207 effects on the alleles H or F, to explain this unpredictable male offspring (Carbonneau 1983).

208 However, is worth to notice that male offspring between hermaphrodites plants were only

209 observe in two different crosses involving four different varieties of which the result offspring

210 from self-pollination is unknown. Therefore, this model has not been tested so far. Instead, the

211 segregation patterns observed in the majority of studies of sex inheritance and recent studies on

212 sex determinism markers appear to support Valleu, Oberle and Antcliff models (Antcliff 1980;

213 Avramov et al. 1967; Dalbó et al. 2000; Fechter et al. 2012; Hedrick & Anthony 1915; Marguerit

214 et al. 2009a; Oberle 1938).

215 Wild vines of *V. rotundifolia* are dioecious, while cultivated varieties are hermaphrodite or

216 female, similar to the flower types of cultivated vinifera and wild sylvestris. There are two

217 sources of hermaphroditism in *muscadinia*, named H1 and H2. The H1 hermaphrodite plant was

218 achieved through the cross of the *muscadinia* female variety Eden and the male variety Mission

219 Male while the H2 hermaphrodite was the result of the cross between the female Scuppernog and

220 the male New Smyrna. These two hermaphrodite plants are the origin of hermaphrodite *V.*

221 *rotundifolia* (Loomis & Williams 1957). The H1 when self-pollinated originates hermaphrodite

222 and female plants in a 3:1 ratio, which mean that the H1 must be heterozygous HF or *so Sp/ so*

223 *sp*, according to Antcliff (1980) or Oberle (1938) respectively. However the H2 plant when self-

224 pollinated originates hermaphrodite, female and male plants in a 9:3:4 ratio (Conner et al. 2017;

225 Loomis & Williams 1957). Studies of the records which originate both H1 and H2, lead to the

226 conclusion that they were genetically similar and therefore, H2 could be a mutation of a

227 staminate plant (Loomis & Williams 1957). In *vinifera* the source of hermaphroditism is largely

228 unknown, although the existence of hermaphroditic males (Negi & Olmo 1970; Negi & Olmo

229 1971) and the hermaphrodite offspring between male and female *V. rotundifolia* raises the

230 possibility that a particularly unknown type of *sylvestris* could be in the origins of a full

231 hermaphrodite *sylvestris* and thus target for domestication.

232

233 **Compromised pollen formation**

234 RNA-Seq studies on *vinifera* and *sylvestris* (male and female) uncovered some genes, whose

235 functionally characterized homologues were previously described to be involved in flower

236 development and organ development, displaying differential expression between male, female

237 and hermaphrodite flower development (Ramos et al. 2014; Ramos et al. 2017). These genes

238 belong to several functional categories and seem to be distributed throughout the 19 grapevine

239 chromosomes. Genes responsible for pollen formation seem to have different expression

240 patterns between female and male plants: *LESS ADHERENT POLLEN* (*LAP3*); a gene annotated

241 as a *Nodulin MtN3,* which is a member of the *Lipoxygenase* (*LO*) gene family; a calose synthase

242 5 (*CalS5*) coding gene; *CYP704B1*; and *ACYL-CoA synthase 5* (*ACOS5*), all have higher

243 expression levels at late development stages of female plants when compared with male or

244 hermaphrodite plants (Ramos et al. 2014; Ramos et al. 2017). *Arabidopsis thaliana* mutants for

245 *LAP3* show induced pollen sterility or abnormal exine patterns (Dobritsa et al. 2009a). Also, in

246 *Arabidopsis thaliana MtN3* has a role in exine pattern and in the cellular integrity of

247 microspores (Guan et al. 2008). *AtCYP704B1* seems to be implied in sporopollenin synthesis

248 (Dobritsa et al. 2009b). Furthermore, mutant plants for *ACOS5* do not produce pollen in late

249 stage of stamens development, as the pollen wall lacks sporopollenin or exine and its formation

250 is thus compromised (Dong et al. 2005). *AtCalS5*, which codes for a protein responsible for

251 depositing callose at microspores, is essential for correct exine formation in the pollen, and

252 knockout mutants for *CalS5* resulted in reduced fertility (Dong et al. 2005; Enns et al. 2005).

253 However, as a whole, the above experiments result in either the absence or defective expression

254 of these genes. In female *sylvestris* the opposite was observed, with the transcripts being highly

255 expressed at late flower development. The effect of over-expression of these genes has not yet

256 been studied, nevertheless, the results might suggest that excess of sporopollennin and exine

257 deposition could result in infertile pollen. A large deposition of these pollen wall elements might

258 lead to small and oval pollen grains lacking pores (Gallardo et al. 2009). In *sylvestris* female

259 pollen is also smaller and fewer grains are formed (Gallardo et al. 2009). This might be due to

260 resource allocation in pollen development, since excessive deposition of sporopollenin and

261 exine might consume more resources, leading to the formation of fewer and smaller pollen

262 grains in female plants. Also, the *VviERECTA* gene seems to have less expression, at late

263 development stages, in female when compared with male and hermaphrodite flowers. In

264 *Arabidopsis*, *ERECTA* mutants are associated with short thick stamens and pedicels (Ferrandiz

265 et al. 1999; Torii et al. 1996). This phenotype is not observed in female *Vitis* plants, although

266 their stamens are shorter than those of male and hermaphrodite plants. Also, *in situ*

267 hybridization targeting *VviERECTA* mRNA showed that this gene is expressed similarly in all

268 three flower types in the stamens and carpels (Coito 2018)

269 Pollen wall appears to be compromised by the over-expression of transcripts involved in its

270 formation. However, transcription factors might also play a role in female stamens phenotype

271 *TRANSCRIPTION FACTOR ABORTED MICROSPORES-LIKE* (*AMS*) (Ramos et al. 2017),

272 which only shows expression in female plants at late developmental stages and thus could

273 contribute to pollen inviability. VIT_212s0057g00440 (Ramos et al. 2017), which is over-

274 expressed in flower plants at stage H, is annotated as an "acyl-n-acyltransferase domain-

275 containing protein" and may also be contributing to the abnormal pollen wall formation and

276 subsequent infertility.

277

278 **The development of the male carpel: possible hormone or timeframe**

279 **control**

280 The RNA-Seq studies (Ramos et al. 2014; Ramos et al. 2017) also showed a small number of

281 genes related to carpel formation differently expressed in late stages of male flower

282 development. While *VviJAGGED* and *VviERECTA* were highly expressed at late development

283 stages in male plants, *VviSHEPHERD* showed the opposite pattern, being less expressed at these

284 stages in male plants when compared to the other flower types. Other gene involved in carpel

285 formation, *VviCRABSCLAW*, seems to be less expressed in late development stages in male

286 flowers. In *Arabidopsis JAGGED* codes a putative C_2H_2 zinc-finger transcription factor (Ohno et

287 al. 2004) and in stamens, *AtJAGGED* expression promotes the growth of the adaxial side, a

288 requisite for microsporangia formation, while in the carpel *AtJAGGED* acts by promoting the

289 growth of the valves that enclose the ovules (Dinneny et al. 2006). *AtERECTA* codes for a

290 leucine-rich receptor-like serine/threonine kinase (Torii et al. 1996) and its function includes

291 specifying aerial organ shape and size, mainly by promoting cell proliferation (van Zanten et al.

292 2009). This is evident in *AtERECTA* mutants, which show a dwarfish morphology (Shpak et al.

293 2004; Shpak et al. 2003). However, as stated previously, *in situ* hybridization targeting

294 *VviERECTA* mRNA showed no differences between flower types (Coito 2018). In *Arabidopsis*,

295 *SHEPHERD* seems to be required for the correct fold of CLAVATA proteins, which in turn

296 promotes the differentiation of peripheral stem cells during organ initiation (Ishiguro et al.

297 2002). However, in *Vitis*, several *CLAVATA* genes seem to be differently expressed during

298 development but not between flower types. *CRABSCLAW* is a member of the YABBY family

299 (Bowman & Smyth 1999; Siegfried et al. 1999) and seems to be required for nectaries

300 development independently of *AGAMOUS* activity (Baum et al. 2001). In *Arabidopsis*,

301 *CRABSCLAW* mutations affect the gynoecium growth resulting in a shorter carpel (Alvarez &

302 Smyth 1999). In grapevine male plants seem to have a shorter carpel, however,

303 *VviCRABSCLAW* is expressed identically in the nectaries and carpel in all three flower types

304 (Coito 2018). This observation shows that differences in carpel formation between male and

305 female plants of *sylvestris* occur very late in flower organ development.

306 A search for transcripts involved in phytohormones synthesis and signalling found several

307 differentially expressed transcripts between flower types during development. The expression of

308 genes involved in hormone biosyntesis and signalling, as well as the expression of *VviJAGGED*,

309 *VviERECTA* and *VviSHEPHERD*, seems to be more associated with the timing of flower

310 development initiation rather than with the development of specific flower organs. However,

311   some hormone related genes could be involved in carpel shape regulation in male flowers, since

312   they are differentially expressed in late stages of flower development. *VviPINOID* and

313   *VviETTIN* were both more expressed in male plants at late flower development stages. In

314   *Arabidopsis, PINOID* codes a serione-threonine kinase and its over-expression is linked to

315   negative regulation of auxin signalling, which results in a dwarf plant morphology (Christensen

316   et al. 2000) and *AtETTIN*, which codes for a protein with a DNA-biding domain highly similar

317   of the *AUXIN RESPONSIVE FACTOR 1* involved in early response to auxin (Ulmasov et al.

318   1997a; Ulmasov et al. 1997b). *ETTIN* defective *Arabidopsis* mutants show enhanced

319   proliferation of style and internodes (Nemhauser et al. 1998). The effects of the over-expression

320   of *ETTIN* are largely unknown; however, it could lead to a diminished proliferation of style or,

321   coupled with higher expression of *PINOID*, could lead to a deficient response to auxin

322   signalling, in turn leading to an abnormal carpel with underdeveloped style and stigma. Thus,

323   these genes could act together through an auxin regulated process in carpel development

324   (Ferrandiz et al. 1999) and it is quite likely that hormones influence carpel morphology. As

325   previously demonstrated, the exogenous application of the synthetic cytokinin BAP

326   (6-benezylamino-9-(2-tetrahydropyranyl)-purine), induces sex reversion of *sylvestris* male

327   flowers into hermaphrodites (Negi & Olmo 1971). Similarly, more recently, the exogenous

328   application of a cytokinin in *Sapium sebiferum* induced a female flower development, instead of

329   a male one (Ni et al. 2018). In *Arabidopsis thaliana*, *AtAPRT1* mutants develop male sterility

330   due to the formation of atypical pollen (Gaillard et al. 1998; Moffatt & Somerville 1988).

331   Taking into consideration the close cross talk between auxins and cytokinins, it is not to exclude

332   that flower type and sex specification may be controlled through hormone regulation. However,

333   it is difficult to distinguish normal floral development pathways from the abnormal carpel

334   formation through this approach, since these pathways seem dependent on an expression balance

335   of hormone related genes.

336

Homeotic genes in grapevine

338   The ABCDE model postulates that genes from three regulatory functions act in concert to confer

339   organ identify during flower development, subdividing the developing flower into whorls (Coen

340   & Meyerowitz 1991). For example, in *Arabidopsis thaliana, APETALA1* and *APETALA2*, both

341   A- function genes, specify sepal identity, while A- function genes combined with B- function

342 genes (*APETALA3* and *PISTILLATA*) confer petal identity. B- function genes together with C-

343 function genes (*AGAMOUS*) confer stamen identify and *AGAMOUS* alone confers carpel

344 identity (Coen & Meyerowitz 1991). The D- function (composed by the genes *SEEDSTICK* and

345 *SHATTERPROOF 1* and *2*) is required for ovule identity (Pinyopich et al. 2003) and E- function

346 genes (conferred by four *SEPALLATA* genes) are redundantly necessary for the specification of

347 the four flower whorls (Ditta et al. 2004; Favaro et al. 2003; Pelaz et al. 2000).

348 In hermaphrodite grapevine, *in-situ* hybridization (Coito et al. 2018) and real-time PCR

349 experiments (Palumbo et al. 2019) showed that B- class genes are expressed in petals, stamens

350 and carpel in the early stages of development but in later stages, *VviPI* seems to stop being

351 expressed in the forth whorl while *VviAP3* and *VviTM6* still display some expression in the

352 carpel. In the case of wild grapevine a possible abnormal expression of ABCDE model genes,

353 particularly those responsible for stamens and carpel organ identity could trigger the abnormal

354 morphologies that distinguish female and male flowers.

355 Upon the emergence of sepal primordia, *VviPI* and *VviAP3* are excluded from the sepals.

356 Afterwards these genes are expressed in the third whorl and continue throughout flower

357 development (Coito et al. 2018). Other important gene to refer is *VviSUPERMAN* (*VviSUP*)

358 which was found to clearly mark the boundary between the third and fourth whorl, equally in

359 both female and male flowers (Coito et al. 2018). Finally, the C- function gene, *VviAG* seems to

360 be expressed at the centre of the flower meristem and during flower development it is gradually

361 excluded from the first and second whorl, while retaining its expression in the stamens and

362 carpel in both female and male plants (Coito et al. 2018).

363 This expression profiles excludes the possibility that B- and C- class genes may be responsible

364 for carpel or stamen abortion and, therefore, controlling flower type specification.

365

366 A small genomic region defining the sex in grapevine

367 Several attempts were made to understand and provide insight into the molecular mechanism

368 regarding the origins of sexual dimorphism present in *sylvestris* individuals. Several genetic

369 mapping studies based on the 8x version of the *Vitis* genome annotation

370 (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/entry_ggb.html) (Dalbó et al. 2000;

371 Marguerit et al. 2009a; Riaz et al. 2006) located the *locus* responsible for sex determination at

372 the vicinity of the genetic markers VviMD34 and VviIB23 on chromosome 2 in the 8x version

373   (http://genomes.cribi.unipd.it/grape/). With the use of these genetic markers and genetic maps,

374   the authors conclude that only one gene would be responsible for the dimorphism in *Vitis* (Dalbó

375   et al. 2000; Marguerit et al. 2009a; Riaz et al. 2006). This conclusion was in accordance with the

376   work of Antcliff (1980) in which *Vitis* flower type trait follows a simple Mendelian inheritance

377   model whit three alleles: male (**M**)*,* female (**F**) and hermaphrodite (**H**).

378   Using these markers putatively linked to the sex *locus*, a new genetic map was developed and

379   refined to restrict the sex *locus* to 143 kb in the chromosome 2, between 4,907,434 bp and

380   5,050,616 bp (Fechter et al. 2012). This region contained 16 genes, including an *ADENINE*

381   *PHOSPHORIBOSYILTRANSFERASE* (*VviAPRT*) which was identified as a possible genetic

382   marker able to discriminate female plants from male and hermaphrodite ones (Fechter et al.

383   2012). The suggestion that *VviAPRT* could potentially be involved in sexual dimorphism in *Vitis*

384   is particularly interesting, since in other species *APRT* homologues code for a key metabolic

385   enzymes participating in cytokinin metabolism (Allen et al. 2002; Mok & Mok 2001). A more

386   recent study, focusing on the 143 kb region of chromosome 2 extended the sex *locus* region to

387   158 kb downstream of the genetic marker VviIB23 and encompassing the previous 143 kb region

388   (Picq et al. 2014). This new *locus* showed haplotype diversity, linkage disequilibrium and several

389   genes segregating to typically associated X-Y sex determining region, with males being XY and

390   females XX with one of them being the previously identified *VviAPRT* (Picq et al. 2014). Also, it

391   has been suggested that many of the genes in the sex *locus* could have homologue sequences

392   located in the grapevine virtual chromosome unknown (Picq et al. 2014), thus potentially being

393   in a heterozygous state in *vinifera* reference genome, suggesting that the reference genome

394   PN40024 could be heterozygous regarding the sex *locus* (Jaillon et al. 2007; Picq et al. 2014).

395   Similar results were found for *V. rotundifolia* (Lewter et al. 2019). Linkage maps were

396   constructed for two F1 populations segregating for flower type and berry colour. Results showed

397   that the putative sex *locus* on *V. mucadinia* mapped on chromosome 2 of *vinifera,* in the previous

398   identified sex *locus* (Battilana et al. 2013; Dalbó et al. 2000; Fechter et al. 2012; Marguerit et al.

399   2009a; Picq et al. 2014; Riaz et al. 2006). This suggests that the sex *locus* might be conserved

400   among *Vitis* species.

401   In 2017, Zhou et al. (2017), using NGS (Next Generation Sequence), identified two regions on

402   the chromosome 2 that might be linked to sex determination in grapevine. One region contained

403   six genes expressed in female *sylvestris,* while the second region is believed to harbour a

404   dominant female sterility gene(s) (Zhou et al. 2017). These results seem to suggest that two

405   factors might be involved in flower type determination in grapevines. A study which though to

406   identify structural variances in *vinifera* (Zhou et al. 2019) shed some light into *Vitis vinifera* sex

407   determination and domestication. Important variations were found between cultivated and wild

408   grapevines in the potential sex *locus*. A phylogeny build on the sex determinant region showed

409   that the hermaphrodite haplotype was closer to the *M* haplotype than to the female. It also

410   showed cultivated varieties homozygous for the hermaphrodite trait, and varieties heterozygous,

411   having the hermaphrodite and female structural variant (Zhou et al. 2019). This corroborates the

412   early Mendelian studies in grapevine which states that some cultivated grapevine varieties could

413   either be *FH* or *HH* (Antcliff 1980; Avramov et al. 1967; Oberle 1938; Valleau 1916). This study

414   also shows that wild grapevine can also be *HM* as proposed by Valleau (1916) and that female

415   grapevines are homozygous for the female trait (Antcliff 1980; Avramov et al. 1967; Oberle

416   1938; Valleau 1916; Zhou et al. 2019).

417   Recently, two genetic markers were found, one marker in the *VviAPRT3* gene and a new marker

418   called *VviFEMALE SEX (VviFSEX)* gene (Coito et al. 2017). *VviFSEX* gene is located in the

419   previously identified putative sex *locus* in the grapevine chromosome 2 (Fechter et al. 2012; Picq

420   et al. 2014), downstream of the *VviAPRT3*. *VviFSEX* is also one of the six genes expressed in one

421   of the regions linked to sex determination in grapevine (Zhou et al. 2017). When used together,

422   the amplification profile of these two genetic markers made possible to distinguish female and

423   male *sylvestris* and hermaphrodite *vinifera*. It was also showed that male plants where

424   heterozygous for both markers, while female plants were homozygous. Hermaphrodite plants

425   were homozygous for the *VviAPRT3* genetic marker but heterozygous for the *VviFSEX* marker

426   (*Fig.* 5). In addition, using markers developed from Picq et al. (2014), (VSVV006 and

427   VSVV009) (Conner et al. 2017) in two F1 populations obtained by crossing three *V. rotundifolia*

428   varieties segregating for flower type and berry colour, it was possible to identify female, male

429   and hermaphrodite plants (Lewter et al. 2019).

430

The molecular validation of the long-sought sex *locus*

432   Two seemingly contrasting hypothesis exist, as previously referred: (1) two linked genes (Oberle

433   1938) and (2) one gene, three alleles (Antcliff 1980). With the second hypothesis being

434   somewhat more accepted in recent works (Dalbó et al. 2000; Fechter et al. 2012; Picq et al.

435 2014) while the first hypothesis is suggested based on the evolutionary assumption that two

436 mutations are required in a hermaphrodite ancestral population in order for male and female

437 individuals to emerge (Charlesworth & Charlesworth 1978) and seems to find some validation

438 on recent works (Coito et al. 2017; Ramos et al. 2014; Zhou et al. 2017). With the development

439 of the two genetic markers (Coito et al. 2017) it became possible to associate these to the

440 previous observations by Oberle (1938), Valleau (1916), Avramov et al. (1967) and Antcliff

441 (1980), therefore validating them by a molecular approach.

442 The combinations of the two genetic markers could represent or hint the *locus* responsible for

443 male and female plants and contributed to the possibility that grapevine sex *locus* does reside in

444 the chromosome 2. In the two linked genes hypothesis (Oberle 1938) the combination *SO/SP*

445 identify a male plant, while the allele combination *so/SP* identify a hermaphrodite plant and the

446 combination *so/sp* identify a female plant. In the recent finding, different combinations of the

447 molecular markers VviAPRT3 and VviFEX can also identify male, female and hermaphrodite

448 plants (*Fig.* 5), however is unlikely that these markers are solely the responsible for sex

449 specification on grapevine, rather, they seem to identify a large *locus*, or genomic region

450 responsible for such dimorphism. Taking this into consideration the combination of both markers

451 can also be applied to the one gene, three alleles hypothesis (Antcliff 1980), where the molecular

452 markers combinations could represent and identify the M, H and F *locus* (*Fig.* 5).

453

454 ***Vitis* flower types: a long road to go**

455 Despite the most recent molecular studies corroborate the Mendelian works on grapevine flower

456 type segregation, the knowledge is still limited. Likewise, there are many gaps in understanding

457 *Vitis* flower types development and regulation. Genetic markers (Battilana et al. 2013 ; Dalbó et

458 al. 2000; Fechter et al. 2012; Marguerit et al. 2009b; Riaz et al. 2006) point to a precise region of

459 chromosome 2 which encompass several genes between the 4,09 and 5.04 Mbp. However,

460 despite the development of genetic markers that can differentiate hermaphrodite, male and

461 female plants (Coito et al. 2017; Conner et al. 2017; Picq et al. 2014), sequence analysis (Fechter

462 et al. 2012; Picq et al. 2014; Ramos et al. 2014; Ramos et al. 2017) has been unable to identify

463 the key players responsible for the regulation of flower types. Therefore it is still unknown which

464 gene or genes, trigger exactly the formation of the different types of flowers. Renewed efforts in

465   sequencing *sylvestris* genome and fine mapping the sex region could shed light regarding the

466   differences between putative key players regulating flower development in male and female.

467   The use of genetic editing technology can also be of assistance in the comprehension of

468   grapevine flower type determination. CRISPR/Cas9 technology was already tested with success

469   in tomato (*Solanum lycopersicum*) targeting the gene *SlAGO7*, whose mutant can be easy

470   identified. Loss of function mutants for *SlAGO7* show leaflets without petioles and later leaves

471   lacking laminae (Brooks et al. 2014). RNA interference (RNAi) is another technology able to

472   explore the putative function and role of candidate genes in grapevine flower type specification.

473   RNA interference has been previously used to down-regulate *OsSQS* which resulted in a reduced

474   stomatal conductance and therefore higher drought resistance (Manavalan et al. 2012). It was

475   used to shut down the gene *Aminocyclopropane-1-carboxylate* in tomato plants and these

476   transgenic plants produced fruits capable of releasing traces of ethylene and so had a longer shelf

477   life (Xiong et al. 2005).

478   Naturally occurring mutations for flowering in grapevines, conversely to *fleshless* berry mutant

479   applied to identify genes putatively involved in the early development of grapevine fruit

480   (Fernandez et al. 2007), are still poorly documented. Although natural mutations are important

481   tools to provide information on *Vitis* flower segregation, few flower mutations have been

482   reported. Flower mutants with abnormal petals and stamens were first reported by Sreekantan et

483   al. (2006). One mutant showed normal sepals and petals, but the carpel structures appeared fused

484   to pseudo-ovules in the place of stamens and lacked ovary in the central whorl. The flowers of

485   this mutant were not functional. The second flower mutant described (Sreekantan et al. 2006),

486   showed normal stamens number but filaments were shorter or absent with only 2 -5% of flower

487   developing into seeded berries. This second flower mutant develops in a similar way of female

488   flowers, however, only *VviPI* was characterized in these mutants (Sreekantan et al. 2006) and no

489   F1 segregation was studied. More six grapevine flower mutants were described and characterized

490   (Chatelet et al. 2007), although the expression of only some homeotic genes was covered in the

491   study. Additionally, two grapevine rootstocks could only be identified through flower

492   morphology (Wolf et al. 2003), variety SO4 which shows only male flowers, and the variety

493   Binova that produces hermaphrodite flowers. AFLP and SAMPL markers failed to distinguished

494   these two rootstocks, leading the authors to the conclusion that Binova could be a somatic mutant

495 of SO4 (Wolf et al. 2003). Unfortunately no self-pollination of Binova was reported so far, and

496 so, it is unknown how a F1 segregates for flower type.

497

498

Conclusions

499

500

501 Sex specification in *Vitis* continues to be an elusive subject, however this review seeks to gather

502 all the information regarding sex specification and early identification in *vinifera*, from the first

503 Mendelian genetic studies to more recent molecular and NGS approaches. It seems that there is a

504 consensus regarding male determinant being dominant over hermaphrodite and female plants,

505 while hermaphrodite determinant seems to be dominant over the female determinant. Mendelian

506 genetic studies regarding grapevine offspring showed that female plants were homozygous while

507 hermaphrodite and male grapevine could either be homozygous or heterozygous. Several studies

508 regarding molecular and NGS approaches resulted in the finding of a potential sex *locus* located

509 in the chromosome 2 and two genetic markers being developed to identify male, female and

510 hermaphrodite plants and validate the previous assumptions regarding the homozygosity or

511 heterozygosity of male, female and hermaphrodite plants. Nevertheless is still unknown if sex

512 specification is due to one or more genes, only that is most likely present in the chromosome 2

513 sex *locus* and that it seem to influence several genes downstream of the ABCDE homeotic genes

514 mainly pollen development genes and hormone signaling genes (*Fig.* 6).

515

References

517 Adam-Blondon AF, Martinez-Zapater JM, and Kole C. 2016. *Genetics, Genomics, and Breeding*

518   *of Grapes*: CRC Press.

519 Allen M, Qin W, Moreau F, and Moffatt B. 2002. Adenine phosphoribosyltransferase isoforms

520   of *Arabidopsis* and their potential contributions to adenine and cytokinin metabolism.

521   *Physiologia Plantarum* 115:56-68.

522 Alvarez J, and Smyth DR. 1999. *CRABS CLAW* and *SPATULA*, two *Arabidopsis* genes that

523   control carpel development in parallel with *AGAMOUS*. *Development* 126:2377-2386.

524 Antcliff AJ. 1980. Inheritance of sex in *Vitis*. *Ann Amélior Plant* 30:113-122.

525 Arroyo-Garcia R, Ruiz-Garcia L, Bolling L, Ocete R, Lopez MA, Arnold C, Ergul A,
526   Soylemezoglu G, Uzun HI, Cabello F, Ibanez J, Aradhya MK, Atanassov A, Atanassov I,
527   Balint S, Cenis JL, Costantini L, Goris-Lavets S, Grando MS, Klein BY, McGovern PE,
528   Merdinoglu D, Pejic I, Pelsy F, Primikirios N, Risovannaya V, Roubelakis-Angelakis
529   KA, Snoussi H, Sotiri P, Tamhankar S, This P, Troshin L, Malpica JM, Lefort F, and
530   Martinez-Zapater JM. 2006. Multiple origins of cultivated grapevine (*Vitis vinifera* L. ssp
531   *sativa*) based on chloroplast DNA polymorphisms. *Molecular Ecology* 15:3707-3714.
532 Avramov L, Jelenkovi G, Jovanovic M, and Rodié Z. 1967. Inheritance of flower type in some
533   grape varieties (*Vitis vinifera* L.). *Vitis* 6: 129-135.
534 Battilana J, Lorenzi S, Moreira FM, Moreno-Sanz P, Failla O, Emanuelli F, and Grando MS.
535   2013. Linkage mapping and molecular diversity at the flower sex locus in wild and
536   cultivated grapevine reveal a prominent SSR haplotype in hermaphrodite plants.
537   *Molecular Biotechnology* 54:1031-1037.
538 Battilana J, Lorenzi S, Moreira FM, Moreno-Sanz P, Failla O, Emanuelli F, and Grando MS.
539   2013 Linkage mapping and molecular diversity at the flower sex locus in wild and
540   cultivated grapevine reveal a prominent SSR haplotype in hermaphrodite plants.
541   *Molecular Biotechnology* 54. 10.1007/s12033-013-9657-5.
542 Baum SF, Eshed Y, and Bowman JL. 2001. The *Arabidopsis* nectary is an ABC-independent
543   floral structure. *Development* 128:4657-4667.
544 Bowman JL, and Smyth DR. 1999. *CRABS CLAW*, a gene that regulates carpel and nectary
545   development in *Arabidopsis*, encodes a novel protein with zinc finger and helix-loop-
546   helix domains. *Development* 126:2387-2396.
547 Bronner A. 1981. Observation de types sexuels males dans la descendance par autofecondation
548   de la variete Muscat Ottonel (Vitis vinifera L.). *Vitis*.
549 Brooks C, Nekrasov V, Lippman ZB, and Van Eck J. 2014. Efficient Gene Editing in Tomato in
550   the First Generation Using the Clustered Regularly Interspaced Short Palindromic
551   Repeats/CRISPR-Associated9 System. *Plant Physiology* 166:1292-1297.
552   10.1104/pp.114.247577
553 Caporali E, Spada A, Marziani G, Failla Q, and Scienza A. 2003. The arrest of development of
554   abortive reproductive organs in the unisexual flower of *Vitis vinifera* ssp. *silvestris*.
555   *Sexual Plant Reproduction* 15:291-300.

556 Carbonneau A. 1983. Male and female sterility in the genus *Vitis*. I: Modeling of their
557   inheritance. *Agronomie* 3:635-644.

558 Charlesworth B, and Charlesworth D. 1978. A Model for the Evolution of Dioecy and
559   Gynodioecy. *The American Naturalist* 112:975-997.

560 Chatelet P, Laucou V, Fernandez L, Sreekantan L, Lacombe T, Martinez-Zapater JM, Thomas
561   MR, and Torregrosa L. 2007. Characterization of *Vitis vinifera* L. somatic variants
562   exhibiting abnormal flower development patterns. *Journal of Experimental Botany*
563   58:4107-4118.

564 Christensen SK, Dagenais N, Chory J, and Weigel D. 2000. Regulation of auxin response by the
565   protein kinase PINOID. *Cell* 100:469-478. 10.1016/S0092-8674(00)80682-0

566 Coen ES, and Meyerowitz EM. 1991. The war of the whorls: genetic interactions controlling
567   flower development. *Nature* 353:31-37. 10.1038/353031a0

568 Coito JL, Ramos M, Cunha JMM, Silva HG, Amâncio S, Costa MMR, and Rocheta MP. 2017.
569   *VviAPRT3* and *VviFSEX*: two genes involved in sex specification able to distinguish
570   different flower types in *Vitis*. *Frontiers in Plant Science* 8:98. 10.3389/fpls.2017.00098

571 Coito JL, Silva H, Ramos MJN, Montez M, Cunha J, Amancio S, Costa MMR, and Rocheta M.
572   2018. Vitis Flower Sex Specification Acts Downstream and Independently of the
573   ABCDE Model Genes. *Frontiers in Plant Science* 9:1029. 10.3389/fpls.2018.01029

574 Coito JLFO. 2018. Vitis vinifera sex genes: from the wild to the domesticated varieties.

575 Conner P, Conner J, Catotti P, Lewter J, Clark JR, and Biasi LA. 2017. Development and
576   Characterization of Molecular Markers Associated with Female Plants in Muscadine
577   Grape. *Journal of the American Society for Horticultural Science* 142:143-+.
578   10.21273/Jashs04012-16

579 Dalbó MA, Ye GN, Weeden NF, Steinkellner H, Sefc KM, and Reisch BI. 2000. A gene
580   controlling sex in grapevines placed on a molecular marker-based genetic map. *Genome*
581   43:333-340. 10.1139/g99-136

582 Dinneny JR, Weigel D, and Yanofsky MF. 2006. *NUBBIN* and *JAGGED* define stamen and
583   carpel shape in *Arabidopsis*. *Development* 133:1645-1655. 10.1242/dev.02335

584 Ditta G, Pinyopich A, Robles P, Pelaz S, and Yanofsky MF. 2004. The *SEP4* gene of
585   *Arabidopsis thaliana* functions in floral organ and meristem identity. *Current Biology*
586   14:1935-1940. 10.1016/j.cub.2004.10.028

587 Dobritsa AA, Nishikawa S, Preuss D, Urbanczyk-Wochniak E, Sumner LW, Hammond A,
588   Carlson AL, and Swanson RJ. 2009a. LAP3, a novel plant protein required for pollen
589   development, is essential for proper exine formation. *Sexual Plant Reproduction* 22:167-
590   177. 10.1007/s00497-009-0101-8

591 Dobritsa AA, Shrestha J, Morant M, Pinot F, Matsuno M, Swanson R, Moller BL, and Preuss D.
592   2009b. CYP704B1 is a long-chain fatty acid omega-hydroxylase essential for
593   sporopollenin synthesis in pollen of *Arabidopsis*. *Plant Physiology* 151:574-589.
594   10.1104/pp.109.144469

595 Doligez A, Adam-Blondon AF, Cipriani G, Di Gaspero G, Laucou V, Merdinoglu D, Meredith
596   CP, Riaz S, Roux C, and This P. 2006. An integrated SSR map of grapevine based on
597   five mapping populations. *Theoretical and Applied Genetics* 113:369-382.
598   10.1007/s00122-006-0295-1

599 Dong XY, Hong ZL, Sivaramakrishnan M, Mahfouz M, and Verma DPS. 2005. Callose synthase
600   (CalS5) is required for exine formation during microgametogenesis and for pollen
601   viability in *Arabidopsis*. *Plant Journal* 42:315-328. 10.1111/j.1365-313X.2005.02379.x

602 Dorsey MJ. 1912. Variation in the floral structures of *Vitis*. *Bulletin of the Torrey Botanical Club*
603   39:37-52.

604 Enns LC, Kanaoka MM, Torii KU, Comai L, Okada K, and Cleland RE. 2005. Two callose
605   synthases, GSL1 and GSL5, play an essential and redundant role in plant and pollen
606   development and in fertility. *Plant Molecular Biology* 58:333-349. 10.1007/s11103-005-
607   4526-7

608 Favaro R, Pinyopich A, Battaglia R, Kooiker M, Borghi L, Ditta G, Yanofsky MF, Kater MM,
609   and Colombo L. 2003. MADS-box protein complexes control carpel and ovule
610   development in *Arabidopsis*. *The Plant Cell* 15:2603-2611. 10.1105/tpc.015123

611 Fechter I, Hausmann L, Daum M, Sorensen TR, Viehover P, Weisshaar B, and Topfer R. 2012.
612   Candidate genes within a 143 kb region of the flower sex locus in *Vitis*. *Molecular*
613   *Genetics and Genomics* 287:247-259. 10.1007/s00438-012-0674-z

614 Fernandez L, Torregrosa L, Terrier N, Sreekantan L, Grimplet J, Davies C, Thomas MR, Romieu
615   C, and Ageorges A. 2007. Identification of genes associated with flesh morphogenesis
616   during grapevine fruit development. *Plant Molecular Biology* 63:307-323.
617   10.1007/s11103-006-9090-2

618 Ferrandiz C, Pelaz S, and Yanofsky MF. 1999. Control of carpel and fruit development in
619   *Arabidopsis*. *Annual Review of Biochemistry* 68:321-354.
620   10.1146/annurev.biochem.68.1.321

621 Gaillard C, Moffatt BA, Blacker M, and Laloue M. 1998. Male sterility associated with APRT
622   deficiency in *Arabidopsis thaliana* results from a mutation in the gene *APT1*. *Molecular*
623   *and General Genetics* 257:348-353.

624 Gallardo A, Ocete R, López MÁ, Lara M, and Riviera D. 2009. Assessment of pollen
625   dimorphism in populations of *Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi in Spain.
626   *Vitis* 48:59-62.

627 Guan YF, Huang XY, Zhu J, Gao JF, Zhang HX, and Yang ZN. 2008. *RUPTURED POLLEN*
628   *GRAIN*1, a member of the MtN3/saliva gene family, is crucial for exine pattern formation
629   and cell integrity of microspores in *Arabidopsis*. *Plant Physiology* 147:852-863.
630   10.1104/pp.108.118026

631 Haas HU, and Alleweldt G. 2000. The karyotype of grapevine (*Vitis vinifera* L.). 528 ed:
632   International Society for Horticultural Science (ISHS), Leuven, Belgium. p 249-258.

633 Hardie WJ. 2000. Grapevine biology and adaptation to viticulture. *Australian Journal of*
634   *Agricultural Research* 6:74-81. 10.1111/j.1755-0238.2000.tb00165.x

635 Hedrick UP, and Anthony RD. 1915. Inheritance of certain characters of grapes. *New York*
636   *Agricultural Experiment Station Technical Bulletin*: 1-19.

637 Iizuka M, and Hashizume T. 1968. Induction of female organsin staminata grape by 6-
638   substituted adenine derivates. *JAPAN J GENETICS* 43:393-394.

639 Ingrouille MJ, Chase MW, Fay MF, Bowman D, Van der Bank M, and Bruijn ADE. 2002.
640   Systematics of Vitaceae from the viewpoint of plastid rbcL DNA sequence data.
641   *Botanical Journal of the Linnean Society* 138:421-432. 10.1046/j.1095-
642   8339.2002.00028.x

643 Ishiguro S, Watanabe Y, Ito N, Nonaka H, Takeda N, Sakai T, Kanaya H, and Okada K. 2002.
644   *SHEPHERD* is the *Arabidopsis GRP94* responsible for the formation of functional
645   CLAVATA proteins. *Embo Journal* 21:898-908. 1093/emboj/21.5.898

646 Jaillon O, Aury JM, Noel B, Policriti A, Clepet C, Casagrande A, Choisne N, Aubourg S, Vitulo
647   N, Jubin C, Vezzi A, Legeai F, Hugueney P, Dasilva C, Horner D, Mica E, Jublot D,
648   Poulain J, Bruyere C, Billault A, Segurens B, Gouyvenoux M, Ugarte E, Cattonaro F,

649   Anthouard V, Vico V, Del Fabbro C, Alaux M, Di Gaspero G, Dumas V, Felice N,

650   Paillard S, Juman I, Moroldo M, Scalabrin S, Canaguier A, Le Clainche I, Malacrida G,

651   Durand E, Pesole G, Laucou V, Chatelet P, Merdinoglu D, Delledonne M, Pezzotti M,

652   Lecharny A, Scarpelli C, Artiguenave F, Pe ME, Valle G, Morgante M, Caboche M,

653   Adam-Blondon AF, Weissenbach J, Quetier F, Wincker P, and French-Italian Public

654   Consortium for Grapevine Genome C. 2007. The grapevine genome sequence suggests

655   ancestral hexaploidization in major angiosperm phyla. *Nature* 449:463-467.

656   10.1038/nature06148

657 Levadoux L. 1956. Wild and cultivated populations of *Vitis vinifera* L. Annales de

658   l'Amelioration des Plantes. p 59-118.

659 Lewis WH. 1979. Polyploidy in angiosperms: dicotyledons. *Basic Life Sciences* 13:241-268.

660 Lewter J, Worthington ML, Clark JR, Varanasi AV, Nelson L, Owens CL, Conner P, and

661   Gunawan G. 2019. High-density linkage maps and loci for berry color and flower sex in

662   muscadine grape (Vitis rotundifolia). *Theoretical and Applied Genetics* 132:1571-1585.

663   10.1007/s00122-019-03302-7

664 Loomis N, and Williams C. 1957. A new genetic flower type of the muscadine grape. *Journal of*

665   *Heredity* 48:294-304.

666 Lowe KM, and Walker MA. 2006. Genetic linkage map of the interspecific grape rootstock cross

667   Ramsey (*Vitis champinii*) x Riparia Gloire (*Vitis riparia*). *Theoretical and Applied*

668   *Genetics* 112:1582-1592. 10.1007/s00122-006-0264-8

669 Manavalan LP, Chen X, Clarke J, Salmeron J, and Nguyen HT. 2012. RNAi-mediated disruption

670   of squalene synthase improves drought tolerance and yield in rice. *Journal of*

671   *Experimental Botany* 63:163-175. 10.1093/jxb/err258

672 Marguerit E, Boury C, Manicki A, Donnart M, Butterlin G, Nemorin A, Wiedemann-Merdinoglu

673   S, Merdinoglu D, Ollat N, and Decroocq S. 2009a. Genetic dissection of sex

674   determinism, inflorescence morphology and downy mildew resistance in grapevine.

675   *Theoretical and Applied Genetics* 118:1261-1278. 10.1007/s00122-009-0979-4

676 Marguerit E, Boury C, Manicki A, Donnart M, Butterlin G, Némorin A, Wiedemann-Merdinoglu

677   S, Merdinoglu D, Ollat N, and Decroocq S. 2009b. Genetic dissection of sex

678   determinism, inflorescence morphology and downy mildew resistance in grapevine.

679   *Theoretical and Applied Genetics* 118:1261-1278.

680   McGovern P, Jalabadze M, Batiuk S, Callahan MP, Smith KE, Hall GR, Kvavadze E,
681   Maghradze D, Rusishvili N, Bouby L, Failla O, Cola G, Mariani L, Boaretto E, Bacilieri
682   R, This P, Wales N, and Lordkipanidze D. 2017. Early Neolithic wine of Georgia in the
683   South Caucasus. *Proceedings of the National Academy of Sciences of the United States of*
684   *America* 114:E10309-E10318. 10.1073/pnas.1714728114

685   McGovern PE. 2004. *Ancient wine: the search for the origins of viniculture*: Princeton
686   University Press.

687   Moffatt B, and Somerville C. 1988. Positive selection for male-sterile mutants of *Arabidopsis*
688   lacking adenine phosphoribosyl transferase activity. *Plant Physiology* 86:1150-1154.

689   Mok DWS, and Mok MC. 2001. Cytokinin metabolism and action. *Annual Review of Plant*
690   *Physiology and Plant Molecular Biology* 52:89-118.

691   Negi SS, and Olmo HP. 1970. Studies on sex conversion in male *Vitis vinifera* L. (*sylvestris*).
692   *Vitis* 9:89-96.

693   Negi SS, and Olmo HP. 1971. Conversion and determination of sex in *Vitis vinifera* L.
694   (*sylvestris*). *Vitis* 9:265-279.

695   Nemhauser JL, Zambryski PC, and Roe JL. 1998. Auxin signaling in *Arabidopsis* flower
696   development? *Current Opinion in Plant Biology* 1:531-535.

697   Ni J, Shah FA, Liu W, Wang Q, Wang D, Zhao W, Lu W, Huang S, Fu S, and Wu L. 2018.
698   Comparative transcriptome analysis reveals the regulatory networks of cytokinin in
699   promoting the floral feminization in the oil plant Sapium sebiferum. *BMC Plant Biology*
700   18:96. 10.1186/s12870-018-1314-5

701   Oberle GD. 1938. A genetic study of variations in floral morphology and function in cultivated
702   forms of *Vitis*. *N Y AgrExpt Sta Tech Bul* 250:3-32.

703   Ohno CK, Reddy GV, Heisler MGB, and Meyerowitz EM. 2004. The *Arabidopsis JAGGED*
704   gene encodes a zinc finger protein that promotes leaf tissue development. *Development*
705   131:1111-1122. 10.1242/dev.00991

706   Olien WC. 1990. The Muscadine grape - botany, viticulture, history, and current industry.
707   *Hortscience* 25:732-739.

708   Palumbo F, Vannozzi A, Magon G, Lucchin M, and Barcaccia G. 2019. Genomics of Flower
709   Identity in Grapevine (Vitis vinifera L.). *Frontiers in Plant Science* 10.
710   10.3389/fpls.2019.00316

711 Patel GI, and Olmo HP. 1955. Cytogenetics of *Vitis:* I. the hybrid *V. Vinifera* x *V. Rotundifolia*.
712   *American Journal of Botany* 42:141-159. 10.2307/2438464

713 Pelaz S, Ditta GS, Baumann E, Wisman E, and Yanofsky MF. 2000. B and C floral organ
714   identity functions require *SEPALLATA* MADS-box genes. *Nature* 405:200-203.
715   10.1038/35012103

716 Picq S, Santoni S, Lacombe T, Latreille M, Weber A, Ardisson M, Ivorra S, Maghradze D,
717   Arroyo-Garcia R, Chatelet P, This P, Terral JF, and Bacilieri R. 2014. A small XY
718   chromosomal region explains sex determination in wild dioecious *V. vinifera* and the
719   reversal to hermaphroditism in domesticated grapevines. *BMC Plant Biology* 14:229.
720   10.1186/s12870-014-0229-z

721 Pinyopich A, Ditta GS, Savidge B, Liljegren SJ, Baumann E, Wisman E, and Yanofsky MF.
722   2003. Assessing the redundancy of MADS-box genes during carpel and ovule
723   development. *Nature* 424:85-88. 10.1038/nature01741

724 Popescu CF, Dejeu LC, and Ocete RR. 2013. Preliminary characterization of wild grapevine
725   populations *(Vitis vinifera* ssp . *sylvestris*) grown along the Danube river. *Not Bot Horti*
726   *Agrobo* 41:472-477.

727 Ramos MJ, Coito JL, Silva HG, Cunha J, Costa MM, and Rocheta M. 2014. Flower development
728   and sex specification in wild grapevine. *BMC Genomics* 15:1095. 10.1186/1471-2164-
729   15-1095

730 Ramos MJN, Coito J, Fino J, Cunha J, Silva H, de Almeida PG, Costa MMR, Amancio S, Paulo
731   OS, and Rocheta M. 2017. Deep analysis of wild *Vitis* flower transcriptome reveals
732   unexplored genome regions associated with sex specification. *Plant Molecular Biology*
733   93:151-170.

734 Riaz S, Krivanek AF, Xu K, and Walker MA. 2006. Refined mapping of the Pierce's disease
735   resistance locus, *PdR1*, and sex on an extended genetic map of *Vitis rupestris* x *V.*
736   *arizonica*. *Theoretical and Applied Genetics* 113:1317-1329. 10.1007/s00122-006-0385-
737   0

738 Royer C. 1888. Mouvement historiques de la vigne dans le monde. In: La Vigne et le Vin. Paris:
739   La Manufacture. 15-25.

740 Shpak ED, Berthiaume CT, Hill EJ, and Torii KU. 2004. Synergistic interaction of three
741   ERECTA-family receptor-like kinases controls *Arabidopsis* organ growth and flower

742 development by promoting cell proliferation. *Development* 131:1491-1501.
743 10.1242/dev.01028
744 Shpak ED, Lakeman MB, and Torii KU. 2003. Dominant-negative receptor uncovers
745 redundancy in the *Arabidopsis* ERECTA leucine-rich repeat receptor-like kinase
746 signaling pathway that regulates organ shape. *The Plant Cell* 15:1095-1110.
747 10.1105/tpc.010413
748 Siegfried KR, Eshed Y, Baum SF, Otsuga D, Drews GN, and Bowman JL. 1999. Members of the
749 YABBY gene family specify abaxial cell fate in *Arabidopsis*. *Development* 126:4117-
750 4128.
751 Soejima A, and Wen J. 2006. Phylogenetic analysis of the grape family (Vitaceae) based on three
752 chloroplast markers. *American Journal of Botany* 93:278-287. 10.3732/ajb.93.2.278
753 Sreekantan L, Torregrosa L, Fernandez L, and Thomas MR. 2006. VvMADS9, a class B MADS-
754 box gene involved in grapevine flowering, shows different expression patterns in mutants
755 with abnormal petal and stamen structures. *Functional Plant Biology* 33:877-886.
756 10.1071/FP06016
757 Stout AB. 1921. Types of flowers and intersexes in grapes with reference to fruit development.
758 *New York Agricultural Experiment Station Technical Bulletin* 82:1-16.
759 Tiffney BH, and Barghoorn ES. 1976. Fruits and Seeds of Brandon Lignite .1. Vitaceae. *Review*
760 *of Palaeobotany and Palynology* 22:169-191. 10.1016/0034-6667(76)90001-4
761 Torii KU, Mitsukawa N, Oosumi T, Matsuura Y, Yokoyama R, Whittier RF, and Komeda Y.
762 1996. The *Arabidopsis ERECTA* gene encodes a putative receptor protein kinase with
763 extracellular leucine-rich repeats. *The Plant Cell* 8:735-746. Doi 10.1105/Tpc.8.4.735
764 Ulmasov T, Hagen G, and Guilfoyle TJ. 1997a. *ARF1*, a transcription factor that binds to auxin
765 response elements. *Science* 276:1865-1868.
766 Ulmasov T, Murfett J, Hagen G, and Guilfoyle TJ. 1997b. Aux/IAA proteins repress expression
767 of reporter genes containing natural and highly active synthetic auxin response elements.
768 *The Plant Cell* 9:1963-1971. 10.1105/tpc.9.11.1963
769 Valleau WD. 1916. Inheritance of sex in the grape. *The American Naturalist* 50:554-564.
770 van Zanten M, Snoek LB, Proveniers MCG, and Peeters AJM. 2009. The many functions of
771 ERECTA. *Trends in Plant Science* 14:214-218. 10.1016/j.tplants.2009.01.010

772   Wolf T, Cabezas J-A, Martinez-Zapater J-M, and Cervera M-T. 2003. Genetic Characterization

773   of Closely Related Rootstocks Varieties Based on AFLP and SAMPL Markers. *Acta*

774   *horticulturae*.

775   Xiong AS, Yao QH, Peng RH, Li X, Han PL, and Fan HQ. 2005. Different effects on ACC

776   oxidase gene silencing triggered by RNA interference in transgenic tomato. *Plant Cell*

777   *Reports* 23:639-646. 10.1007/s00299-004-0887-7

778   Zhou Y, Minio A, Massonnet M, Solares E, Lv Y, Beridze T, Cantu D, and Gaut BS. 2019.

779   Structural variants, hemizygosity and clonal propagation in grapevines. *bioRxiv*:508119.

780   10.1101/508119

781   Zhou YF, Massonnet M, Sanjak JS, Cantu D, and Gaut BS. 2017. Evolutionary genomics of

782   grape (*Vitis vinifera* ssp. *vinifera*) domestication. *Proceedings of the National Academy*

783   *of Sciences of the United States of America* 114:11715-11720. 10.1073/pnas.1709257114

784   Zohary D, Hopf M, and Weiss E. 2000. *Domestication of plants in the Old World.*: New York:

785   Oxford University Press.

786
787

Figure 1

The most striking morphological aspect of both flower types.

Dissected flowers from *Vitis vinifera sylvestris.* (A and B) Male and female flower of *V. v. sylvestris* without petals. (B) In male flowers, it is possible to observe the undeveloped pistil and in (C) females the reflex stamens, that produce infertile pollen. Photo credit: Margarida Rocheta.

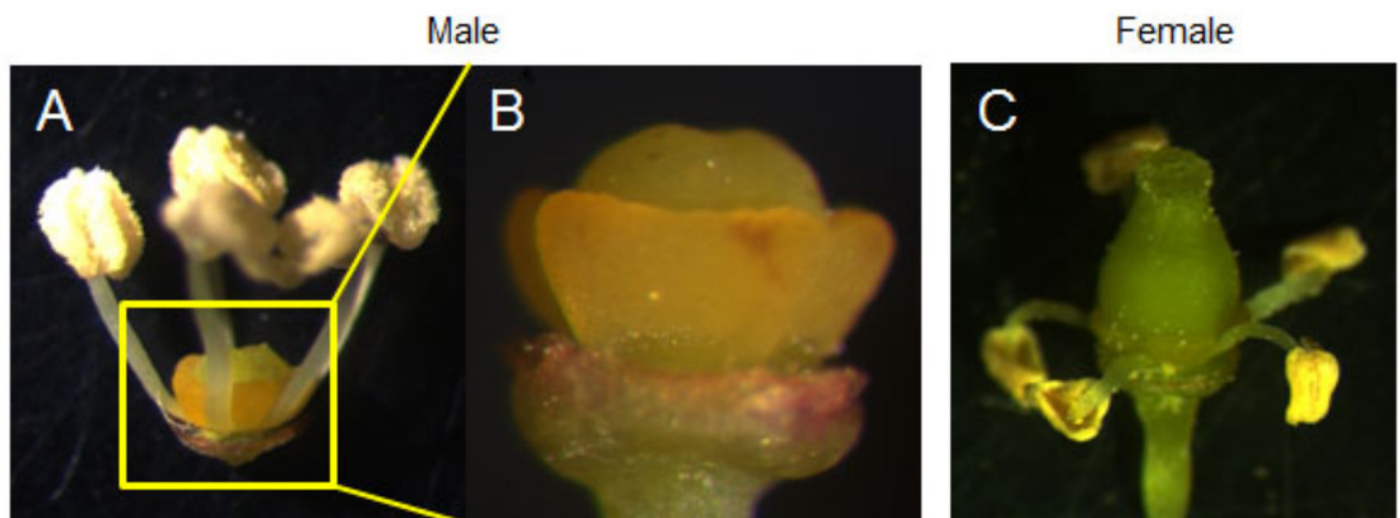



Figure 2

Variations in stamens and pistils in *Vitis* flower types

Scheme of some possible morphologic variations in stamens and pistils on hermaphrodite, female and male flowers (according to Stout (1921)). (A, B, C) Hermaphrodite plants hold both functional organs but might show variation in (B) stamens and (C) pistil size. (D to H) Female flowers show a functional pistil but several types of non-functioning stamens (producing infertile pollen). From (D) erect but crinkled stamens, (E and F) to semi-reflexed stamens, (G and H) and reflexed stamens. (I and J) Male plants exhibit several variations of non-functional pistils but fully functional stamens with variable size.

Hermaphrodite

A   B   C

Female

D

Erect crinkled stamens

E   F

Semi- reflexed stamens

G   H

Reflexed stamens

Male

I   J

Figure 3

Differences in the pistil of male plants

Diagram of the morphologic differences in the pistil of male flowers (according to Stout (1921)). (A) Include well-formed but non-functional pistils, (B and C) pistils with different sizes, and (D) a non-functional pistil with the complete absence (X) of style and stigma.

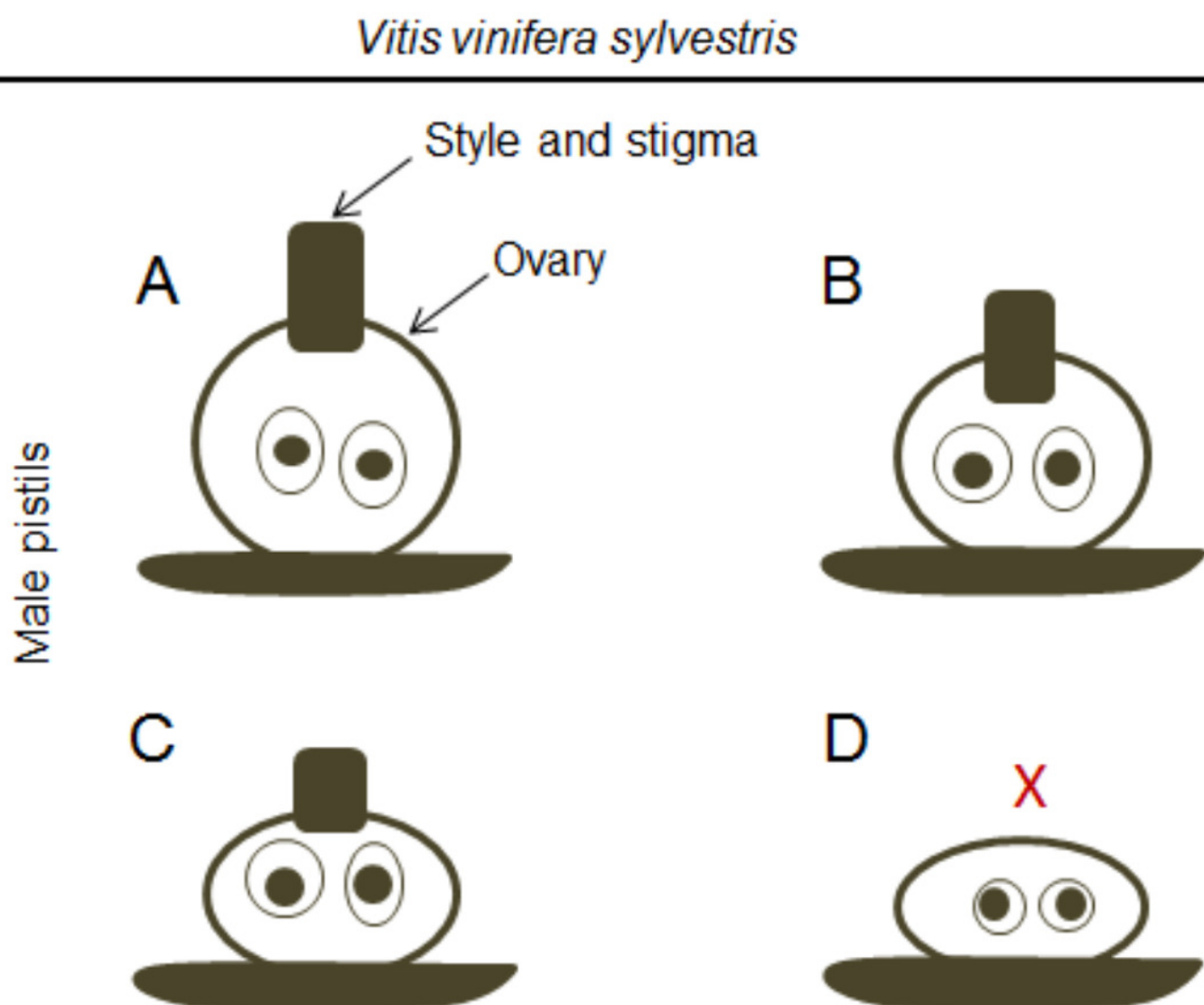



Figure 4

Diagram illustrating the digenic determinants of sex inheritance in *Vitis*

Illustration of the digenic determinants for flower sex in *Vitis* according to Oberle (1938), using the *So/so* and *Sp/sp* genes and assuming a homozygous form for female plants (*so sp/so sp*) and heterozygous for male (*So Sp/so sp* and *So Sp/so Sp*) and hermaphrodite (*so Sp/so sp* and *so Sp/so Sp*) plants.

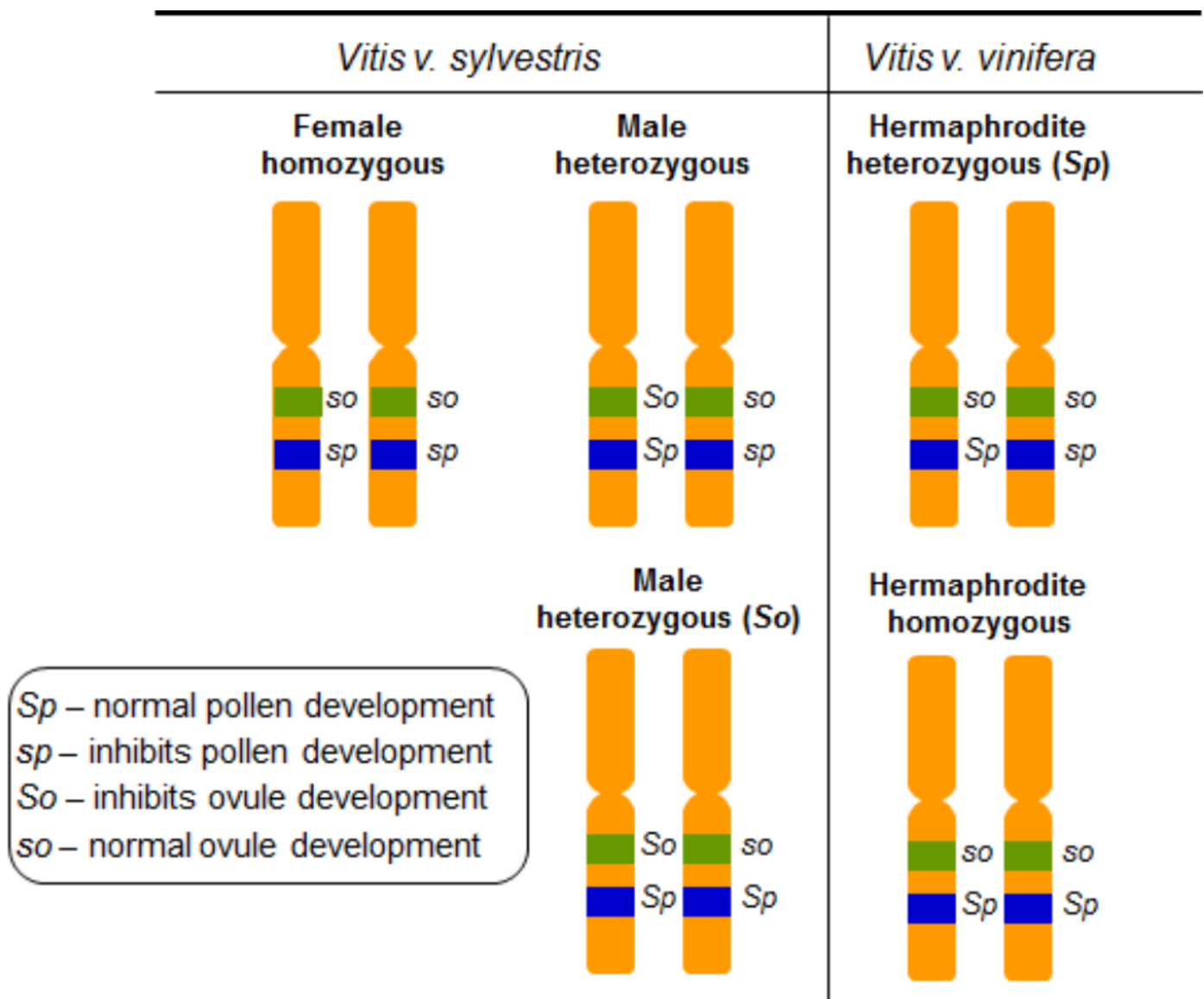
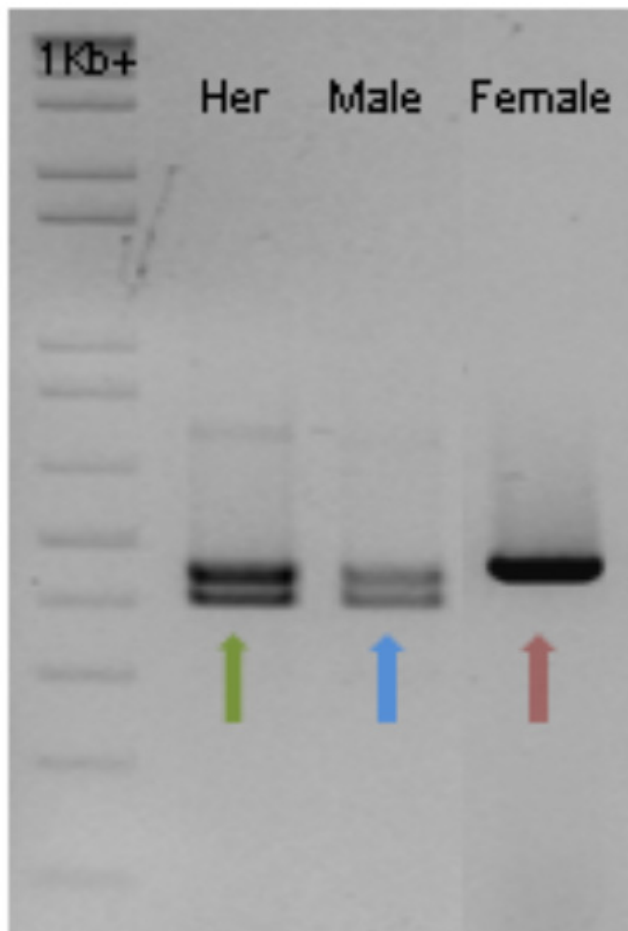
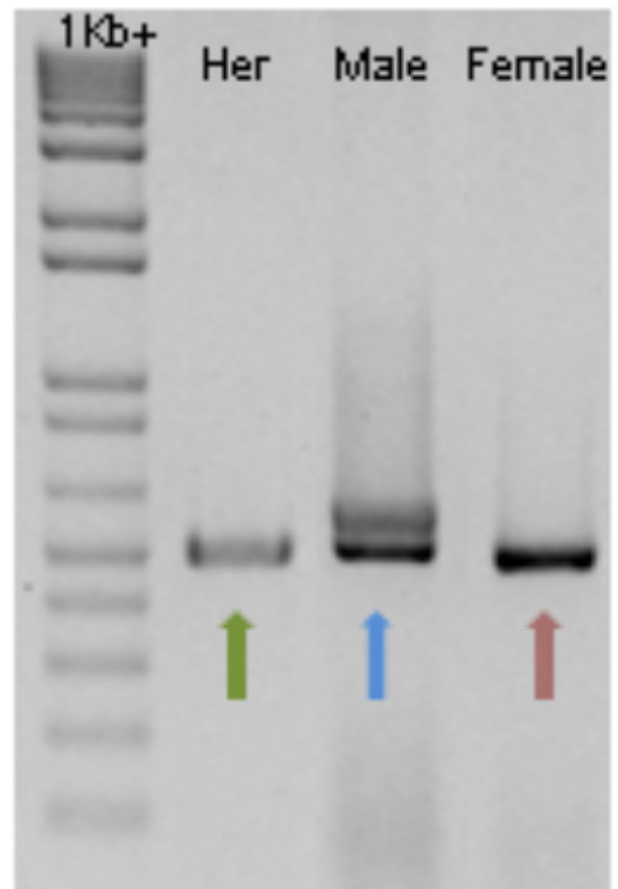



Figure 5

Schematic of the amplification of *VviFSEX* and *VviAPRT3*

Hermaphrodite (Her) plants show only one amplification fragment of *VviAPRT3* but two fragments of *VviFSEX*, male plants show two fragments of both *VviAPRT3* and *VviFSEX*, while female plants only show one amplification fragment of each genetic marker, adapted from Coito et al. (2017). According to Oberle (1938), and through this amplification profile, Her plants would be homozygous recessive for *SO* (*so so*) and heterozygous for *SP* (*Sp sp*), or HF, according to Antcliff (1980), likewise male plants would be heterozygous for both *SO* (*So so*) and *SP* (*SP sp)* or MF. Finally female plants would be homozygous recessive for both *SO* (*so so*) and *SP* (*sp sp*), or FF).

VviFSEX

VviAPRT3

→ Her   *so Sp / so sp* - HF

→ Male   *So Sp / so sp* - MF

→ Female *so sp / so sp* - FF

off

offoff

offoffoffoffoffoffoffoffoffoff

Chromosome 2
small determining sex
region

Genetic markers:
VviAPRT3 and *VviFSEX*

Regulators/
Key players

ETTIN

PINOID

APRT3

?

Repressed
Pistil

ACOS5

VIT_212s0057g00440

CalS5

?

LAP3

ERECTA

Nodulin
MtN3

AMS

Abnormal Stamens
Infertile Pollen

Male
Flower

Female
Flower

Sex determinants.

Sex determinants of hermaphrodites, males and females plants of grapevine according to several authors.

Table 1:

Sex determinants.

Sex determinants of hermaphrodites, males and females plants of grapevine according to several authors.

	Valleau, 1916	Oberle, 1938	Avramov, 1967	Negi and Olso, 1971	Antcliff, 1983
Hermaphrodite	HF HH	*so Sp/so sp* *so Sp/so Sp*	$S^h S^h$ $S^h S^f$	-	H
Male	MH MF	*So Sp/so Sp* *So Sp/so sp*	-	Su^F/Su^F Su^F/Su^m	*M*
Female	FF	*so sp/so sp*	S^f	Su^m/Su^m	*F*