

1 **Environmental conditions and agronomic practices induce consistent global changes in DNA**
2 **methylation patterns in grapevine (*Vitis vinifera* cv Shiraz).**

3

4 Huahan Xie^{1,2‡}, Moumouni Konate^{1,2‡}, Na Sai^{1,2,3}, Kiflu Gebremicael Tesfamicael^{1,2}, Timothy
5 Cavagnaro², Matthew Gilliham^{2,3}, James Breen^{4,5}, Andrew Metcalfe⁶, Roberta DeBei², Cassandra
6 Collins², Carlos Marcelino Rodriguez Lopez^{1,2*}

7

8 ¹Environmental Epigenetics and Genetics Group, University of Adelaide, Waite Campus, PMB1
9 Glen Osmond, SA, 5064.

10 ²The Waite Research Institute and The School of Agriculture, Food and Wine, University of
11 Adelaide, Waite Campus, PMB1 Glen Osmond, SA, 5064

12 ³The ARC Centre of Excellence in Plant Energy Biology, University of Adelaide, Waite Campus,
13 PMB1 Glen Osmond, SA, 5064.

14 ⁴Robinson Research Institute, University of Adelaide, Adelaide 5005, Australia

15 ⁵University of Adelaide Bioinformatics Hub, School of Biological Sciences, University of Adelaide,
16 Adelaide 5005, Australia

17 ⁶School of Mathematical Sciences, University of Adelaide, Adelaide 5005, Australia

18 [‡]Both authors contributed equally.

19

20 **Corresponding author*

21 **Correspondence:**

22 Dr. Carlos Marcelino Rodriguez Lopez

23 University of Adelaide, Plant Research Centre

24 School of Agriculture, Food and Wine, Waite Campus, Adelaide, SA, 5064, Australia.

25 carlos.rodriguezlopez@adelaide.edu.au

26 **Abstract**

27 Fruit attributes that affect wine quality are thought to be largely driven by the interaction of
28 grapevine's genetic characteristics with environmental factors (i.e. climate, soil and topography) and
29 vineyard management. All these variables, in conjunction with the wine making process, give a wine
30 its distinctive character. Understanding how grapevines perceive and adapt to a changing
31 environment will provide us with an insight into how to better manage crop quality. Mounting
32 evidence suggests that epigenetic mechanisms are a key interface between the environment and the
33 genotype that ultimately affect the plant's phenotype. Moreover, it is now widely accepted that
34 epigenetic mechanisms are a source of useful variability during crop varietal selection that could
35 affect crop performance. While the contribution of DNA methylation to plant performance has been
36 extensively studied in other major crops, very little work has been done in grapevine. Here we used
37 Methylation Sensitive Amplified Polymorphisms to obtain global patterns of DNA methylation, and
38 to identify the main drivers of epigenetic diversity across 22 vineyards planted with the cultivar
39 Shiraz in six distinctive wine areas of a major wine zone, The Barossa, South Australia. The
40 observed epigenetic profiles showed a high level of differentiation that grouped vineyards by their
41 area of provenance despite the low genetic differentiation between vineyards and sub-regions.
42 Furthermore, pairwise epigenetic distances between vineyards with similar management systems
43 showed a significant correlation with geographic distance. Finally, methylation sensitive Genotyping
44 By Sequencing identified 3,598 differentially methylated genes that were assigned to 1,144 unique
45 GO terms of which 8.6% were associated with response to environmental stimulus. Taken together,
46 our results indicate that the intensity and directionality of DNA methylation differentiation between
47 vineyards and wine sub-regions within The Barossa are driven by management and local growing
48 conditions. Finally, we discuss how epigenetic variability can be used as a tool to understand and
49 potentially modulate terroir in grapevine.

50 **Introduction**

51 The ability of plants to produce alternative phenotypes in response to changing environments is
52 known as phenotypic plasticity (Pigliucci, 2005). Genotypes with this characteristic are able to
53 produce a variety of phenotypes including improved growth and reproduction (Lacaze et al., 2009).
54 Grapevine (*Vitis vinifera* L.) is a highly-plastic crop that exhibits large differences in fruit quality
55 from a given variety depending upon the environmental conditions of the vineyard of origin (Dal
56 Santo et al., 2016). Fruit traits that affect wine quality are thought to be largely driven by the
57 interaction of a grapevine's genetic characteristics with environmental factors (i.e. climate, soil and
58 topography) and vineyard management (Robinson et al., 2012). All these variables, in conjunction
59 with the wine making process, give a wine its distinctive character. The impact of the environment on
60 grape quality and subsequent wine excellence has given rise to the concept of 'terroir', a French term
61 referring to *terre*, "land" (Fanet and Brutton, 2004).

62
63 Terroir is defined as the interaction between the physical and biological environment and applied
64 viticultural and oenological practices that lead to unique characteristics in a final wine (Seguin,
65 1986). Extensive studies have been published on terroir, but generally, these focus on a single
66 parameter such as climatic factors, soil structure or soil microbiology (Harrison, 2000; Tonietto and
67 Carbonneau, 2004;). However, studying only one environmental parameter does not provide an entire
68 understanding of how wine quality is influenced by terroir (Van Leeuwen et al., 2004). A significant
69 amount of work has also been published on the genetic basis of fruit quality in grapevines (eg.
70 Doligez et al, 2002). Despite these insights, further research is required on the molecular changes that
71 are involved in the vine interaction with its environment.

72
73 One of the molecular changes worth investigating relates to environmentally induced epigenetic
74 modifications. In fact, phenotypic plasticity has been previously associated to epigenetic variation

75 (Vogt, 2015). Interestingly, analysis of epigenetic diversity has been shown to be more effective in
76 discriminating inter-clonal variability in grapevine than the use of purely genetic molecular markers
77 such as simple sequence repeats (SSRs) or amplified fragment length polymorphisms (AFLPs)
78 (Imazio et al., 2002; Schellenbaum et al., 2008; Ocaña et al., 2013).

79

80 Epigenetic mechanisms refer to molecular changes that affect gene expression without changing the
81 organism DNA sequence (Jaenisch and Bird, 2003; Haig, 2004). Epigenetic mechanisms act as an
82 interface between the environment and the genotype by regulating gene expression in response to
83 development and environmental cues and, ultimately affect the plant's phenotype (Tricker et al.,
84 2012; Kumar et al., 2016). Epigenetic priming is an adaptive strategy by which plants use their
85 memory of the environment to modify their phenotypes to adapt to subsequent conditions (Kelly et
86 al., 2012; Tricker et al., 2013a; 2013b, Vogt, 2015). It is now also widely accepted that epigenetic
87 mechanisms have been the source of useful variability during crop varietal selection (Amoah et al.,
88 2012; Bloomfield et al., 2014; Rodríguez López and Wilkinson, 2015). Of the known epigenetic
89 mechanisms, cytosine methylation (5mC) is arguably the best understood (Goldberg et al., 2007). In
90 plants, 5mC occurs at different cytosine contexts (CpG, CpHpG or CpHpH) (H = A, T or C)
91 (Richards, 1997) and it is induced, maintained or removed by different classes of methyltransferase
92 in conjunction with environmental and developmental cues (Jaenisch and Bird, 2003). It is
93 commonly accepted that DNA methylation constitutes an adaptation strategy to the environment
94 (YunLei et al., 2009), and that changes in DNA methylation can produce altered phenotypes (Zhang
95 et al., 2006; Herrera and Bazaga, 2011; Iqbal et al., 2011). To this extent, there have been extensive
96 studies establishing a link between DNA methylation in plants and environmental conditions
97 (Fonseca Lira-Medeiros et al., 2010; Herrera and Bazaga, 2010; Alonso et al., 2016).

98

99 The contribution of DNA methylation to plant performance has been extensively studied in model
100 organisms and some annual crops (Rodríguez López and Wilkinson, 2015). However, we are only
101 beginning to understand how long-living plants, such as grapevines, use epigenetic mechanisms to
102 adapt to changing environments (Fortes and Gallusci, 2017). Effects of environmental conditions on
103 non-annual crops performance can be very difficult to evaluate since many environmental factors
104 interact over the life of the plant to ultimately contribute towards the plant's phenotype (Fortes and
105 Gallusci, 2017). Although epigenetic mechanisms have been shown to act as a system that allows
106 information storage in organisms across all kingdoms (Levenson and Sweatt, 2005), very few studies
107 have focussed on DNA methylation changes in grapevine. The few known studies in this field used
108 Methylation Sensitive Amplified Polymorphisms (MSAPs) (Reyna-López et al., 1997) for the
109 detection of *in vitro* culture induced epigenetic somaclonal variability (Baránek et al., 2015), and the
110 identification of commercial clones (Imazio et al., 2002; Schellenbaum et al., 2008; Ocaña et al.,
111 2013). However, these studies did not investigate the molecular drivers of terroir.

112 In this study, we hypothesize that DNA methylation can play a role in defining terroir. To test this
113 hypothesis we investigated the effect of environmental and management conditions on DNA
114 methylation variation in grapevine cultivar Shiraz across 22 vineyards representative of The Barossa
115 wine zone (Australia) (Robinson and Sandercock, 2014) using MSAPs. Finally, we used methylation
116 sensitive Genotyping By Sequencing to characterize the genomic context of the observed regional
117 genetic and epigenetic variability.

118

119 **Material and Methods**

120 **Vineyard selection and plant material**

121 Vines from 22 commercial vineyards located in the iconic Barossa wine zone (The Barossa hereafter)
122 (Australia) were included in this study. Vineyards were selected to be representative of the two
123 Barossa Regions as described by the Barossa Grounds Project (Robinson and Sandercock, 2014) (i.e.
124 Eden Valley (three vineyards) and Barossa Valley (19 vineyards which included vineyards in the five
125 distinctive sub-regions within the Barossa Valley Region: Northern Grounds (four vineyards),
126 Central Grounds (four vineyards), Eastern Edge (four vineyards), Western Ridge (four vineyards),
127 Southern Grounds (three vineyards)) (Table S1). To simplify the nomenclature, the Eden Valley
128 region, Northern, Central, Southern Grounds, Eastern Edge and Western Ridge will be defined as
129 sub-regions hereafter. All vineyards were planted with own-rooted vines of the cv Shiraz. Ten
130 vineyards were planted with clone SA 1654 (Whiting, 2003), one with clone BVRC30 (Whiting,
131 2003), one with clone PT15 Griffith (Farquhar, 2005) and 10 of ‘unknown’ clonal status (Table S1).

132

133 A total of 198 plants (nine plants per vineyard) were selected to capture the diversity from each
134 vineyard. Leaf samples (first fully expanded leaf at bud burst, E-L 7) (Coombe, 1995) were collected
135 from three nodes per plant and pooled into a single sample per plant. All samples were taken before
136 dawn (between 10:00 pm and sunrise) to minimize variability associated with differences in plant
137 water status (Williams and Araujo, 2002). Samples were immediately snap-frozen in liquid nitrogen
138 in the vineyard and stored at -80°C until DNA extraction.

139

140 **DNA Isolation**

141 Genomic DNA (gDNA) extractions from all 198 samples were performed using the three pooled
142 leaves per plant powdered using an automatic mill grinder (Genogrinder). The obtained frozen
143 powder was used for DNA extraction using the Oktopure automated DNA extraction platform (LGC
144 Genomics GmbH) according to the manufacturer’s instructions. Isolated DNA was quantified using

145 the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). DNA final
146 concentrations were normalised to 20 ng/µl using nanopure water (Eppendorf, Germany).

147

148 **Analysis of genetic/epigenetic variability using MSAP**

149 MSAP analysis was performed as described by Rodríguez López *et al.*, (2012). In brief, genomic
150 DNA from 88 plants (four plants per vineyard) was digested with a combination of the restriction
151 enzymes *EcoRI* and one of two DNA methylation sensitive isoschizomers (*HpaII* or *MspI*). Double
152 stranded DNA adapters (See Table S2 for the sequence of all oligonucleotides used) containing co-
153 adhesive ends complementary to those generated by *EcoRI* and *HpaII/MspI* were ligated to the
154 digested gDNA and then used as a template for the first of two consecutive selective PCR
155 amplifications in which the primers were complementary to the adaptors but possessed unique 3'
156 overhangs. The second selective PCR amplification used primers containing 3' overhangs previously
157 tested on grapevine (Baránek *et al.*, 2015). *HpaII/MspI* selective primer was 5' end-labelled using a
158 6-FAM reporter molecule for fragment detection using capillary electrophoresis on a ABI PRISM
159 3130 (Applied Biosystems, Foster City, CA) housed at the Australian Genome Research Facility Ltd,
160 Adelaide, South Australia.

161

162 Generated electropherograms were visualized using GeneMapper Software v4 (Applied Biosystems,
163 Foster City, CA). A binary matrix containing presence (1) absence (0) epilocus information was
164 generated for each enzyme combination (i.e. *EcoRI/HpaII* and *EcoRI/MspI*). MSAP fragment
165 selection was limited to allelic sizes between 95 and 500 bp to reduce the potential impact of size co-
166 migration during capillary electrophoresis (Caballero *et al.*, 2008). Different levels of hierarchy were
167 used to group the samples. Samples were first grouped according to vineyard of origin. Then,
168 samples were divided into their sub-regions of origin. Finally, samples were further separated into

169 groups according to clones and the vineyard management systems (i.e. pruning system used in their
170 vineyard of origin) (Table S1).

171

172 *HpaII* and *MspI* binary matrices were then used to compute Shannon's Diversity Index implemented
173 using *msap* R package (v. 1.1.8) (Perez-Figueroa, 2013) and Principal Coordinate Analysis (PCoA)
174 was estimated in all regions to determine and visualize the contribution to the observed molecular
175 variability within regions of non-methylated polymorphic loci (NML) and of methylation sensitive
176 polymorphic loci (MSL) (genetic and epigenetic variability respectively) (Smouse et al., 2015).

177

178 GenAlex v 6.5 software (Peakall and Smouse, 2006) was used for Principal Coordinate Analysis
179 PCoA in order to visualise the molecular differentiation between Barossa sub-regions detected using
180 MSAP profiles generated after the restriction of gDNA with *HpaII* or *MspI*. We then used analysis of
181 molecular variance (AMOVA) to determine the structure of the observed variability using PCoA.
182 Molecular differences between vineyards and regions was inferred as pairwise PhiPT distances
183 (Michalakis and Excoffier, 1996).

184

185 Mantel test analysis (Hutchison and Templeton, 1999) was used to estimate the correlation between
186 the calculated pairwise molecular distances with 1. the geographic distance (GeoD) (i.e.
187 $\text{Log}(1+\text{GeoD (km)})$) and 2. differences in environmental variables among vineyards (i.e, vineyard
188 altitude, regional average annual rainfall, regional growing season rainfall, regional mean January
189 temperature, regional growing season temperature and growing degree days). Mantel test was
190 implemented in Genalex v 6.5 as described by Rois et al., (2013) and significance was assigned by
191 random permutations tests (based on 9,999 replicates).

192

193 **Characterization of genetic/epigenetic variability using msGBS**

194 msGBS was performed as described by Kitimu et al (2015). In brief, 200ng of genomic DNA from 9
195 samples from Northern, Central and Southern Grounds (vineyards 1-4, 5-8 and 13-15 respectively)
196 were digested using 8U of HF-*EcoRI* and 8 U of *MspI* (New England BioLabs Inc., Ipswich, MA,
197 USA) in a volume of 20 μ l containing 2 μ l of NEB Smartcut buffer at 37 $^{\circ}$ C for 2 h followed by
198 enzyme inactivation at 65 $^{\circ}$ C for 10 min. Sequencing adapters were ligated by adding 0.1 pmol of the
199 *MspI* adapters (uniquely barcoded for each of the 198 samples) and 15 pmol of the common *EcoRI* Y
200 adapter (See Table S2 for the sequence of all oligonucleotides used), 200 U of T4 Ligase and T4
201 Ligase buffer (New England BioLabs Inc., Ipswich, MA, USA) in a total volume of 40 μ l at 24 $^{\circ}$ C for
202 2 h followed by an enzyme inactivation step at 65 $^{\circ}$ C for 10 min. Excess adapters were removed from
203 ligation products using Agencourt AMPure XP beads (Beckman Coulter, Australia) at the ratio of
204 0.85 and following manufacturer's instructions. Single sample msGBS libraries were then quantified
205 using Qbit 3 (Thermofisher). A single library was generated by pooling 25 ng of DNA from each
206 sample. Library was then amplified in 8 separate PCR reactions (25 μ l each) containing 10 μ l of
207 library DNA, 5 μ l of 5x Q5 high fidelity buffer, 0.25 μ l polymerase Q5 high fidelity, 1 μ l of each
208 forward and reverse common primers at 10 μ M, 0.5 μ l of 10 μ M dNTP and 7.25 μ l of pure sterile
209 water. PCR amplification was performed in a BioRad T100 thermocycler consisting of DNA
210 denaturation at 98 $^{\circ}$ C (30 s) and 10 cycles of 98 $^{\circ}$ C (30 s), 62 $^{\circ}$ C (20 s) and 72 $^{\circ}$ C (30 s), followed by
211 72 $^{\circ}$ C for 5 minutes. PCR products were then re-pooled and DNA fragments ranging between 200 and
212 350 bp in size were captured using the AMPure XP beads following manufacturer's instructions.
213 Libraries were sequenced using an Illumina NextSeq High Output 75bp pair-end run (Illumina Inc.,
214 San Diego, CA, USA) at the Australian Genome Research Facility (AGRF, Adelaide, Australia).

215

216 **msGBS Data analysis**

217 Analysis of genetic diversity between regions was performed by single nucleotide polymorphism
218 (SNP) calling using TASSEL 3 (Bradbury et al, 2007) on msGBS sequencing results. Only SNPs
219 present in at least 80% of the samples were considered for analysis. Principal component
220 analysis (PCA) was implemented on TASSEL 3 using the selected SNPs. To identify any possible
221 geographical genetic structure, the optimal number of genetic clusters present in the three regions
222 were computed using Bayesian Information Criterion (BIC) as effected by Discriminant Analysis of
223 Principal Components (DAPC) using adegenet 2.0.0 (<http://adegenet.r-forge.r-project.org/>).

224

225 Identification of significant differentially methylated markers (DMMs) between regions was then
226 computed using the package *msgbsR* (<https://github.com/BenjaminAdelaide/msgbsR>), accessed on
227 26/08/2016). In brief, raw sequencing data was first demultiplexed using GBSX (Herten et al, 2015)
228 and filtered to remove any reads that did not match the barcode sequence used for library
229 construction. Following demultiplexing, paired-end reads were merged using *bbmerge* in *bbtools*
230 package (Bushnell, 2016). Merged reads were next aligned to the 12X grapevine reference genome
231 (http://plants.ensembl.org/Vitis_vinifera/). Alignment BAM files were then used to generate a read
232 count matrix with marker sequence tags, and used as source data to perform subsequent analyses
233 using *msgbsR* in the R environment (R Core Team, 2015). Finally, the presence of differential
234 methylation between regions was inferred from the difference in the number of read counts from all
235 sequenced *MspI* containing loci that had at least 1 count per million (CPM) reads and present in at
236 least 20 samples per region. Significance threshold was set at Bonferroni adjusted P-value (or false
237 discovery rate, FDR) < 0.01 for difference in read counts per million. The *logFC* (logarithm 2 of fold
238 change) was computed to evaluate the intensity and direction of the region specific DNA methylation
239 polymorphism.

240

241 To determine how the observed changes in DNA methylation between sub-regions were associated to
242 protein coding genes, the distribution of DMMs was assessed around such genomic features, as
243 defined in Ensembl database (<http://plants.ensembl.org/biomart/martview/>), by tallying the number of
244 DMMs between the transcription start site (TSS) and the transcription end site (TES) and within five
245 1 Kb windows before the TSS and after TES of all *V. Vinifera* genes, using *bedtools* (Quinlan and
246 Hall, 2010).

247

248 Genes within 5 Kb of a DMM were referred to as differentially methylated genes (DMGs). DMGs in
249 each pairwise regional comparison were grouped into those showing hypermethylation or
250 hypomethylation, and were next used separately for GO terms enrichment, using the R packages:
251 *GO.db* (Carlson, 2016) and *annotate* (Gentlemen, 2016). Significant GO terms were selected based
252 on Bonferroni adjusted P-values at significance threshold of 0.05. Finally, GO terms containing
253 DMGs in all three pairwise comparisons were visualized using Revigo (Supek et al, 2011).

254

255 **Results**

256 Analysis of MSAP profiles obtained from DNA extractions of the first fully expanded leaf of 88
257 individual vines selected from 22 commercial vineyards within the six Barossa sub-regions (Figure 1;
258 Table S1) yielded 215 fragments comprising 189 from *MspI* and 211 from *HpaII*, of which 80% and
259 84% respectively, were polymorphic (i.e. not present in all the analysed samples/replicates when
260 restricted with one of the isoschizomers).

261

262 **Analysis of genome/methylome differences within wine sub-regions in The Barossa.**

263 PCoA of the MSAP profiles generated from non-methylated polymorphic loci (NML) (genetic
264 variability) and by methylation sensitive polymorphic loci (MSL) (epigenetic variability) (Pérez-
265 Figueroa, 2013) revealed a higher separation between vineyards when using epigenetic information
266 than when using genetic (Figure S1). The capacity of both types of variability to differentiate
267 between vineyards was more evident on vineyards in the Southern Grounds (Figure 2 C-D). Both
268 PCoA analysis and Shannon's diversity index showed significantly higher epigenetic than genetic
269 diversity for all sub-regions (Figure S2, Table 1). Among sub-regions, Southern Grounds had the
270 highest epigenetic diversity (0.581 ± 0.124) and Western Ridge the lowest (0.536 ± 0.143). Genetic
271 diversity showed the highest value in the Southern Grounds (0.374 ± 0.143) and the lowest in the
272 Northern Grounds (0.240 ± 0.030).

273

274 **Analysis of genome/methylome differences between wine sub-regions in The Barossa.**

275 We used analysis of the molecular variance (AMOVA) (Table 2) to obtain an overview of the
276 molecular variability between all the studied sub-regions. Overall, MSAP profiles generated using
277 restriction enzyme *MspI* achieved better separation between sub-regions than those generated using
278 *HpaII*. Of all 30 calculated molecular pairwise distances between sub-regions (PhiPTs), 25 were
279 significant ($P < 0.05$) (Table 2). Calculated PhiPT values ranged from 0.115 (PhiPT of Northern
280 Grounds vs Southern Grounds calculated using *MspI*) and 0.012 (PhiPT of Central Grounds vs
281 Eastern Edge calculated using *HpaII*).

282

283 AMOVA on MSAP profiles indicates that the majority of the observed variability is explained by
284 differences within vineyards (81% using profiles generated with *MspI* and 91% with *HpaII*). A
285 significant proportion of the total variability detected was associated to differences between
286 vineyards (17% with *MspI* and 8% with *HpaII*) and 2% and 1% was due to differences between sub-
287 regions (*MspI* and *HpaII* respectively).

288

289 **Effect of vineyard location on methylome differentiation**

290 To determine if environmental differences between vineyards influenced the observed epigenetic
291 differences we studied the vineyards' pairwise geographic and molecular distances correlation.
292 Vineyards located on the North-South axis of the Barossa Valley (i.e, vineyards 1, 2, 3 and 4
293 (Northern Grounds), 5, 6, 7 and 8 (Central Grounds), and 13, 14 and 15 (Southern Grounds)) (Figure
294 2A) were selected as Northern and Southern Grounds showed the greatest epigenetic differentiation
295 (Table 2). PCoA analysis showed that Central Grounds samples occupied an intermediate Eigen
296 space between Northern and Southern Grounds samples with coordinate 1 (24% of the observed
297 variability) representing the North-South axis (Figure 2B). Moreover, Mantel test showed a
298 significant ($P = 0.0003$) positive correlation ($R^2=0.3066$) between pairwise vineyard epigenetic and
299 geographic distances (Figure 2C). Then, Mantel test analysis was implemented to compare the
300 observed molecular differences against environmental variables. Differences in vineyard altitude
301 showed a small but significant positive correlations ($R^2=0.1615$, $P=0.013$) with PhiPT values
302 between vineyards (Figure S3). We then investigated if clone and vineyard management systems
303 could be contributing to this correlation, by comparing the epigenetic/geographic distances
304 correlation of 10 vineyards planted with clone 1654 (vineyards 1 and 4 (Northern Grounds), 7
305 (Central Grounds), 9 and 12 (Eastern Ridge), 15 (Southern Grounds) 16, 17, 18 and 19 (Western
306 Ridge) (Figure 3A) and of 6 vineyards planted with the same clone (1654) and trained using the same
307 pruning system (i.e. spur pruning) (vineyards 1 (Northern Grounds), 7 (Central Grounds), 9 (Eastern
308 Ridge), 15 (Southern Grounds) 16 and 19 (Western Ridge) (Figure 4A)). Again, PCoA shows that the
309 main contributor (23-24%) to the detected variability is associated to the distribution of the vineyards
310 on the N-S axis. Mantel test showed a positive correlation for both epigenetic/geographic distance
311 comparisons, however, although both correlations were significant ($P < 0.05$), the correlation among

312 vineyards pruned using the same system (Figure 4B-C) was higher than that observed when all
313 pruning systems were incorporated in the analysis (Figure 3B-C).

314

315 **msGBS analysis of genome/methylome differentiation between Northern, Central and Southern** 316 **Grounds**

317 TASSEL 3 was then implemented on msGBS data for single nucleotide polymorphism (SNP) calling
318 from 99 samples collected in 11 vineyards in the Northern, Central and Southern Grounds sub-
319 regions. This generated a total of 8,139 SNPs of which 4,893 were present in at least 80% of the
320 sequenced samples. PCA analysis using filtered SNPs showed very low level of genetic structure,
321 with only five plants from vineyard 3 (Northern Grounds) separating from the rest (Figure S4A).
322 However, this clustering was not supported by DAPC (i.e. the optimal clustering solution should
323 correspond to the lowest BIC) which indicated the optimal number of clusters for this data set is 1
324 (Figure S4B) suggesting a lack of genetic structure in the vineyards/regions analysed.

325 PC-LDA analysis was then used to visualize differences in DNA methylation detected using msGBS.
326 DNA methylation profiles clustered samples by their sub-region of origin, with Northern and Central
327 Grounds being separated by differential factor (DF1) from Southern Grounds while DF2 separated
328 Northern from Central Grounds (Figure 5). These results were supported by the higher number of
329 DMMs found when comparing samples from Southern to samples from Central or Northern Grounds
330 than when comparing Northern to Central (Table 3).

331 We next investigated the association of the detected DMMs to annotated protein-coding genes in the
332 grapevine genome by surveying their location and density within and flanking such genomic features.
333 A total of 3,598 genes were deemed differentially methylated (i.e. presented one or more DMMs
334 within 5kb of the TSS or the TSE) or within genes (Table 3). Quantification of such DNA
335 methylation changes showed that, in average, methylation levels are higher in the northern most

336 region in each comparison (i.e. NG > CG > SG) (Figure 6A). The majority of detected DMMs
337 associated to a gene were present in the body of the gene and the number of DMMs decreased
338 symmetrically with distance from the TSS and the TES (Figure 6B and Tables S3A-C). Finally, as
339 observed with all DMMs, the comparison between Northern and Central Grounds samples showed
340 the lowest number of DMGs (Table 3, Figure 6C and Supplementary Tables S3A-C).

341 To gain further insight into the functional implications of the DNA methylation differences
342 detected between sub-regions, we used *GO.db* (Carlson, 2016) and *annotate* (Gentleman, 2016) to
343 assign 1144 unique GO terms to the observed DMGs (Adjusted P value <0.05). As observed with
344 DMMs and DMGs the comparison between Northern and Central Grounds samples showed the
345 lowest number of GO terms containing differentially methylated genes (DMGOs) (Table 3, Figure
346 6C and Tables S3A-C). REViGO semantic analysis of GO terms shared by all 3 pairwise regional
347 comparisons (Figure 7) showed an increase of gene enrichment (i.e. a decrease in adjusted P values)
348 with geographic distance (e.g. see Figure 7 for comparisons between Northern Grounds and Southern
349 Grounds (A-B) and Central Grounds and Northern Grounds (C-D). 311 DMGs (8.6% of the total)
350 were allocated in GO terms associated to response to environmental stimulus (161 and 150 abiotic
351 and biotic challenges respectively) (Figure 7, Tables S4A-B), which included GO terms in the
352 semantic space of plant response to light, temperature, osmotic/salt stress and defence to biotic
353 stimulus.

354

355 **Discussion**

356 **Grapevine DNA methylation patterns are region specific**

357 Analysis of *HpaII* and *MspI* generated MSAP profiles showed that the methylation profiles of the six
358 different sub-regions were significantly different (P<0.05) in 25 of the 30 possible pairwise

359 comparisons (Table 2). Variability among vineyards and sub-regions was higher in *MspI* generated
360 profiles (17 and 2%) than in *HpaII* profiles (8 and 1%), indicating that the detected regional
361 epigenetic differences are, at least partially, sequence context specific (Tricker et al., 2012; Meyer,
362 2015). Calculated PhiPT values showed low levels of molecular differentiation between sub-regions,
363 even when those differences were statistically significant (Table 2). This could be explained by the
364 high proportion of the total variability associated to differences between individual plants (81-91%)
365 compared to 1-2% associated to differences between sub-regions. Such high levels of molecular
366 differentiation between individuals could be due to the random accumulation of somatic variation
367 with age, which can be genetic or epigenetic in nature. PCA of genetic polymorphisms detected using
368 msGBS results showed a high level of genetic variability between plants (Figure S4A) which is
369 characteristic of long living plants in general (Baali-Cherif and Besnard, 2005) and in grapevine in
370 particular (Torregrosa et al., 2011). However, Discriminant Analysis of Principal Components did
371 not detect any sample clustering associated to their origin (Figure S4B) indicating that genetic
372 diversity is not structured in a geographic manner. Although both genetic and epigenetic somatic
373 variation can be random (Vogt, 2015), different growing conditions will differentially affect the DNA
374 methylation profiles of otherwise genetically identical individuals (Consuegra and Rodríguez López,
375 2016) as previously shown on clonally propagated *Populus alba* (Guarino et al., 2015). It is,
376 therefore, not surprising to find that epigenetic profiling was a better predictor of sample origin than
377 genetic profiling alone both using MSAP data (Table 2, Figure S1) or msGBS data (Figure 5-7 and
378 Figure S4). This suggests that although genetic differences between regions or vineyards can partly
379 contribute to the observed molecular differentiation between vineyards/sub-regions, epigenetic
380 differences are the major driver of such differentiation.

381

382 Samples collected from vineyards in the Southern Grounds presented the highest levels of both
383 genetic and epigenetic diversity (Table 1). These vineyards also presented higher levels of
384 differentiation when inter-vineyard variability was analysed (Figure 2G-H), suggesting a major
385 contributor to the observed molecular variability between vines in the Southern Grounds is linked to
386 the vineyard of origin. Taken collectively, these results suggest that the specific growing conditions
387 from each subregion impose DNA methylation patterns on grapevine plants specific for each region
388 as previously shown both in cultivated (Guarino et al., 2015) and wild plant populations (Fonseca
389 Lira-Medeiros et al., 2010). Not surprisingly, and contrary to what has been shown in natural plant
390 populations (Fonseca Lira-Medeiros et al., 2010; Róis et al., 2013), no clear negative correlation
391 between genetic and epigenetic diversity was observed in the studied vineyards. This is most
392 probably due to the intensive phenotypic selection to which grapevine cultivars have been under
393 since domestication and the relative low levels of environmental disparity to which vines growing in
394 the same vineyard are exposed to.

395

396 **Environmental and vineyard management differences are drivers of regional epigenetic** 397 **differentiation**

398 Principal coordinate and Mantel test analysis showed that the correlation between epigenetic and
399 geographic distance between vineyards on the North-South axis of the Barossa Valley (Figure 2A)
400 was significant ($P=0.0003$) (Figure 2C) and that the main contributor to the observed epigenetic
401 differences was the position of the studied vineyards along the N-S axis (Figure 2B). This suggests
402 that environmental differences between locations could be contributing to the observed molecular
403 differences between sub-regions or vineyards (Figure 3). Moreover, the correlation ($R^2 = 0.3066$)
404 between epigenetic and geographic distance among vineyards planted with clone 1654 on the N-S
405 axis (Figure 2) supports the Shannon diversity analysis that indicate that the different genetic

406 backgrounds used in this study do not greatly affect the epigenetic differences observed between
407 regions (Table 1). Conversely, differences in vineyard altitude appear to be a contributor to the
408 detected epigenetic differentiation between vineyards (Figure S3). Previous work has shown that sun
409 exposure can have significant effects both in berry metabolomic profiles (Son et al., 2009; Tarr et al.,
410 2013) and on the epigenetic profiles of plants growing in different environments (Guarino et al.,
411 2015). Although altitude does not necessarily affect sun exposure, it can have a profound effect on
412 the UV levels experienced by plants (approximately 1% increase every 70 m gain in altitude). Our
413 results indicate that, although DNA methylation in and around genes changes in both directions
414 (hyper- and hypo-methylation), on average, it increases with altitude (i.e. NG > CG > SG (vineyard
415 average altitude 301, 277 and 236 m respectively) (Figure 6A). Functional analysis of the DMGs
416 between sub-regions generated GO terms associated to plant response to light stimulus (Table S4B).
417 More importantly, the number of genes associated to such GO terms was higher in comparison
418 between regions with bigger differences in altitude (74 and 46 genes in comparison SG vs NG (65 m
419 difference) and SG vs CG (41 m) respectively) than in the pairwise comparison with lower difference
420 in altitude (6 genes NG vs CG (24 m)). Although this positive polynomial grade 2 correlation ($R^2=1$)
421 was generated using only three data points, it is tempting to speculate that differences in light
422 incidence due to differences in altitude are triggering the observed changes in DNA methylation in
423 response to light stimulus genes. Especially when previous work has shown that, in grapevine leaves,
424 increased UV levels trigger the synthesis of non-flavonoid phenolics such as resveratrol (Sbaghi et
425 al., 1995; Teixeira et al., 2013). Interestingly, DNA methylation has been previously linked to the
426 regulation of the gene VaSTS10, which controls the synthesis of resveratrol in *Vitis amurensis*
427 (Tyunin et al., 2013; Kiselev et al., 2013).

428

429 To our knowledge the effect of pruning has not yet been studied at an epigenetic level. However
430 Herrera and Bazaga (2011) showed that long term defoliation by herbivory does have an effect on the

431 DNA methylation patterns of predated plants. The correlation between epigenetic and geographic
432 distances observed between vineyards planted with clone 1654 and pruned with the same method
433 (spur pruning) (Figure 4) was reduced when all vineyards planted with clone 1654 were considered
434 irrespectively of the pruning system used (Figure 3). This concerted epigenetic response of plants
435 growing in different environments towards the same human stimulus is consistent with the
436 hypothesis that differences in cultural practices (e.g. pruning method) act together with
437 environmental conditions as major drivers of the epigenetic differences observed between vineyards
438 and sub-regions in this study.

439

440 Vintage, geographic location and vineyard management have been shown to influence both
441 vegetative growth (Jackson and Lombard 1993) and fruit quality in grapevine (Roullier-Gall et al.,
442 2014). In light of the results shown here, we propose that epigenetic processes in general and DNA
443 methylation in particular, could constitute an important set of molecular mechanisms implicated in
444 the effect that provenance and vintage has, not only on plant vegetative growth, but also on fruit and
445 wine quality. It is also tempting to speculate that in long living crops, such as grapevine, epigenetic
446 priming (Tricker et al., 2013) could allow for the storage of environmental information that would
447 ultimately contribute, at least partially, to the uniqueness of wines produced in different regions.

448

449 **Author contributions**

450 HX and MK carried out the experiments and contributed to data analysis. NS performed gene
451 ontology analysis on msGBS data. KGT performed TASSEL analysis on msGBS data. TC, MG, JB,
452 and AM contributed to the design of the research project. RD and CC contributed to the design of the
453 research project, site selection and collection of material. CMRL contributed to the design of the

454 research project, data analysis and drafted the manuscript. All authors read and contributed to the
455 final manuscript.

456

457 **Acknowledgements**

458 The authors would like to gratefully acknowledge the Barossa Grounds Project and in particular the
459 growers that allowed us to sample material from their properties and supplied information about their
460 vineyards and management strategies. Dr Kendall R. Corbin performed DNA extractions from all
461 samples used in this study. Personnel in the viticulture group, Dr Sandra Milena Mantilla, Annette
462 James, and Valentin Olek contributed to collection of material.

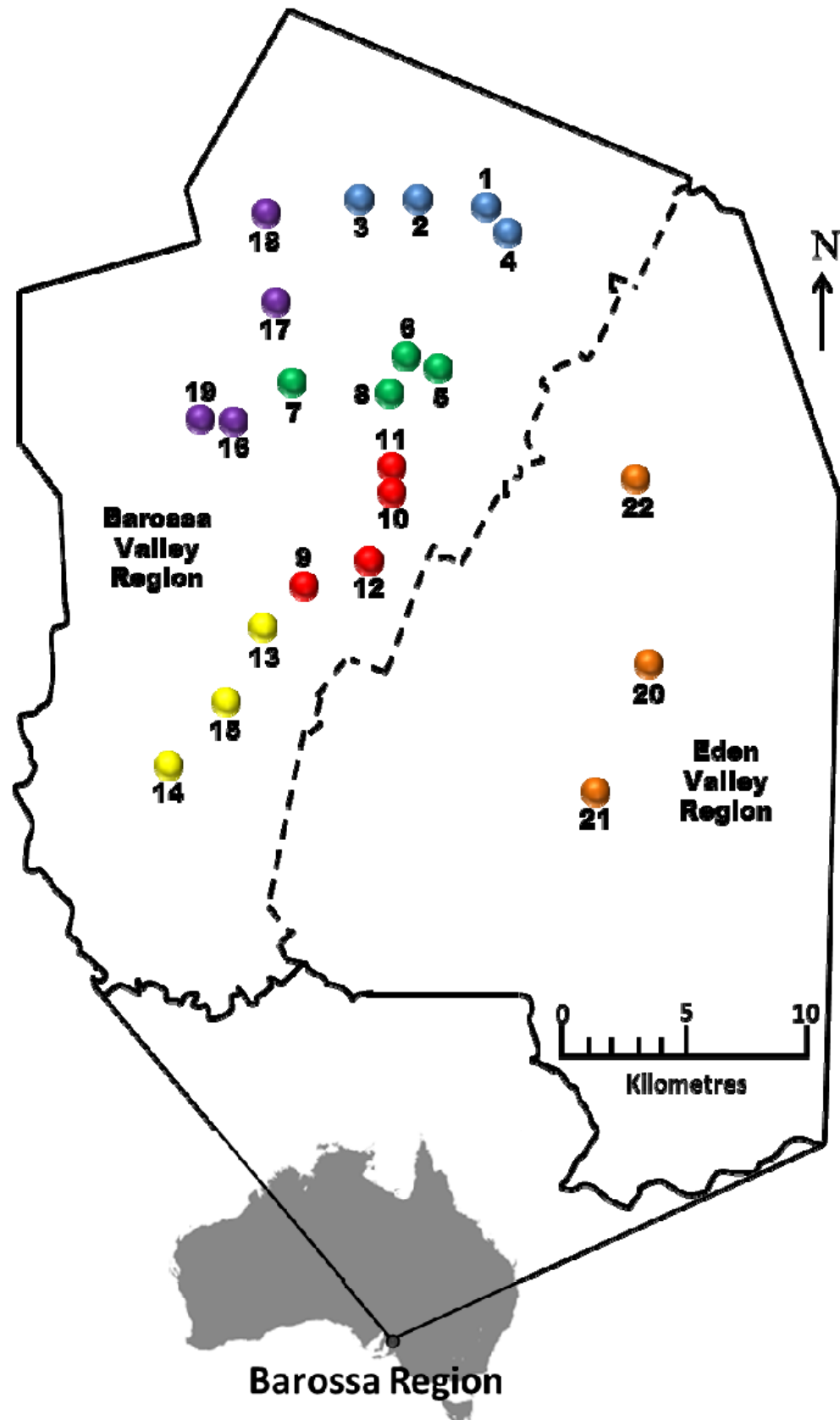
463

464 **Funding statements**

465 This study was funded through a Pilot Program in Genomic applications in Agriculture and
466 Environment Sectors jointly supported by the University of Adelaide and the Australian Genome
467 Research Facility Ltd (Adelaide node). MK was supported by the Australian Agency for International
468 Development (AusAID) PhD scholarship. CMRL is supported by a University of Adelaide Research
469 Fellowship. MG is supported by the Australian Research Council through Centre of Excellence
470 (CE1400008) and Future Fellowship (FT130100709) funding.

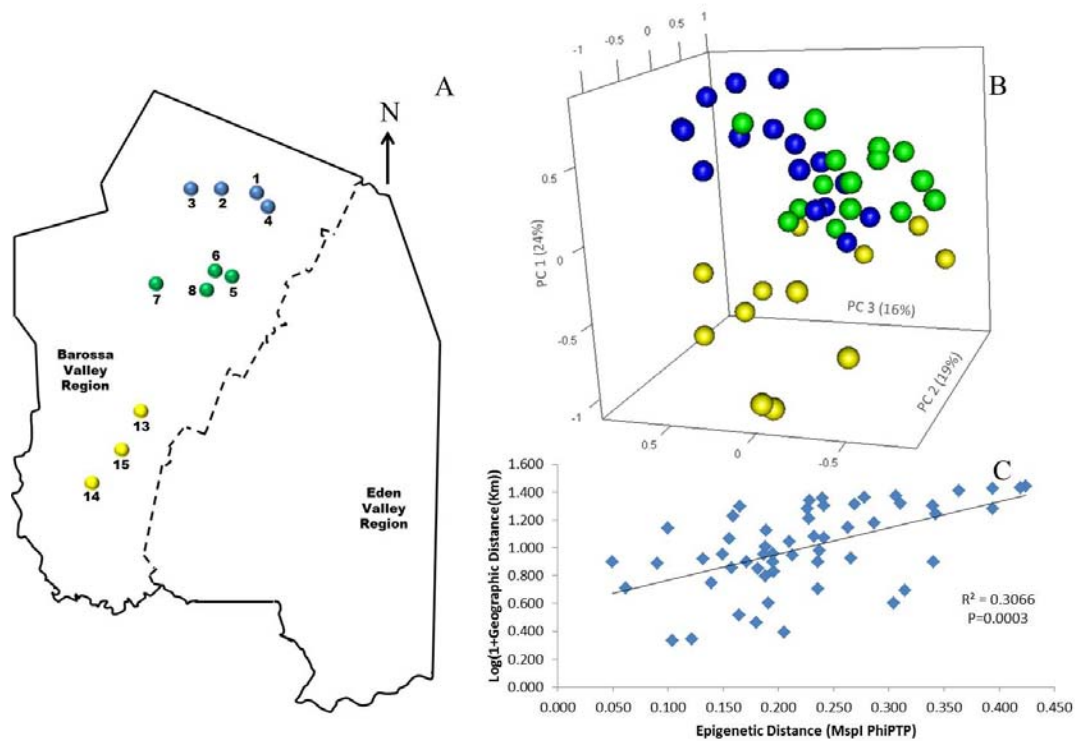
471

472 **Figures and Tables**



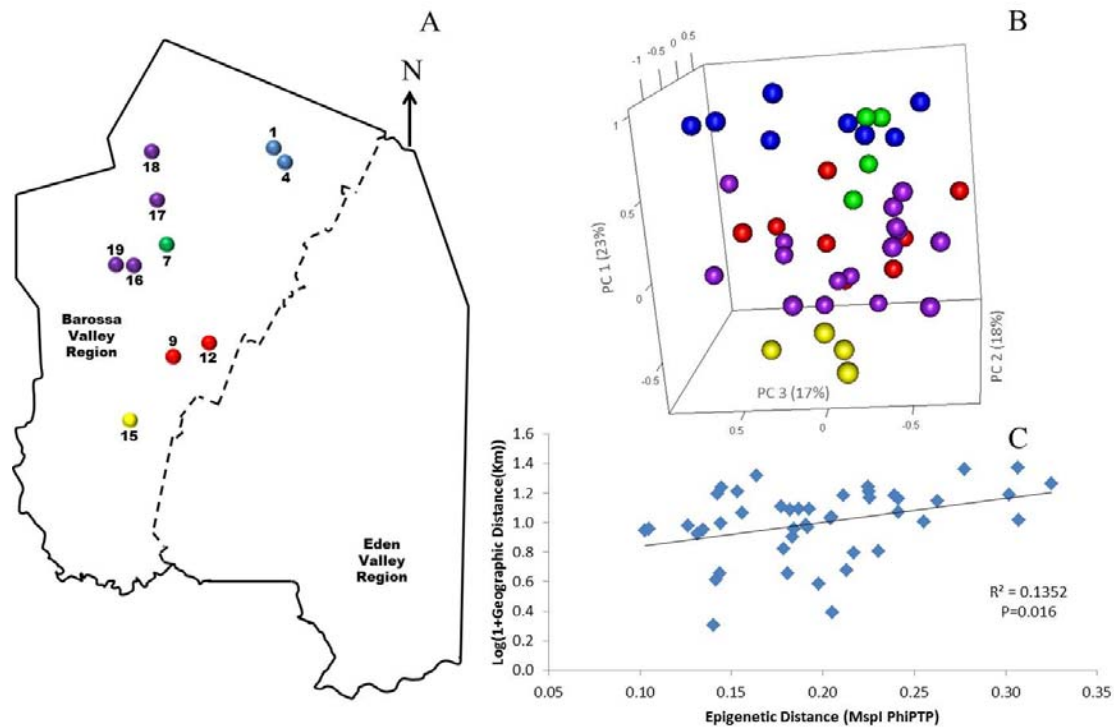
473

474 **Figure 1: Selected Barossa Region vineyard sites.** Northern Grounds: Blue, Southern Grounds:
475 Yellow, Central Grounds: Green, Eastern Edge: Red, Western Ridge: Purple, Eden Valley:
476 Orange. Arrow indicates geographic north.
477



478

479 **Figure 2. Analysis of the correlation between epigenetic differentiation and geographic distance**
480 **of vineyards planted along the Barossa Valley North-South axis:** (A) Location of the Barossa
481 Valley vineyards from the three sub-regions distributed along the Barossa Valley North-South axis;
482 Northern Grounds (blue), Central Grounds (Green) and Southern Grounds (Yellow). Arrow indicates
483 the direction of geographic North. (B) Principal Coordinate Analysis (PCoA) representing epigenetic
484 differences between leaf samples collected from 4 plants/vineyard. Percentage of the variability
485 capture by each Principal Coordinate (PC) is shown in parenthesis. (C) Correlation between pairwise
486 epigenetic distance (*MspI* PhiPT) and geographical distance ($\text{Log}(1+\text{GeoD (km)})$) between vineyards.
487 Shown equations are the correlation's R^2 and the Mantel test significance (P value was estimated
488 over 9,999 random permutations tests). PCoA and PhiPT for Mantel test were based on
489 presence/absence of 215 loci obtained from MSAP profiles generated using *MspI*.



490

491 **Figure 3. Analysis of the correlation between epigenetic differentiation and geographic distance**

492 **of vineyards planted with clone 1654 in the Barossa Region: (A)** Location of the selected Barossa

493 Valley vineyards from the three sub-regions distributed along the Barossa Valley North-South axis

494 Northern Grounds (blue), Central Grounds (Green), Eastern Edge (red), Southern Grounds (Yellow)

495 and Western Ridge (Purple). Arrow indicates the direction of geographic North. **(B)** Principal

496 Coordinate Analysis (PCoA) representing epigenetic differences between leaf samples collected from

497 4 plants/vineyard. Percentage of the variability captured by each Principal Coordinate (PC) is shown

498 in parenthesis. **(C)** Correlation between pairwise epigenetic distance (*MspI* PhiPT) and geographical

499 distance (Log(1+GeoD (km))) between vineyards. Shown equations are the correlation's R^2 and the

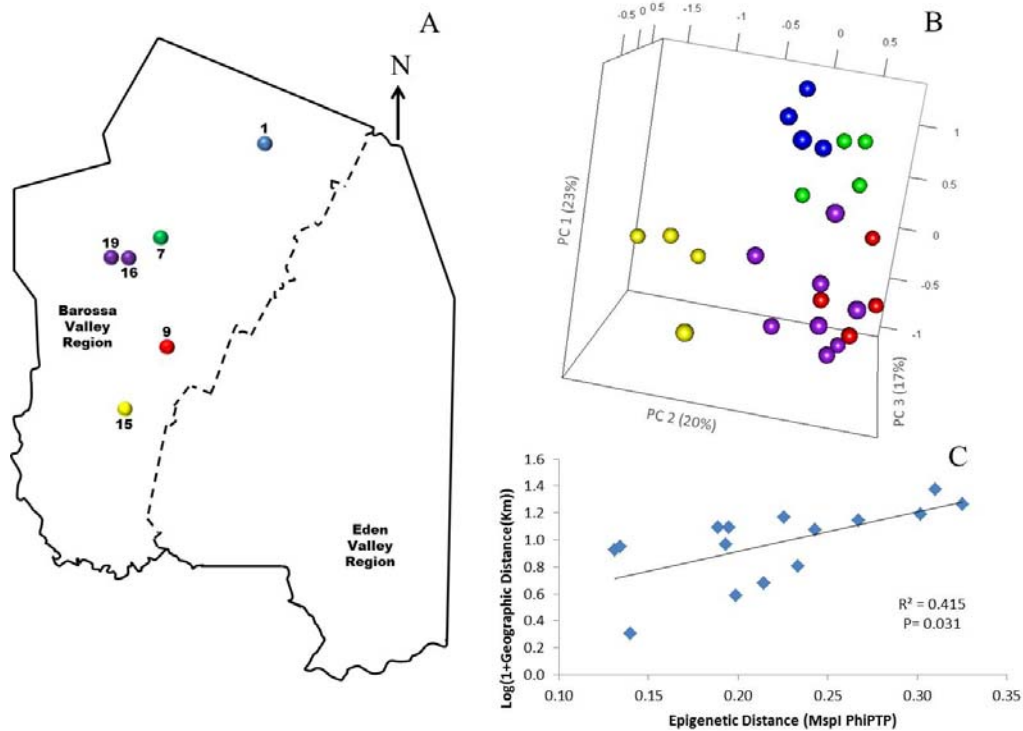
500 Mantel test significance (P value was estimated over 9,999 random permutations tests). PCoA and

501 PhiPT for Mantel test were based on presence/absence of 215 loci obtained from MSAP profiles

502 generated using *MspI*.

503

504



505

506 **Figure 4. Analysis of the correlation between epigenetic differentiation and geographic distance**

507 **of vineyards planted with clone 1654 in the Barossa Region and trained using the spur pruned**

508 **method: (A)** Location of the selected Barossa Valley vineyards: Northern Grounds (blue), Central
509 Grounds (Green), Eastern Edge (red), Southern Grounds (Yellow) and Western Ridge (Purple).

510 Arrow indicates the direction of geographic North. **(B)** Principal Coordinate Analysis (PCoA)

511 representing epigenetic differences between leaf samples collected from 4 plants/vineyard.

512 Percentage of the variability capture by each Principal Coordinate (PC) is shown in parenthesis. **(C)**

513 Correlation between pairwise epigenetic distance (*MspI* PhiPT) and geographical distance

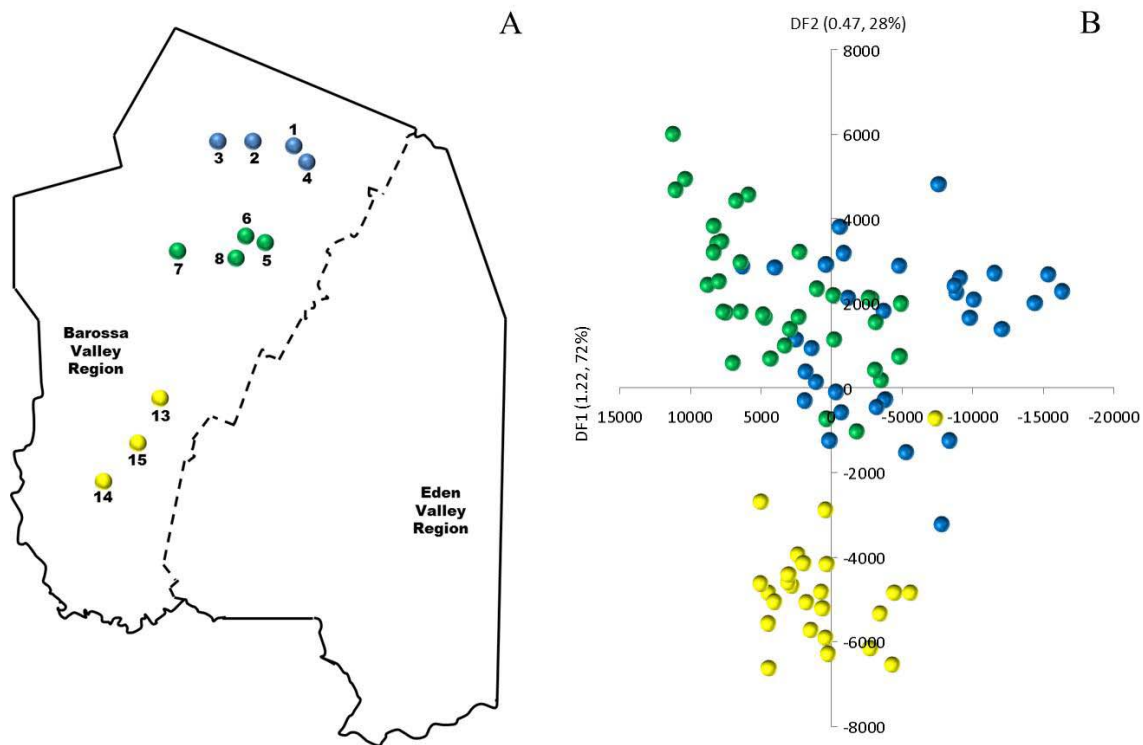
514 (Log(1+GeoD (km))) between vineyards. Shown equations are the correlation's R^2 and the Mantel test

515 significance (P value was estimated over 9,999 random permutations tests). PCoA and PhiPT for

516 Mantel test are based on presence/absence of 215 loci obtained from MSAP profiles generated

517 using *MspI*.

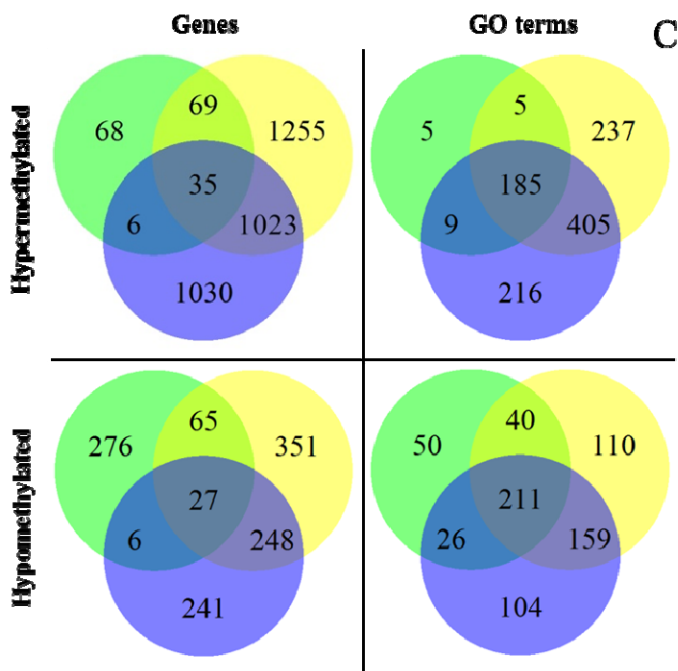
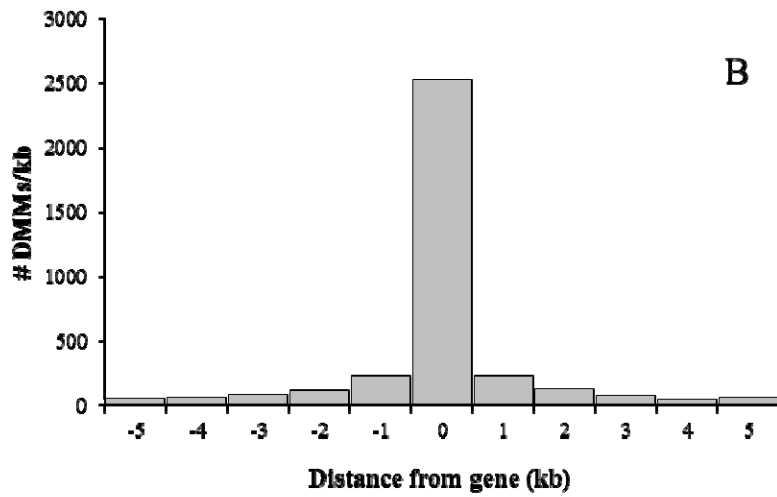
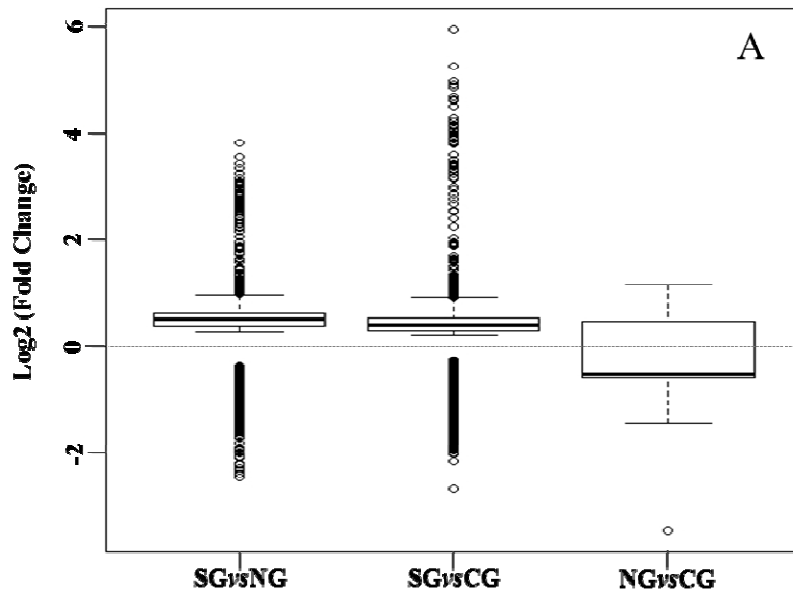
518



519

520 **Figure 5. Analysis of the correlation between epigenetic differentiation and geographic distance**
521 **of vineyards planted along the Barossa Valley North-South axis: (A)** Location of the Barossa
522 Valley vineyards from the three sub-regions distributed along the Barossa Valley North-South axis;
523 Northern Grounds (blue), Central Grounds (Green) and Southern Grounds (Yellow). Arrow
524 indicates the direction of geographic North. **(B)** Principal Components-Linear Discriminant Analysis
525 (PC-LDA) representing epigenetic differences between leaf samples collected from 9
526 plants/vineyard. Percentage of the variability capture by each differential factor (DF) is shown in
527 parenthesis. PC-LDA were based on read number of loci obtained from msGBS profiles.

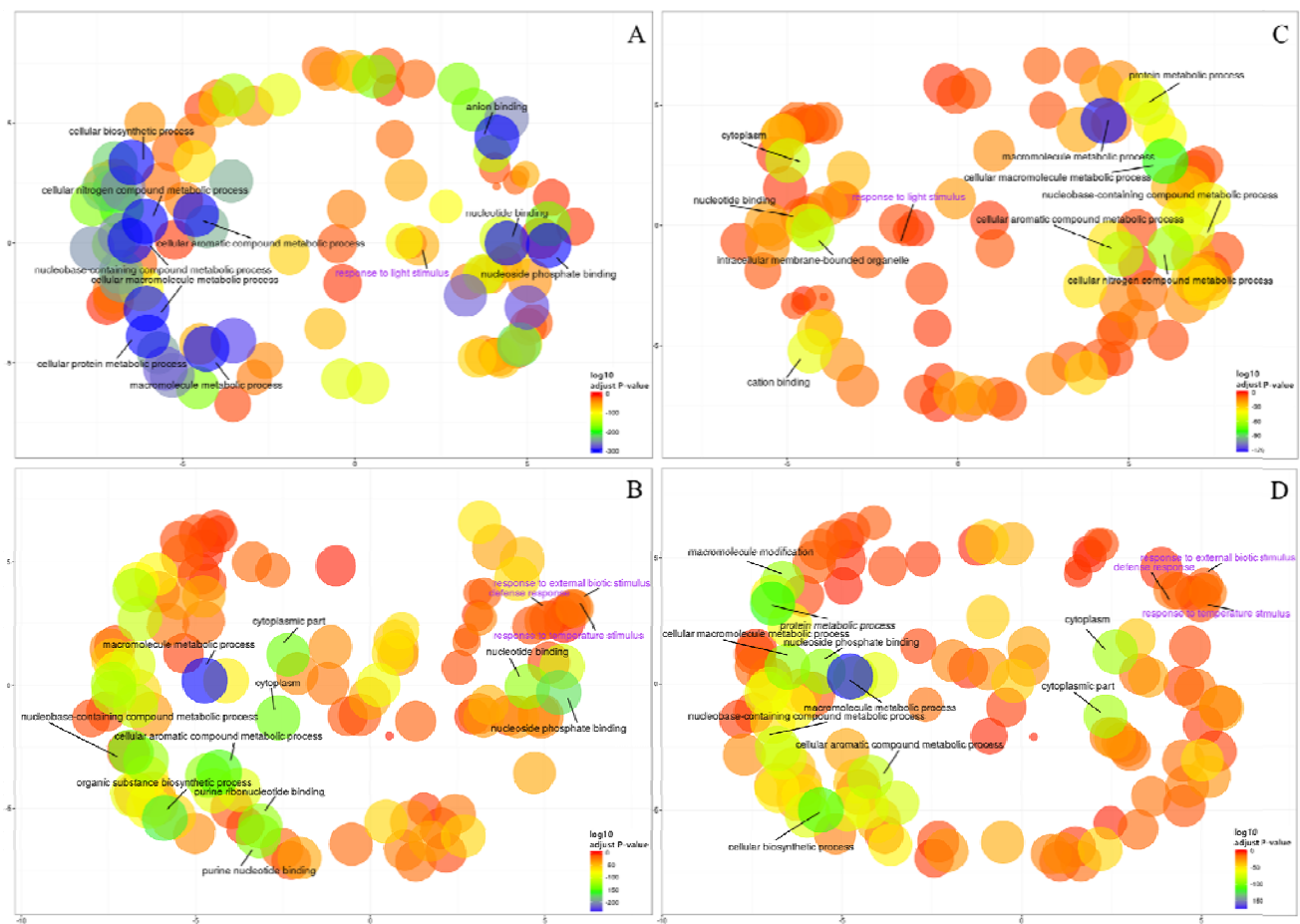
528



530 **Figure 6. Analysis of differentially methylated genes (DMGs) and GO terms (DMGOs) among**
531 **three wine sub-regions in Barossa Shiraz.** Genes were considered differentially methylated if
532 located within 5kb of at least one differentially methylated marker (DMM) (FDR < 0.01). DMMs
533 were generated using msGBS on 9 plants per vineyard (Northern Grounds: four vineyards, Central
534 Grounds: four vineyards and Southern Grounds; three vineyards). **(A)** Directionality of methylation
535 differences between regions. Boxplots show the distribution of the intensity of changes in DNA
536 methylation level between regions, represented here as the fold-change ($2^{\text{power } \log_2\text{FC}}$) in read
537 counts for a given msGBS markers between two regions. Median shows the direction of the
538 methylation flux at a whole genome level in each region comparison (i.e. positive medians indicate a
539 global increase in DNA methylation (hypermethylation) while negative medians indicate a global
540 decrease in DNA methylation (hypomethylation) in the second region in the comparison (e.g.
541 Northern Grounds is hypermethylated compared to Southern Grounds). **(B)** Distribution of 3598
542 region specific DMMs around genes. Columns -5 to -1 and 1 to 5 represents the number of DMMs
543 per Kb around *V. vinifera* genes. Column 0 indicates the number of DMMs within the coding
544 sequence (i.e. between the transcription start and end sites) of *V. vinifera* genes. **(C)** Shared DMGs
545 and differentially methylated Gene Ontology Terms (DMGOs) between regional comparisons. Venn
546 diagrams show the number of unique and shared DMGs and DMGOs between each regional pairwise
547 comparison (i.e. Blue: hyper/hypomethylated genes and GOs in Northern Grounds compared to
548 Southern Grounds; Yellow: in Central Grounds compared to Southern Grounds; and Green: in
549 Central Grounds compared to Northern Grounds).

550

551



552
 553 **Figure 7: REViGO semantic analysis of differentially methylated GO terms shared by all three**
 554 **regional pairwise comparisons.** Functional enrichment of GO-terms was carried out for the genes
 555 deemed differentially methylated (DMGs) hypermethylated (185) (A-C) or hypomethylated (211) (B-
 556 D) in Northern Grounds compared to Southern Grounds (A-B) and Central Grounds compared to
 557 Northern Grounds (C-D) using *GO.db* and *annotate* and summarized using REViGO. Bubble color
 558 indicates the p-value for the false discovery rates (the first 10 terms are labelled with legends in
 559 black. A detailed list of all GO terms containing DMGs has been supplied as a Tables S3 and S4);
 560 circle size indicates the frequency of the GO term in the underlying GO database (bubbles of more
 561 general terms are larger).

562

Region	Shannon Index	
	MSL	NML
Northern Grounds	0.542 (0.119)	0.240 (0.030)
Central Grounds	0.552 (0.124)	0.242 (0.035)
Eastern Edge	0.547 (0.138)	0.244 (0.038)
Southern Grounds	0.581 (0.124)	0.374 (0.143)
Western Ridge	0.536 (0.133)	0.250 (0.048)
Eden Valley	0.573 (0.095)	0.287 (0.000)

563 **Table 1. Analysis of genetic (NML) and epigenetic (MSL) diversity within the six Barossa**
564 **Valley wine growing regions:** Shannon diversity indices are reported as mean (\pm Standard
565 Deviation). Wilcoxon rank test provides statistical support for all Shannon diversity indices ($P <$
566 0.0001).

567

568

	North	South	Central	East	West	Eden
North	–	0.115(1e-04)	0.043(8e-04)	0.062(2e-04)	0.082(1e-04)	0.069(0.001)
South	0.028(0.0059)	–	0.064(1e-04)	0.042(0.001)	0.027(0.024)	0.024(0.073)
Central	0.012(0.1085)	0.025(0.0079)	–	0.043(1e-04)	0.060(1e-04)	0.067(2e-04)
East	0.025(0.0043)	0.012(0.0712)	0.015(0.0474)	–	0.029(0.004)	0.038(0.0011)
West	0.039(2e-04)	0.018(0.0426)	0.033(0.0001)	0.013(0.0651)	–	0.024(0.024)
Eden	0.056(0.0001)	0.043(4e-04)	0.015(0.0601)	0.031(0.0023)	0.031(0.0016)	–

569

570 **Table 2: Molecular distances (PhiPT) between Barossa Valley wine producing sub-regions.**

571 PhiPT values were calculated using MSAP profiles generated from 88 grapevine plants (4 individual
572 plants per vineyard) using restriction enzyme combinations *MspI/EcoRI* (above diagonal) and
573 *HpaII/EcoRI* (below diagonal). P-values (shown in parenthesis) were calculated based on 9,999
574 permutations. Pairwise regional comparisons showing not significantly different PhiPT values are
575 highlighted in bold. A total of 22 vineyards were included in the analysis: Northern Grounds (4),
576 Central Grounds (4), Southern Grounds (3), 4 vineyards in Eastern Edge (4), 4 vineyards in Western
577 Ridge (4) and Eden valley (3).

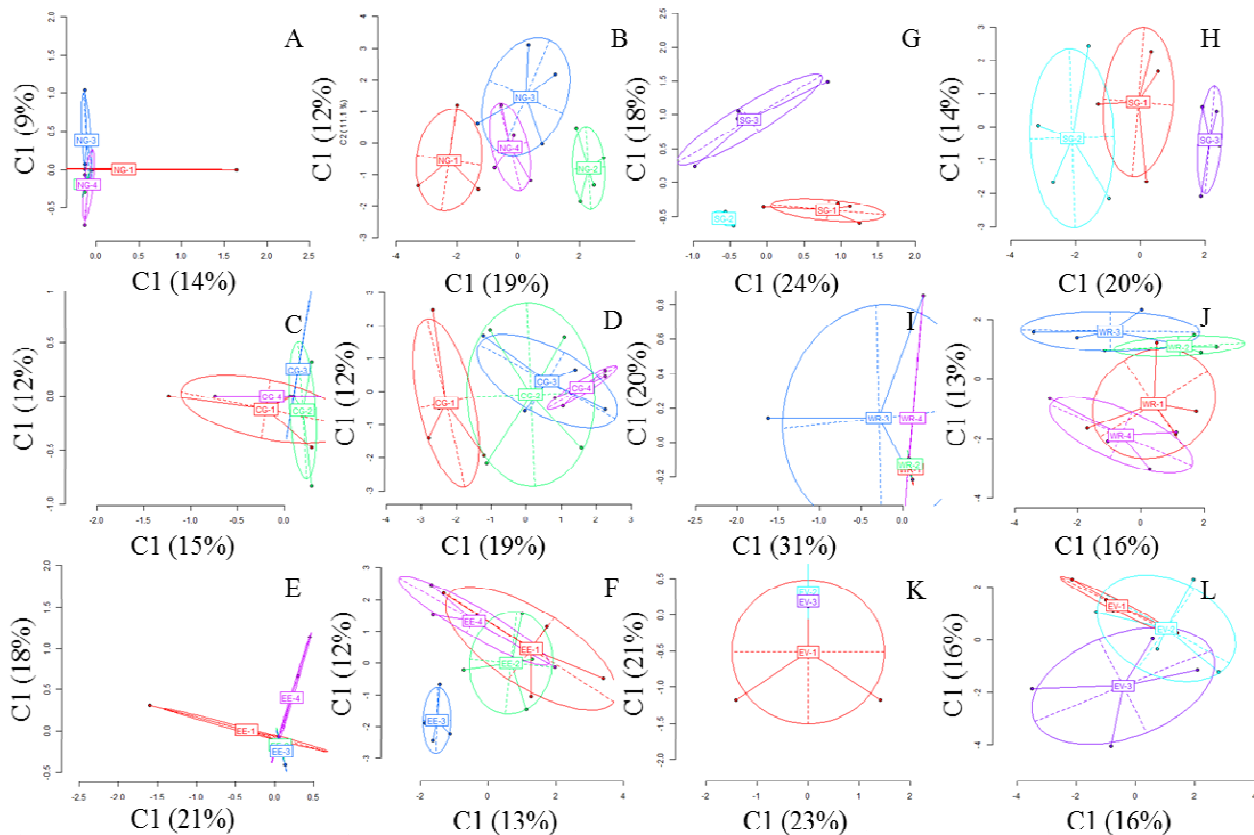
578

579

	Differentially methylated markers	Differentially methylated genes		Differentially methylated GO terms	
		Hypometh Genes	Hypermeth Genes	Hypometh GO terms	Hypermeth GO terms
NG vs CG	7465	374	178	327	204
SG vs CG	15276	691	2382	520	832
SG vs NG	12911	522	2094	500	815

580 **Table 3: Identification of differentially methylated markers (DMMs), genes (DMGs) and GO**
581 **terms (DMGOs) between sub-regions in Barossa Shiraz.** Cells contain the number of DMMs,
582 DMGs or DMGOs detected in each pairwise comparison. Differential methylation between sub-
583 regions was calculated using msGBS data from 9 Shiraz plants per vineyard (Northern Grounds
584 (NG): four vineyards, Central Grounds (CG): Four vineyards and Southern Grounds (SG); Three
585 vineyards). Directionality of methylation (i.e. Hyper/hypomethylation) indicates an increase or
586 decrease in DNA methylation in the second region compared to the first region in each pairwise
587 comparison.

588



589

590 **Figure S1: Analysis of genetic and epigenetic differences within Barossa Valley sub-regions:**

591 PCoAs represent variability of non-methylated polymorphic loci (Genetic variability) (A, C, E, G, I

592 **and K)** and of methylation sensitive polymorphic loci (Epigenetic variability) (B, D, F, H J and L)

593 as classified by the msap (v. 1.1.8) software (Pérez-Figueroa, 2013) of leaf samples in vineyards from

594 Northern Grounds (A-B), and the Barossa Valley's Western Ridge Central Grounds (C-D), Eastern

595 Edge (E-F) and Southern Grounds (G-H), Western Ridge (I-J) and Eden valley (K-L). Coordinates 1

596 and 2 are shown with the percentage of variance explained by them. Points represent individuals

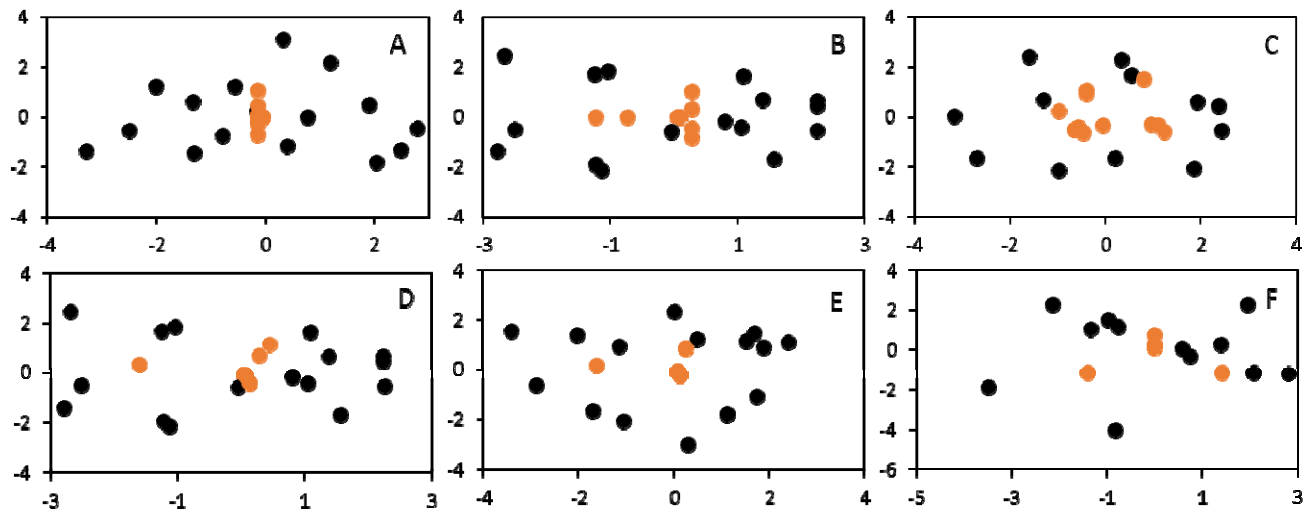
597 from each vineyard. Vineyard code (NG, CG, EE, SG, WR and EV) is shown as the centroid for each

598 vineyard. Ellipses represent the average dispersion of those pointst around their centre. The long axis

599 of the ellipse shows the direction of maximum dispersion and the short axis, the direction of

600 minimum dispersion.

601

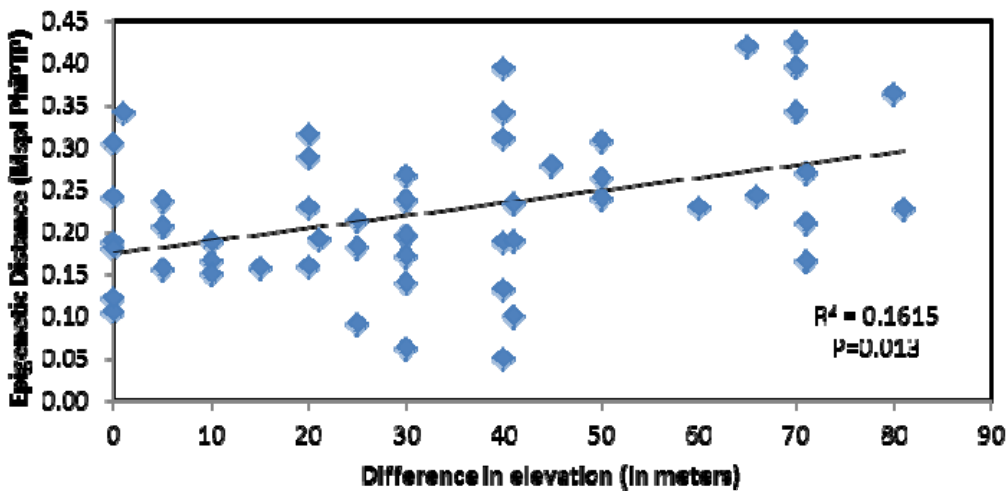


602

603 **Figure S2. Analysis of regional genetic and epigenetic diversity.** Red symbols indicate samples
604 analysed using genetic information only, black symbols represent samples analysed using epigenetic
605 information only according to the R package for Statistical analysis of Methylation-Sensitive
606 Amplification Polymorphism data “msap”. PCoAs were calculated using MSAP profiles generated
607 from gDNA extracted from E-L 7 stage leaves (Coombe, 1995) of 88 grapevine plants grown in
608 vineyards located in the six Barossa Valley wine sub-regions (**A** Northern Grounds, **B** Central
609 Grounds, **C** Southern Grounds, **D** Eastern Edge, **E** Western Ridge, **F** Eden valley) (4 individual
610 plants per vineyard) using restriction enzyme combinations *MspI/EcoRI* and *HpaII/EcoRI*.

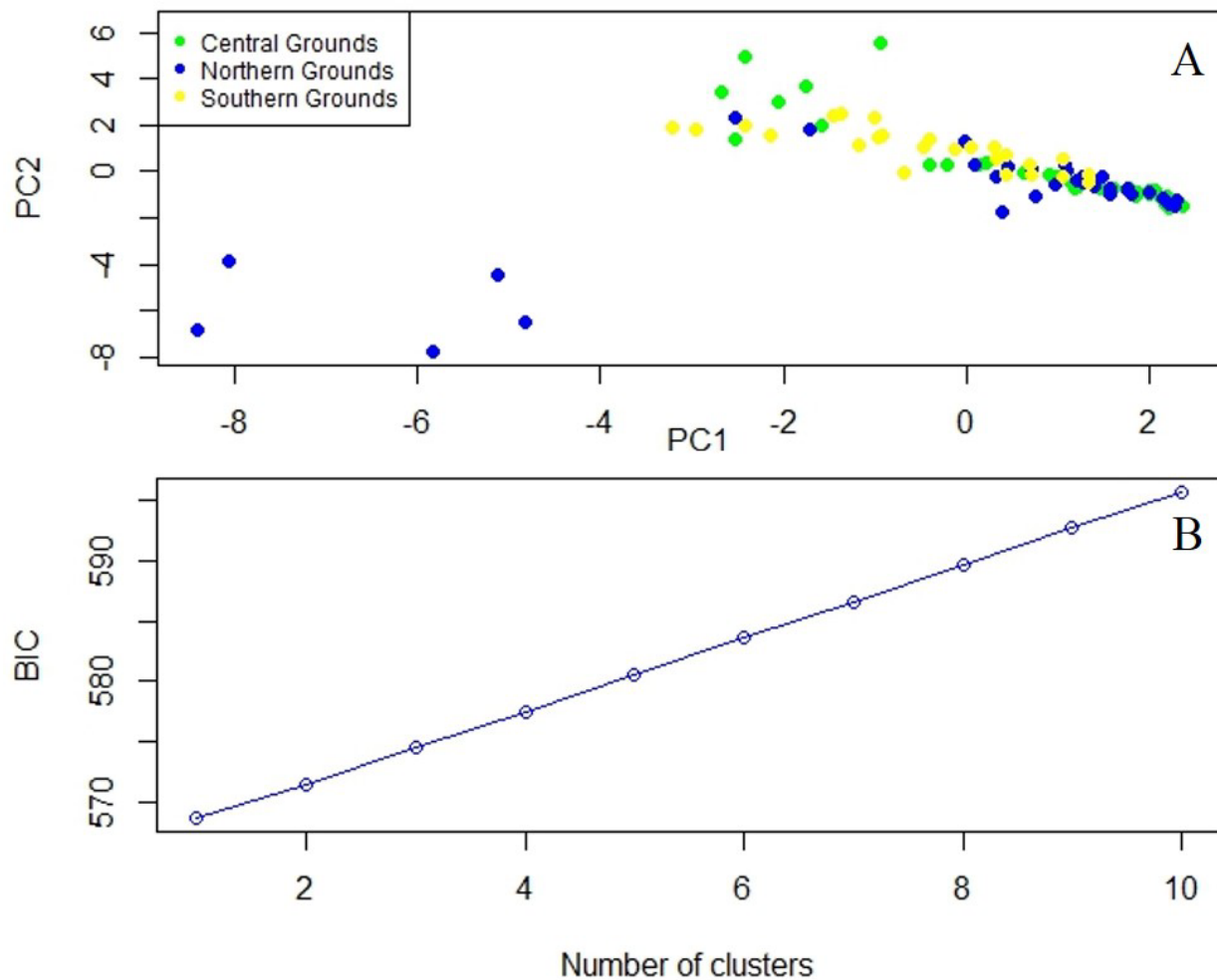
611

612



613

614 **Figure S3. Analysis of the correlation between epigenetic differentiation and environmental**
615 **differences among vineyards planted along the Barossa Valley North-South axis: Mantel test**
616 analysis of the correlation between pairwise epigenetic distance (MspI PhiPT) and differences in
617 altitude between vineyards. Shown equations are the correlation's R^2 and the Mantel test significance
618 (P value was estimated over 9,999 random permutations tests). PhiPT values were based on
619 presence/absence of 215 loci obtained from MSAP profiles generated using *MspI*.



620

621 **Figure S4: Analysis of the grapevine genetic diversity in vineyards planted along the Barossa**
622 **Valley North-South axis: (A)** Principal Component Analysis (PCA) representing genetic structure
623 calculated using 4893 high quality SNPs (i.e. present in at least 80% of the samples) in genomic
624 DNA collected from 11 vineyards (Northern Grounds (blue) 4 vineyards, Central Grounds (Green) 4
625 vineyards, and Southern Grounds (Yellow) 3 vineyards (9 plants/vineyard)). **(B)** Identification of the
626 optimal number of genetic clusters present within the three sub-regions compared using Bayesian
627 Information Criterion (BIC) as implemented by Discriminant Analysis of Principal Components
628 (DAPC) using adegenet 2.0.0 (i.e. the optimal clustering solution should correspond to the lowest
629 BIC).

630 **Table S2:** Sequences of oligonucleotide used for MSAP. Selective bases in the primers used during
 631 the preselective and selective amplifications are highlighted in bold. Unique msBGS barcode bases
 632 are represented as X.

Oligo name	Function	Sequence
<i>HpaII/MspI</i> adaptor	Reverse Adaptor	CGCTCAGGACTCAT
<i>HpaII/MspI</i> adaptor	Forward Adaptor	GACGATGAGTCCTGAG
<i>EcoRI</i> adaptor	Reverse Adaptor	AATTGGTACGCAGTCTAC
<i>EcoRI</i> adaptor	Forward Adaptor	CTCGTAGACTGCGTACC
Pre- <i>EcoRI</i>	Preselective primer	GACTGCGTACCAATTCA
Pre- <i>HpaII/MspI</i>	Preselective primer	GATGAGTCCTGAGCGGC
<i>EcoRI</i> Selective Primer	Selective primer	GACTGCGTACCAATT CACG
<i>HpaII/MspI</i> Selective Primer	Selective primer	GATGAGTCCTGAGCGG CAA
<i>MspI</i> msBGS barcoded adaptor	Reverse Adaptor	CGXXXXAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
<i>MspI</i> msBGS barcoded adaptor	Forward Adaptor	ACACTCTTTCCTACACGACGCTCTCCGATCTXXXXX
<i>EcoRI</i> msBGS Y adaptor	Reverse Adaptor	CGAGATCGGAAGAGCGGTTTCAGCAGGAATGCCGAG
<i>EcoRI</i> msBGS Y adaptor	Forward Adaptor	CTCGGCATTCTGCTGAACCGCTCTCCGATCT
<i>MspI</i> primer	Sequencing library primers	AATGATACGGCGACCACCGAGATCTACACTCTTTCCTACACGACGCTCTCCGATCT
<i>EcoRI</i> primer		CAAGCAGAAGACGGCATAACGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTCCGATCT

633

634

635

636

637 Bibliography

- 638 Alonso, C., Pérez, R., Bazaga, P., Medrano, M., and Herrera, C. M. (2016). MSAP markers and
639 global cytosine methylation in plants: a literature survey and comparative analysis for a wild-
640 growing species. *Mol Ecol Resour* 16, 80–90. doi:10.1111/1755-0998.12426.
- 641 Amoah, S., Kurup, S., Rodriguez Lopez, C. M., Welham, S. J., Powers, S. J., Hopkins, C. J.,
642 Wilkinson, M. J., and King, G. J. (2012). A hypomethylated population of *Brassica rapa* for
643 forward and reverse epi-genetics. *BMC Plant Biol* 12, 193. doi:10.1186/1471-2229-12-193.
- 644 Baali-Cherif, D., and Besnard, G. (2005). High genetic diversity and clonal growth in relict
645 populations of *Olea europaea* subsp. *laperrinei* (Oleaceae) from Hoggar, Algeria. *Ann Bot* 96,
646 823–830. doi:10.1093/aob/mci232.
- 647 Baránek, M., Čechová, J., Raddová, J., Holleínová, V., Ondrušíková, E., and Pidra, M. (2015).
648 Dynamics and Reversibility of the DNA Methylation Landscape of Grapevine Plants (*Vitis*
649 *vinifera*) Stressed by In Vitro Cultivation and Thermotherapy. *PLoS ONE* 10, e0126638.
650 doi:10.1371/journal.pone.0126638.
- 651 Bloomfield, J. A., Rose, T. J., and King, G. J. (2014). Sustainable harvest: managing plasticity for
652 resilient crops. *Plant Biotechnol J* 12, 517–533. doi:10.1111/pbi.12198.
- 653 Bradbury PJ, Zhang Z, Kroon DE, Casstevens TM, Ramdoss Y, Buckler ES. (2007) TASSEL:
654 Software for association mapping of complex traits in diverse samples. *Bioinformatics* 23,
655 2633-2635
- 656 Bushnell, B. (2016). BBMap short read aligner. University of California, Berkeley, California. URL
657 <http://sourceforge.net/projects/bbmap>.
- 658 Caballero, A., Quesada, H., and Rolán-Alvarez, E. (2008). Impact of amplified fragment length
659 polymorphism size homoplasy on the estimation of population genetic diversity and the
660 detection of selective loci. *Genetics* 179, 539–554. doi:10.1534/genetics.107.083246.

- 661 Carlson M (2016). *GO.db*: A set of annotation maps describing the entire Gene Ontology. R package
662 version 3.4.0. (<https://bioconductor.org/packages/release/data/annotation/html/GO.db.html>).
- 663 Cazzonelli, C. I., Nisar, N., Roberts, A. C., Murray, K. D., Borevitz, J. O., and Pogson, B. J. (2014).
664 A chromatin modifying enzyme, SDG8, is involved in morphological, gene expression, and
665 epigenetic responses to mechanical stimulation. *Front Plant Sci* 5, 533.
666 doi:10.3389/fpls.2014.00533.
- 667 Consuegra, S., and Rodríguez López, C. M. (2016). Epigenetic-induced alterations in sex-ratios in
668 response to climate change: An epigenetic trap? *Bioessays*. doi:10.1002/bies.201600058.
- 669 Coombe, B. G. (1995). Growth Stages of the Grapevine: Adoption of a system for identifying
670 grapevine growth stages. *Australian journal of grape and wine research* 1, 104–110.
671 doi:10.1111/j.1755-0238.1995.tb00086.x.
- 672 Doligez, A., Bouquet, A., Danglot, Y., Lahogue, F., Riaz, S., Meredith, C., Edwards, K., This, P.
673 (2002). *Theoretical and Applied Genetics*, 105, 780-795. doi: 10.1007/s00122-002-0951-z
- 674 Fanet, J., and Brutton, F. (2004). *Great wine terroirs*. (cloth: alk. paper). Berkeley: University of
675 California Press, c2004.: University of California Press.
- 676 Farquhar, W. (2005). Which Shiraz clones are best? *The Australian & New Zealand Grapegrower*
677 *and Winemaker*, 31–33.
- 678 Fonseca Lira-Medeiros, C., Parisod, C., Fernandes, R. A., Mata, C. S., Cardoso, M. A., and Ferreira,
679 P. C. (2010). Epigenetic variation in mangrove plants occurring in contrasting natural
680 environment. *PLoS ONE* 5, e10326. doi:10.1371/journal.pone.0010326.
- 681 Fortes, A. M., and Gallusci, P. (2017). Plant stress responses and phenotypic plasticity in the
682 Epigenomics era: perspectives on the grapevine scenario, a model for perennial crop plants.
683 *Front Plant Sci* 8:82. doi: 10.3389/fpls.2017.00082.
- 684 Gentleman R (2016). Annotate: Annotation for microarrays. R package version 1.52.1
685 (<https://bioconductor.org/packages/release/bioc/html/annotate.html>).

- 686 Goldberg, A. D., Allis, C. D., and Bernstein, E. (2007). Epigenetics: a landscape takes shape. *Cell*
687 128, 635–638. doi:10.1016/j.cell.2007.02.006.
- 688 Guarino, F., Cicatelli, A., Brundu, G., Heinze, B., and Castiglione, S. (2015). Epigenetic Diversity of
689 Clonal White Poplar (*Populus alba* L.) Populations: Could Methylation Support the Success
690 of Vegetative Reproduction Strategy? *PLoS ONE* 10, e0131480.
691 doi:10.1371/journal.pone.0131480.
- 692 Haig, D. (2004). Genomic imprinting and kinship: how good is the evidence? *Annu Rev Genet* 38,
693 553–585. doi:10.1146/annurev.genet.37.110801.142741.
- 694 Harrison, D. J. (2000). Terroir: the role of geology, climate and culture in the making of French wine.
695 *Quarterly Journal of Engineering Geology and Hydrogeology* 33, 350–350.
696 doi:10.1144/qjegh.33.4.350.
- 697 Herrera, C. M., and Bazaga, P. (2010). Epigenetic differentiation and relationship to adaptive genetic
698 divergence in discrete populations of the violet *Viola cazorlensis*. *New Phytol* 187, 867–876.
699 doi:10.1111/j.1469-8137.2010.03298.x.
- 700 Herrera, C. M., and Bazaga, P. (2011). Untangling individual variation in natural populations:
701 ecological, genetic and epigenetic correlates of long-term inequality in herbivory. *Mol Ecol*
702 20, 1675–1688. doi:10.1111/j.1365-294X.2011.05026.x.
- 703 Herten, K., Hestand, M.S., Vermeesch, J.R., Van Houdting, J.K.J. (2015). GBSX: a toolkit for
704 experimental design and demultiplexing genotyping by sequencing experiments. *BMC*
705 *Bioinformatics*, 16:73. doi: 10.1186/s12859-015-0514-3.
- 706 Hutchison, D., and Templeton, A. (1999). Correlation of pairwise genetic and geographic distance
707 measures: inferring the relative influences of gene flow and drift on the distribution of genetic
708 variability. *Evolution* 53(6), 1898-1914.

- 709 Imazio, S., Labra, M., Grassi, F., Winfield, M., Bardini, M., and Scienza, A. (2002). Molecular tools
710 for clone identification: the case of the grapevine cultivar 'Traminer'. *Plant Breeding* 121,
711 531–535. doi:10.1046/j.1439-0523.2002.00762.x.
- 712 Iqbal, K., Jin, S.-G., Pfeifer, G. P., and Szabó, P. E. (2011). Reprogramming of the paternal genome
713 upon fertilization involves genome-wide oxidation of 5-methylcytosine. *Proc Natl Acad Sci U*
714 *S A* 108, 3642–3647. doi:10.1073/pnas.1014033108.
- 715 Jaenisch, R., and Bird, A. (2003). Epigenetic regulation of gene expression: how the genome
716 integrates intrinsic and environmental signals. *Nat Genet* 33 Suppl, 245–254.
717 doi:10.1038/ng1089.
- 718 Kelly, S. A., Panhuis, T. M., and Stoehr, A. M. (2012). Phenotypic plasticity: molecular mechanisms
719 and adaptive significance. *Compr Physiol* 2, 1417–1439. doi:10.1002/cphy.c110008.
- 720 Kitimu S.R., Taylor J., March T.J., Tairo F., Wilkinson M.J. & Rodriguez Lopez C.M. (2015)
721 Meristem micropropagation of cassava (*Manihot esculenta*) evokes genome-wide changes in
722 DNA methylation. *Front Plant Sci* 6, 590. doi: 10.3389/fpls.2015.00590
- 723 Kiselev, K. V., Tyunin, A. P., and Zhuravlev, Y. N. (2013). Involvement of DNA methylation in the
724 regulation of STS10 gene expression in *Vitis amurensis*. *Planta* 237, 933–941.
725 doi:10.1007/s00425-012-1806-8.
- 726 Kumar, G., Rattan, U. K., and Singh, A. K. (2016). Chilling-Mediated DNA Methylation Changes
727 during Dormancy and Its Release Reveal the Importance of Epigenetic Regulation during
728 Winter Dormancy in Apple (*Malus x domestica* Borkh.). *PLoS ONE* 11, e0149934.
729 doi:10.1371/journal.pone.0149934.
- 730 Lacaze, X., Hayes, P. M., and Korol, A. (2009). Genetics of phenotypic plasticity: QTL analysis in
731 barley, *Hordeum vulgare*. *Heredity* 102, 163–173. doi:10.1038/hdy.2008.76.

- 732 Leeuwen, C. v, Friant, P., Choné, X., Tregoat, O., Koundouras, S., and Dubourdieu, D. (2004).
733 Influence of Climate, Soil, and Cultivar on Terroir. *American Journal of Enology and*
734 *Viticulture* 55: 207-217.
- 735 Levenson, J. M., and Sweatt, J. D. (2005). Epigenetic mechanisms in memory formation. *Nat Rev*
736 *Neurosci* 6, 108–118. doi:10.1038/nrn1604.
- 737 Meyer, P. (2015). Epigenetic variation and environmental change. *J Exp Bot* 66, 3541–3548.
738 doi:10.1093/jxb/eru502.
- 739 Michalakis, Y., and Excoffier, L. (1996). A generic estimation of
740 population subdivision using distances between alleles with special reference for
741 microsatellite loci. *Genetics* 142, 1061–1064.
- 742 Ocaña, J., Walter, B., and Schellenbaum, P. (2013). Stable MSAP markers for the distinction of *Vitis*
743 *vinifera* cv Pinot noir clones. *Mol Biotechnol* 55, 236–248. doi:10.1007/s12033-013-9675-3.
- 744 Pérez-Figueroa, A. (2013). msap: a tool for the statistical analysis of methylation-sensitive amplified
745 polymorphism data. *Mol Ecol Resour* 13, 522–527. doi:10.1111/1755-0998.12064.
- 746 Pigliucci, M. (2005). Evolution of phenotypic plasticity: where are we going now? *Trends Ecol Evol*,
747 20, 481–486. doi:10.1016/j.tree.2005.06.001.
- 748 Quinlan, A.R. and Hall, I.M. (2010). BEDTools: a flexible suite of utilities for comparing genomic
749 features. *Bioinformatics*. 2010 Mar 15; 26(6): 841–842. doi: 10.1093/bioinformatics/btq033.
- 750 R Core Team (2015). R: A language and environment for statistical computing. R Foundation for
751 Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>.
- 752 Reyna-López, G. E., Simpson, J., and Ruiz-Herrera, J. (1997). Differences in DNA methylation
753 patterns are detectable during the dimorphic transition of fungi by amplification of restriction
754 polymorphisms. *Mol Gen Genet* 253, 703–710.
- 755 Richards, E. J. (1997). DNA methylation and plant development. *Trends Genet* 13, 319–323.
doi:10.1016/S0168-9525(97)01199-2.

- 756 Robinson, A. L., Adams, D. O., Boss, P. K., Heymann, H., Solomon, P. S., and Trengove, R. D.
757 (2012). Influence of Geographic Origin on the Sensory Characteristics and Wine Composition
758 of *Vitis vinifera* cv. Cabernet Sauvignon Wines from Australia. *American journal of enology*
759 *and viticulture* 63, 467–476. doi:10.5344/ajev.2012.12023.
- 760 Robinson, S., and Sandercock, N. (2014). An analysis of climate, soil and topographic information to
761 aid the understanding of barossa terroir.
- 762 Rodríguez López, C. M., Morán, P., Lago, F., Espiñeira, M., Beckmann, M., and Consuegra, S.
763 (2012). Detection and quantification of tissue of origin in salmon and veal products using
764 methylation sensitive AFLPs. *Food Chem* 131, 1493–1498.
765 doi:10.1016/j.foodchem.2011.09.120.
- 766 Rodríguez López, C. M., and Wilkinson, M. J. (2015). Epi-fingerprinting and epi-interventions for
767 improved crop production and food quality. *Front Plant Sci* 6, 397.
768 doi:10.3389/fpls.2015.00397.
- 769 Róis, A. S., Rodríguez López, C. M., Cortinhas, A., Erben, M., Espírito-Santo, D., Wilkinson, M. J.,
770 and Caperta, A. D. (2013). Epigenetic rather than genetic factors may explain phenotypic
771 divergence between coastal populations of diploid and tetraploid *Limonium* spp.
772 (*Plumbaginaceae*) in Portugal. *BMC Plant Biol* 13, 205. doi:10.1186/1471-2229-13-205.
- 773 Dal Santo, S., Fasoli, M., Negri, S., D’Incà, E., Vicenzi, N., Guzzo, F., Tornielli, G. B., Pezzotti, M.,
774 and Zenoni, S. (2016). Plasticity of the Berry Ripening Program in a White Grape Variety.
775 *Front Plant Sci* 7, 970. doi:10.3389/fpls.2016.00970.
- 776 Schellenbaum, P., Mohler, V., Wenzel, G., and Walter, B. (2008). Variation in DNA methylation
777 patterns of grapevine somaclones (*Vitis vinifera* L.). *BMC Plant Biol* 8, 78.
778 doi:10.1186/1471-2229-8-78.
- 779 Seguin G (1986) ‘Terroirs’ and pedology of wine growing. *Experientia* 42, 861-873.

- 780 Smouse, P. E., Whitehead, M. R., and Peakall, R. (2015). An informational diversity framework,
781 illustrated with sexually deceptive orchids in early stages of speciation. *Mol Ecol Resour* 15,
782 1375–1384. doi:10.1111/1755-0998.12422.
- 783 Supek, F., Bosnjak, M., Skunca, N. & Smuc, T. REVIGO summarizes and visualizes long lists of
784 gene ontology terms. *PLoS One* 6, e21800 (2011).
- 785 Tonietto, J., and Carbonneau, A. (2004). A multicriteria climatic classification system for grape-
786 growing regions worldwide. *Agricultural and Forest Meteorology* 124, 81–97.
787 doi:10.1016/j.agrformet.2003.06.001.
- 788 Torregrosa, L., Fernandez, L., Bouquet, A., Boursiquot, J.-M., Pelsy, F., and Martínez-Zapater, J.-M.
789 (2011). “Origins and Consequences of Somatic Variation in Grapevine,” in Genetics,
790 Genomics, and Breeding of Grapes, ed. C. Kole (Science Publishers), 68–92.
791 doi:10.1201/b10948-4.
- 792 Tricker, P. J., Gibbings, J. G., Rodríguez López, C. M., Hadley, P., and Wilkinson, M. J. (2012). Low
793 relative humidity triggers RNA-directed de novo DNA methylation and suppression of genes
794 controlling stomatal development. *J Exp Bot* 63, 3799–3813. doi:10.1093/jxb/ers076.
- 795 Tricker, P. J., López, C. M. R., Gibbings, G., Hadley, P., and Wilkinson, M. J. (2013a).
796 Transgenerational, dynamic methylation of stomata genes in response to low relative
797 humidity. *Int J Mol Sci* 14, 6674–6689. doi:10.3390/ijms14046674.
- 798 Tricker, P., Rodríguez López, C., Hadley, P., Wagstaff, C., and Wilkinson, M. (2013b). Pre-
799 conditioning the epigenetic response to high vapor pressure deficit increases the drought
800 tolerance of *Arabidopsis thaliana*. *Plant Signal Behav* 8. doi:10.4161/psb.25974.
- 801 Tyunin, A. P., Kiselev, K. V., and Karetin, Y. A. (2013). Differences in the methylation patterns of
802 the VaSTS1 and VaSTS10 genes of *Vitis amurensis* Rupr. *Biotechnol Lett* 35, 1525–1532.
803 doi:10.1007/s10529-013-1235-1.

- 804 Unwin, T. (2012). “Terroir: At the Heart of Geography,” in *The Geography of Wine*, ed. P. H.
805 Dougherty (Dordrecht: Springer Netherlands), 37–48. doi:10.1007/978-94-007-0464-0_2.
- 806 Vogt, G. (2015). Stochastic developmental variation, an epigenetic source of phenotypic diversity
807 with far-reaching biological consequences. *J Biosci* 40, 159–204. doi:10.1007/s12038-015-
808 9506-8.
- 809 Whiting, J. (2003). Selection of grapevine rootstocks and clones for greater Victoria. Victoria.
810 Department of Primary Industries.
- 811 Williams, L. E., and Araujo, F. J. (2002). Correlations among predawn leaf, midday leaf, and midday
812 stem water potential and their correlations with other measures of soil and plant water status
813 in *Vitis vinifera*. *Journal of the American Society of Horticultural Science* 127, 448–454.
- 814 YunLei, Z., WuWei, Y., JunJuan, W., BaoXiang, F., and LiYan, S. (2009). Review of DNA
815 methylation and plant stress-tolerance. *Acta Botanica Boreali-Occidentalia Sinica* 29, 1479–
816 1489.
- 817 Zhang, X., Yazaki, J., Sundaresan, A., Cokus, S., Chan, S. W.-L., Chen, H., Henderson, I. R., Shinn,
818 P., Pellegrini, M., Jacobsen, S. E., et al. (2006). Genome-wide high-resolution mapping and
819 functional analysis of DNA methylation in arabidopsis. *Cell* 126, 1189–1201.
820 doi:10.1016/j.cell.2006.08.003.
- 821
822

823

824