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Canonical correlations reveal adaptive loci and phenotypic responses to climate in perennial ryegrass

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11

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Accepted Article

36 **Abstract**

37 Germplasm from perennial ryegrass (*Lolium perenne* L.) natural populations is useful for breeding because
38 of its adaptation to a wide range of climates. Climate-adaptive genes can be detected from associations
39 between genotype, phenotype and climate but an integrated framework for the analysis of these three
40 sources of information is lacking.

41 We used two approaches to identify adaptive loci in perennial ryegrass and their effect on phenotypic traits.
42 First, we combined Genome-Environment Association (GEA) and GWAS analyses. Then, we implemented
43 a new test based on a Canonical Correlation Analysis (CANCOR) to detect adaptive loci. Furthermore, we
44 improved the previous perennial ryegrass gene set by *de novo* gene prediction and functional annotation of
45 39,967 genes.

46 GEA-GWAS revealed eight outlier loci associated with both environmental variables and phenotypic traits.
47 CANCOR retrieved 633 outlier loci associated with two climatic gradients, characterized by cold-dry vs
48 mild-wet winter and long rainy season vs long summer, and pointed out traits putatively conferring
49 adaptation at the extremes of these gradients. Our CANCOR test also revealed the presence of both
50 polygenic and oligogenic climatic adaptations. Our gene annotation revealed that 374 of the CANCOR
51 outlier loci were positioned within or close to a gene. Co-association networks of outlier loci revealed a
52 potential utility of CANCOR for investigating the interaction of genes involved in polygenic adaptations.

53 The CANCOR test provides an integrated framework to analyze adaptive genomic diversity and phenotypic
54 responses to environmental selection pressures that could be used to facilitate the adaptation of plant
55 species to climate change.

56

57 **Keywords:** Adaptation, Agriculture, Climate Change, Ecological Genetics, Landscape Genetics,
58 Quantitative Genetics.

59

60 **Introduction**

61 It is now widely acknowledged that droughts and heatwaves will become more frequent and more intense
62 in Europe with climate change (Samaniego et al., 2018; Teuling, 2018) and that rising global temperature
63 will have a profound effect on natural plant populations and crops (Mora et al., 2015; Thuiller, Lavorel,
64 Araújo, Sykes, & Prentice, 2005; Travis, 2016). Climate change will cause an increasing number of hot
65 summers, will lengthen the growing season at high latitudes such as in the Nordic countries and will
66 shorten it at southern latitudes such as in the Mediterranean region (Dai, 2013). Climate extremes, changes
67 in the length of the growing season and their interaction constitute complex challenges for biodiversity
68 conservation and plant breeding (Savolainen, Lascoux, & Merilä, 2013).

69 Natural plant populations have used diverse survival strategies to adapt to a variety of climates at variable
70 temporal and spatial scales (Brozynska, Furtado, & Henry, 2016; Godfray et al., 2010; Henry & Nevo, 2014;
71 Hodgkin & Bordoni, 2012). However, plant breeding has only used a tiny fraction of the genetic diversity
72 available within the entire gene pool of the species and the genetic diversity in cultivated gene pools is low
73 compared to that harbored by natural populations (Blanco-Pastor et al., 2019; Brozynska et al., 2016;
74 Redden et al., 2015; Warschefsky, Penmetsa, Cook, & von Wettberg, 2014). As a consequence, natural
75 populations are one of the most critical assets to address climate change adaptation of species used in
76 agriculture. As wild plant populations have evolved to cope with changes in their environment by means of
77 natural selection, they constitute useful sources of diversity that can be used to improve crop resistance to
78 extreme climatic conditions (FAO, 2015; Redden et al., 2015; Vincent et al., 2013; Warschefsky et al.,
79 2014).

80 One promising strategy to create plant genotypes adapted to extreme climatic conditions is to identify loci
81 responsible for adaptation in stress-tolerant natural populations. This strategy has become feasible thanks
82 to recent advances in the development of genomic tools (Bansal, Lenka, & Mondal, 2014; de la Peña,
83 Ebert, Gniffke, Hanson, & Symonds, 2011) and predictive statistical approaches (Manel et al., 2018).
84 However, the agronomic performance of natural populations is in general lower than that of commercial
85 varieties. It would thus be particularly challenging to create improved gene pools from natural populations
86 that would combine adaptation to particular climatic conditions and sufficient value for cultivation. Often, a
87 compromise needs to be found between climatic adaptation and agronomic value, as an adaptive gene
88 might have negative pleiotropic effects on other traits or might be in linkage disequilibrium with genes of
89 agronomic interest (i.e. linkage drag, Zamir, 2001). To account for these issues, a primary step in breeding
90 programs that focus on adaptation to climatic threats is the identification of the genomic diversity
91 responsible for adaptation and the documentation of its relationship with phenotypes (Shukla & Mattoo,
92 2013). This documentation is essential because phenotypic variation is the ultimate driver of both climate
93 adaptation and agronomic value (Rieseberg, Widmer, Arntz, & Burke, 2002; Sampoux, Barre, & Litrico,
94 2014). But it is also challenging because adaptive phenotypic responses may be determined by a large
95 number of loci of small effect (polygenic traits) that are difficult to identify with current analytical approaches
96 (Berg & Coop, 2014; Berg et al., 2019; Pritchard & Di Rienzo, 2010; Santure & Garant, 2018; Savolainen et
97 al., 2013).

98 The combination of the univariate association methods Genome-Wide Association Studies (GWAS) and
99 Genome-Environment Association analyses (GEA) has proven to be an effective approach to reveal the
100 genomic determinism of phenotypic traits and its relationship with climate adaptation (Anderson, Kono,
101 Stupar, Kantar, & Morrell, 2016; Atwell et al., 2010; Contreras-Moreira et al., 2019; Fournier-Level et al.,
102 2011; Talbot et al., 2017). These methods have however some limitations. GWAS and GEA can only detect
103 adaptive loci whose effects are not hidden by the confounding effect of neutral genetic structure (but see
104 Caye, Jumentier, Lepeule, & François, 2019; Frichot & François, 2015; Frichot, Schoville, Bouchard, &
105 François, 2013; Price, Lopez, Platts, & Lasky, 2020). More importantly, they only provide a partial discovery
106 of adaptive diversity, as local adaptation can be largely determined by coordinated shifts in allele

107 frequencies from multiple loci that are ignored when single-locus analyses are used (Berg & Coop, 2014;
108 Exposito-Alonso et al., 2018; Josephs, Berg, Ross-Ibarra, & Coop, 2019). Although recent reviews have
109 stressed the relevance of multivariate analyses that integrate environmental, genotypic and phenotypic
110 data to uncover adaptive loci with small-effect while reducing the number of false positives (Barrett &
111 Hoekstra, 2011; Hoban et al., 2016), a relatively small number of studies have achieved this integration so
112 far (but see Berg & Coop, 2014; Exposito-Alonso et al., 2018). In that sense, ordination-based multivariate
113 methods show promise as they can effectively detect multilocus selection by analyzing how groups of
114 markers covary in response to multiple predictors (Forester, Lasky, Wagner, & Urban, 2018).

115 Grassland ecosystems are ubiquitous across temperate and tropical regions. They constitute the most
116 extensive semi-natural habitat type accounting for 37% of the terrestrial land cover (Loveland et al., 2000).
117 They are essential for the maintenance of biodiversity, for carbon sequestration and for the functioning of
118 soil biogeochemical cycles (Hejcman, Hejcmanová, Pavlů, & Beneš, 2013; Jones & Donnelly, 2004;
119 Tilman, Wedin, & Knops, 1996). In Europe, they cover 45% of the total agricultural area (Eurostat, 2017).
120 Perennial ryegrass (*Lolium perenne* L.) is one of the most prevalent grass species in natural and semi-
121 natural permanent grasslands across Europe. Its high nutritive value for herbivores and its relatively good
122 adaptation to grazing and trampling have long been recognized. Perennial ryegrass has thus extensively
123 been bred during the past fifty years to deliver improved commercial varieties to sow and regenerate
124 meadows as well as to set up and repair turf areas (Sampoux et al., 2013, 2011). While domestication of
125 major crops started ca. 10000 years ago (Zohary, Hopf, & Weiss, 2012), conscious breeding in perennial
126 ryegrass was initiated only during the last century (Blanco-Pastor et al., 2019; Humphreys, Feuerstein,
127 Vandewalle, & Baert, 2010; Sampoux et al., 2013, 2011). Because of this recent start of human selection,
128 wild populations of perennial ryegrass may still contain potentially useful genetic resources that could be
129 easily incorporated into breeding programs. With that regard, wild populations have extensively been
130 collected in the last decades (Sampoux et al., 2014).

131 Perennial ryegrass natural populations colonized Europe during the Quaternary glacial cycles while
132 adapting to a wide range of environmental conditions (Barre et al., 2017; Blanco-Pastor et al., 2019).
133 Natural ecotypes of perennial ryegrass are today present over a wide range of climatic conditions across
134 Europe and the Near-East (Blanco-Pastor et al., 2019). Cold, heat and drought stresses in the latitudinal
135 and longitudinal extremes of Europe have likely led to the evolution of seasonal acclimation processes
136 regulating climate adaptation in perennial ryegrass (Ergon et al., 2018; Thomas & James, 1999; Zhang,
137 Fei, Arora, & Hannapel, 2010). Consequently, the extant wide natural diversity of perennial ryegrass should
138 represent a valuable genetic resource for its adaptation to climate change (Sampoux et al., 2014). Past
139 breeding activities in perennial ryegrass have mainly focused on improving forage yield, disease resistance
140 and seed yield for the seed industry (Humphreys et al., 2010; Wilkins & Humphreys, 2003). In contrast,
141 there have been fewer efforts to improve resistance to cold, heat and drought stresses (Charmet,
142 Balfourier, Ravel, & Denis, 1993) and the specific phenotypic traits linked to climatic adaptation remain
143 insufficiently documented (Barre et al., 2017; Ergon et al., 2018; but see Kovi et al., 2015).

144 We used genomic and phenotypic data from 469 perennial ryegrass natural populations collected across
145 the natural distribution range of the species (427 genebank accessions and 42 populations collected in situ)
146 (see Blanco-Pastor et al., 2019). We combined Genotyping-by-Sequencing (GBS) and Highly Multiplexed
147 Amplicon Sequencing (HiPlex) pool-Seq genotyping data, extensive phenotyping characterization in three
148 experimental gardens in France, Belgium and Germany and fine-resolution environmental data at
149 population collection sites. We implemented two data-driven analytical approaches. First, we used GEA
150 combined with GWAS to identify putative climate adaptive loci. In a second approach, we implemented a
151 statistical test that used the output of a Canonical Correlation Analysis (CANCOR). The CANCOR (also
152 abbreviated CCorA) is a multivariate analysis that reveals the co-inertia between two tables that describe
153 the same set of observations (here the SNPs) with two different sets of possibly covarying variables (here
154 environmental and phenotypic variables). This approach analyzed simultaneously the environment at sites
155 of origin of populations, their phenotype assessed in experimental gardens and the allelic frequencies of
156 populations in order to identify the environmental variables imposing selection, the adaptive phenotypic
157 responses and the adaptive loci.

158 An extensively annotated gene set can help to identify climate adaptive genes and gene functions under
159 selection. In view of that, we also improved the published perennial ryegrass gene set (Byrne et al., 2015)
160 by *de novo* gene prediction and functional annotation of our genomic dataset. The CANCOR test and the
161 new functional annotation provided a list of loci and molecular functions putatively linked to environmental
162 adaptation that could be used in breeding programs to adapt perennial ryegrass to climate change.

163

164 **Materials and Methods**

165 Genetic material

166 We examined 469 natural populations of perennial ryegrass that were either obtained from genebanks of
167 agronomic research institutes from multiple countries or sampled *in situ* (Fig. 1 and Table S1). They were
168 chosen to capture the extant natural genetic diversity of perennial ryegrass across its natural distribution
169 range (Europe and the Near East). Full description of this set of populations can be found in Blanco-Pastor
170 *et al.* (2019) named as the '*L. perenne set*'.

171

172 Genotype data

173 The genetic data was generated using a Genotyping-by-Sequencing (GBS) pool-Seq protocol
174 (Blanco-Pastor et al., 2019) based on the protocol of Byrne *et al.* (2013). We also re-sequenced from same
175 pools 185 genomic regions of 80-140 bp positioned in, or near, 42 candidate genes putatively involved in
176 environmental adaptation using Highly Multiplexed Amplicon Sequencing (HiPlex set) (see gene
177 descriptions in Supporting Information, Table S2, and further information in Supporting Information,
178 Methods S1). For the GBS and HiPlex genotyping methods, balanced leaf material from c.a. 300

179 individuals per population were pooled before DNA extraction. Variants were called using the draft
180 reference genome sequence of Byrne et al. (2015). Further details are available in the Supporting
181 Information of Blanco-Pastor *et al.* (2019). We merged the two datasets (GBS and HiPlex) for analyses and
182 performed a stringent filter on the minor allele frequency (MAF) to reduce the proportion of low frequency
183 alleles. We retained SNP loci if their MAF was greater than 5% in at least 10 populations. The final merged
184 dataset comprised alternative allele frequencies (AAFs) of 189,968 SNP loci in the 469 natural populations
185 (Data S1 in Blanco-Pastor et al., 2020). The genotype data included 7.81% missing values that were
186 imputed by using the mean allele frequency across populations. To avoid the effect of linkage
187 disequilibrium in outlier discovery, we calculated the kinship-corrected correlation decay with increasing
188 base pair distance for SNP markers belonging to a same scaffold. Based on the squared correlation decay
189 curve, and in line with results from Keep et al. (2020), we considered that two loci were linked when the
190 correlation between their alternative allele frequencies corrected for kinship was larger than 0.4. In such
191 case, we only kept the locus displaying the best association with a phenotypic trait (lowest p-value in
192 independent GWAS analyses).

193

194 Environmental variables

195 We collected a set of 112 variables documenting environmental conditions at sites of origin of the 469
196 studied populations: bioclimatic indices, Climate Change Detection Indices (ETCCDI), ecophysiological
197 indices relevant to the life cycle of perennial ryegrass and soil data derived from the European Soil
198 Database. An exhaustive overview of the environmental variables used is provided in Supporting
199 Information, Table S3 and Methods S2.

200

201 Phenotypic traits

202 For the needs of phenotyping, 385 of the 469 perennial ryegrass populations were sown in experimental
203 gardens in three locations: Poel Island (PO) in Germany on April 2015, Melle (ME) in Belgium on October
204 2015 and Lusignan (LU) in France on April 2015. In each of these three locations, each population was
205 sown in three 1m² micro-swards (small plots sown as to reach plant density similar to real grasslands)
206 arranged in three replicated blocks. Trials were monitored until end of 2017 at PO and ME and until end of
207 2018 at LU. Micro-swards were cut (all aerial biomass higher than 7 cm above ground surface) regularly as
208 to simulate common cutting regime of meadows used for green forage production or grazing. Weather
209 conditions experienced at each trial location are displayed per season of each year in Supporting
210 Information, Table S4. LU was characterized by severe water stress in summer. At PO, water stress was
211 negligible in summer but cold stress was experienced during winter periods. ME was characterized by cool
212 summer and mild winter conditions. Scores or measurements of phenotypic traits were recorded at the
213 level of 1 m² micro-swards over all plants. A set of 145 phenotypic traits were recorded for 385 of the 469

214 perennial ryegrass populations. This set included traits related to vigor after sowing, morphology of plants,
215 sward density, phenology, investment in sexual reproduction, dynamics of vegetative growth in spring,
216 summer and autumn, regrowth after cutting, abiotic and biotic stresses related traits, dynamics of
217 persistency, biochemistry of aerial biomass and leaf lamina traits. An exhaustive overview of the recorded
218 phenotypic traits is provided in Supporting Information, Table S5 and Methods S3.

219

220 The GEA-GWAS approach

221 We performed a “triangulation” of association analyses (e.g. Talbot et al., 2017) to detect putative adaptive
222 loci (Fig. 2a). In this approach, we looked for strong significant environment-genotype (GEA), phenotype-
223 genotype (GWAS) and direct environment-phenotype associations to investigate whether putative adaptive
224 loci were also potentially involved in the determinism of potentially adaptive traits.

225 To identify putative adaptive loci, we used a GEA linear mixed model similar to that of Yoder *et al.* (2014)
226 (Supporting Information, Methods S4). We additionally used a GWAS linear mixed model to assess
227 individual locus effect on a given phenotypic trait (Supporting Information, Methods S5). Both GEA and
228 GWAS were run using the *GWAS* function of the ‘rrBLUP’ R package (Endelman, 2011). Among the
229 significant loci revealed by the GEA analysis, we only considered as GEA-GWAS outlier those also
230 significantly associated with a phenotypic trait in GWAS. We used a liberal threshold of False Discovery
231 Rate (FDR) of 0.2 in both GEA and GWAS because a SNP needed to be significant in the two independent
232 analyses to be considered as outlier. But we also report results using the more conservative FDR = 0.1.
233 The final step of the “triangulation” approach was the assessment of direct correlations between
234 environmental variables and phenotypic traits significantly associated with a same locus (direct correlations
235 significant at p -value < 0.05).

236

237 The CANCOR test

238 As an alternative to investigate adaptive diversity, we implemented a Canonical Correlation Analysis
239 (CANCOR) (Hotelling, 1936) (Fig. 2b) that analyzed simultaneously the association of genomic
240 polymorphisms with environmental variables and phenotypic traits. The CANCOR multivariate analysis
241 aims to reveal the co-inertia between two sets of possibly co-varying variables that describe the same set
242 of experimental units (or observations). It looks for successive pairs of linear combinations from each set
243 (canonical variables) that are maximally correlated (canonical correlations). Successive canonical variables
244 in each set are constrained as to be uncorrelated. In a preliminary step, univariate regression models were
245 implemented to regress the population alternative allele frequency (AAF) of each genotyped SNP locus on
246 each environmental variable (values at sites of origin of populations) and on each phenotypic trait (mean
247 values of populations). The CANCOR was then performed by considering the loci as the experimental units
248 and the two sets of regression slopes of alternative allele frequencies, on environmental variables on the

249 one hand (Y, Fig. 2b) and on phenotypic traits on the other hand (X, Fig. 2b), as the two sets of input
250 variables to analyze (Supporting Information, Methods S6).

251 We also ran an additional CANCOR in order to discern the general structure of correlation between
252 environmental and phenotypic variables at the population level. In this analysis, the populations were
253 considered as the experimental units and the value of environmental variables at sites of origin of
254 populations on the one hand, and the population means of phenotypic traits on the other hand, as the two
255 sets of input variables to analyze.

256 We ran the CANCOR analysis using the R package 'vegan' (Oksanen et al., 2018). We tested the
257 significance of outlier loci using a χ^2 test on Mahalanobis distances following the method of Luu et al.
258 (2017) and Capblancq et al. (2018), which we call hereafter CANCOR test (Supporting Information,
259 Methods S6), and a locus was considered as outlier at FDR = 0.1.

260 To relate CANCOR outliers to putative adaptive traits and selective environmental variables, we first
261 selected the CANCOR input variables best represented in the first two canonical dimensions, which were
262 the only two retained by our CANCOR test. To simplify results we selected only input variables with
263 projection norms larger than 0.95 and 0.90 in the first environmental and phenotypic canonical planes,
264 respectively (thresholds that returned a similar number of environmental and phenotypic variables). We
265 finally retained the corresponding environmental and phenotypic variables if their correlation with the AAFs
266 of at least one CANCOR outlier locus was sufficiently high ($|r| > 0.5$) (see Forester et al., 2018). We also
267 explored the variable heading date (*HEA_avg*) despite its smaller projection norm because of its known
268 importance for adaptation. In order to relate environmental selection pressures to phenotypic responses,
269 we identified those environmental variables sharing their position in the CANCOR first two dimensions with
270 the highest number of phenotypic traits. We further investigated these relationships by performing a linear
271 regression of the phenotypic trait on the environmental variable using the trait mean values of populations
272 and the value of environmental variables at sites of origin of populations.

273

274 Co-association networks

275 To visualize the interaction of SNPs in the bi-dimensional space defined by the CANCOR test, we adapted
276 the approach of Lotterhos et al. (2018). In order to account for the information from both the alternative and
277 reference alleles, we classified CANCOR outliers into two groups according to their position in the four
278 CANCOR quadrants of the first phenotypic canonical plane. We grouped outliers from quadrants I and III
279 since they were expected to be associated to adaptation to the same environmental gradients. For that, we
280 changed the value of the loadings on the CANCOR axes of SNPs in quadrants III to their symmetrical value
281 in quadrant I (negative signs of the loadings on the axes 1 and 2 replaced by positive signs), as the position
282 of SNPs in quadrant I or III only depends on whether the alternative or the reference allele is associated to
283 adaptation. Similarly, we grouped outliers from quadrants II and IV and we changed the value of the
284 loadings of SNPs in quadrants IV to their symmetrical value in quadrant II (positive sign of loadings on the

285 axis 1 replaced by negative sign and negative sign on the axis 2 replaced by positive sign). Then we used
286 these modified canonical loadings to calculate a matrix of pairwise Euclidean distances between SNPs. For
287 each of the two groups, we used undirected graph networks to visualize modules of SNPs. Nodes were
288 connected by edges according to three different thresholds of pairwise Euclidean distances (d) (< 1 , < 0.5
289 and < 0.1). Co-association networks were visualized using the 'igraph' R package (Csardi & Nepusz, 2006).
290 To demonstrate the utility of CANCOR for investigating the genetic basis of complex traits, for each of the
291 two groups of SNPs, we performed two independent Gene Ontology (GO) enrichment analyses in the
292 largest modules obtained with threshold of $d < 0.1$. The GO enrichment analyses were performed with
293 agriGO 2.0 (Tian et al., 2017). We used the Singular Enrichment Analysis (SEA) tool with a customized
294 annotation of GO terms obtained from the new gene prediction and functional annotation (see below) and
295 used the Locus ID (PLAZA3.0) as reference. We applied the Fisher's exact test with the Benjamini-
296 Hochberg FDR correction (FDR < 0.05) (Benjamini & Hochberg, 1995).

297

298 Gene prediction and functional annotation

299 The EvidenceModeler (EVM) (Haas et al., 2008) was used to improve completeness, without losing gene
300 model accuracy, of the previously published set of 28,182 genes annotated on the *L. perenne* draft genome
301 sequence (Byrne et al., 2015). For this, the annotation set was complemented with a less conservative set
302 of gene predictions, orthology-guided transcript assemblies (Ruttink et al., 2013) and aligned proteomes of
303 closely related species (*Brachypodium distachyon*, rice, maize and sorghum). All evidence tracks were
304 generated using the GenomeThreader (Gremme, Brendel, Sparks, & Kurtz, 2005) with default settings and
305 used as input for the EVM. The completeness was estimated using BUSCO (Simão, Waterhouse,
306 Ioannidis, Kriventseva, & Zdobnov, 2015) and the PLAZA 2.5 monocots core gene families (Van Bel et al.,
307 2012). Functional annotation making use of ontologies was generated using InterPro2GO mapping, Gene
308 Ontology (GO) projection between orthologs and MapMan. Additionally, gene descriptions were added
309 using AnnoMine, a homology-based text-mining approach (Van Landeghem, De Bodt, Drebert, Inzé, & Van
310 de Peer, 2013). The gene annotation set has been made publicly available on the PLAZA comparative
311 genomics platform version 4.5 Monocots
312 (https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v4_5_monocots/organism/view/Lolium+perenne).

313

314 Results

315 The GEA-GWAS approach

316 A total of 10,220 and 15,854 loci were found as outliers with the GEA analyses at FDR = 0.1 and FDR =
317 0.2, respectively. A total of 330 and 543 loci were found as outliers with the GWAS analyses at FDR = 0.1
318 and FDR = 0.2, respectively. Among these, only 18 and 49 outliers were significant in both the GEA and
319 GWAS at FDR = 0.1 and FDR = 0.2, respectively (Supporting Information, Table S6 and Table S7).

320 Environmental and phenotypic variables most strongly associated with GEA-GWAS outliers at FDR = 0.1
321 were *bd_subsoil*, *bio.ad.27*, *bio10*, *pet_wi*, *lmts*, *oc_topsoil*, *sis_wi*, *tawc_soil*, *tr_an*; and *AHD_me16*,
322 *AMH_po17*, *CH400h_po16*, *CHs500_me17*, *HFY_lu15*, *HFY_po15*, *HST_lu17*, *SCD_wi1516_po*,
323 *VAC_avg*, *VAC_lu17*, respectively (Table 1 and Supporting Information, Table S7). At FDR = 0.1,
324 phenotypic traits showing strongest association with the highest number of GEA-GWAS outliers were
325 *AMH_po17* (autumn canopy height, 6 outliers) and *CH400h_po16* (spring canopy height 400 growing-
326 degree-days before spike emergence, 5 outliers) (Table 2 and Supporting Information, Table. S7). We
327 found 8 outlier loci significantly associated at FDR = 0.1 with an environmental variable and a phenotypic
328 trait whose direct correlation was significant (p -value < 0.05), and 34 outlier loci at FDR = 0.2. These loci
329 were located in the proximity of 3 (FDR = 0.1) and 16 (FDR = 0.2) independent known genes (Supporting
330 Information, Table S7). The InterPro domains, the Gene Ontology and the functional annotations for these
331 genes are provided in Table S7 and more information on the genome sequence context flanking these
332 genes is available via PLAZA monocots 4.5. Our genotyped loci included SNPs from 185 amplicon regions
333 positioned in, or in the proximity of, 42 candidate genes possibly involved in environmental adaptation
334 (HiPlex set, Supporting Information, Table S2). Of these, the GEA-GWAS approach did not detect any SNP
335 as putatively adaptive at FDR = 0.1. At FDR = 0.2, GEA-GWAS detected 6 SNPs (from two candidate
336 genes) as putatively adaptive but only one showed direct correlation between the associated environmental
337 and phenotypic variables (p -value < 0.05) (Supporting Information, Table S7).

338

339 The CANCOR approach

340 The CANCOR using the loci as experimental units (Fig. 3b-c) found the first 14 canonical correlations
341 ('CanCorr' elements of the CCorA function of the R package 'vegan') larger than 0.9. The environmental
342 input variables (regression slopes of AAFs on environmental variables) were highly correlated ($|r| > 0.7$) to
343 the first and second environmental canonical variates for 44 and 35 environmental variables, respectively.
344 Likewise, the phenotypic input variables (regression slopes of AAFs on phenotypic variables) were highly
345 correlated ($|r| > 0.7$) to the first and the second phenotypic canonical variates for 35 and 37 phenotypic
346 variables, respectively. In contrast, the CANCOR using populations as experimental units (Fig. 3d-f) only
347 found the first two canonical correlations larger than 0.9. None of the correlations between environmental
348 canonical variates and environmental variables were larger than 0.7. Only the first phenotypic canonical
349 variate showed high correlation ($|r| > 0.7$) with four phenotypic traits. Populations from a same geographical
350 origin tended to cluster together on the first phenotypic canonical plane.

351 With the CANCOR test, we observed that the distribution of p -values was correct (flat distribution with
352 enrichment only for the low values) exclusively when only the first two canonical dimensions were
353 considered ($K = 2$) and therefore only the results with $K = 2$ are discussed here (see Supporting Information,
354 Methods S6). At FDR = 0.1, the CANCOR test retrieved 633 outlier loci ("CANCOR outliers") which were
355 located in the proximity of 158 independent known genes (Fig. 3a and Supporting Information, Fig. S2 and
356 Table S8) among which 13 were "HiPlex" loci. The CANCOR test only found four and 10 outlier loci that

357 were also significant outliers in both the GEA and GWAS linear mixed models at FDR = 0.1 and FDR = 0.2,
358 respectively (Supporting Information, Fig. S3). CANCOR outliers showing high correlation ($|r| > 0.5$) with
359 environmental and phenotypic variables well represented in the first two canonical dimensions (projection
360 norm of input variables larger than 0.95 and 0.90, respectively) are shown in Fig. 4.

361 The two main environmental gradients revealed by the CANCOR test are highlighted in Fig. 5. A first
362 gradient opposed the first and third quadrants of the first environmental canonical plane with increasing
363 winter temperature (*tnn_wi*, *txx_wi*, *tasmax_wi*, *tasmin_wi*, *bio3* and *bio6*) and precipitation during the wet
364 season (*rx1day_au*, *sdii_au*, *sdii_sp*, *sdii_wi* and *bio.ad.20*) towards the third quadrant (Fig. 5a) (mild-wet
365 vs cold-dry winter gradient). A second gradient opposed the second and fourth quadrants with increasing
366 duration of summer period (*su_an*), decreasing duration of the rainy periods in autumn (*r01mm_au*) and
367 winter (*r01mm_wi*) and increasing mean diurnal temperature range (*bio2*, *dtr_au*) towards the fourth
368 quadrant (Fig. 5a) (long summer and high diurnal temperature range vs long rainy season and low diurnal
369 temperature range). Note that soil properties were not evidenced to contribute to co-inertia in this CANCOR
370 analysis, at least in the first two canonical dimensions.

371 In the first phenotypic canonical plane, the first quadrant was associated with canopy height during
372 vegetative spring growth in the northernmost experimental garden (*CH300h_po16*, *CH400h_po16*) and with
373 spike emergence date (*HEA_avg*) whereas the third quadrant was associated with winter damage
374 (*WID_po16*) in the northernmost experimental garden (Fig. 5b). The second quadrant was associated with
375 canopy height and canopy growth rate in summer (*SMH_me16*, *SGR_po17* and *SMH_po17*) and in autumn
376 (*AGR_po17* and *AMH_po17*) in the two northern experimental gardens and with good persistency after
377 winter in the northernmost one (*SCD_wi1617_po*) (Fig. 5b). The fourth quadrant was associated with seed
378 production traits, namely aftermath heading (successive recurrent elongation of fertile stems) (*AHD_lu16*
379 and *AHD_po17*) and spike density (*DST_avg*, *DST_lu17* and *resDST_lu17*), with lignin content in
380 vegetative biomass (*ADL_10_me17* and *ADL_avg*) and with canopy growth rate in summer in the
381 southernmost garden (*SGR_lu16*). Among the preceding traits, *HEA_avg*, *ADL_10_me17*, *ADL_avg*,
382 *DST_avg*, *DST_lu17* and *resDST_lu17* were correlated ($|r| > 0.5$) to a small number of outlier loci: 5, 3, 1,
383 3, 20 and 21 respectively (Fig. 4b). Other traits, namely *AHD_lu16*, *AHD_po17*, *WID_po16*, *CH300h_po16*,
384 *CH400h_po16*, *SGR_lu16*, *SGR_po17*, *SMH_po17*, *AGR_po17*, *AMH_po17* and *SCD_wi1617_po* were
385 correlated ($|r| > 0.5$) to a higher number of outlier loci (30 to 289) (Fig. 4b).

386 An environmental variable and a phenotypic trait were considered as associated if they shared position in
387 the first two canonical dimensions (see Fig. 5). Univariate regressions testing the association of pairs of
388 environmental and phenotypic variables located in the same quadrant were highly significant in all cases
389 (p -value < 0.05), with r^2 values ranging between 0.014 and 0.382. Plots of two types of regressions are
390 displayed in Fig. 6: (i) phenotypic traits whose input variable is associated with the first or third quadrants of
391 the first phenotypic canonical plane regressed on minimum temperature of winter period (*tnn_wi*) and (ii)
392 phenotypic traits whose input variable is associated with the second or fourth quadrants regressed on the
393 number of summer days (*su_an*). These regressions confirmed a clear relationship between phenotypic

394 means and values of the climatic variables at sites of origin of populations. Adaptation to cold stress in
395 winter (low *tnn_wi*) was associated with high spring growth in cold conditions, late spike emergence and
396 small damage during cold winters. Adaptation to long summer (high *su_an*), and likely to drought and heat
397 stresses, was associated with high aftermath heading and spike density (reproductive investment), high
398 lignin content in vegetative biomass, high growth in warm summer conditions, but low growth in cool
399 summer and autumn conditions and low persistency after cold winter.

400 Among the HiPlex set, the CANCOR test detected 13 loci within three different known genes as outliers at
401 FDR = 0.1 threshold (Supporting Information, Table S8).

402

403 Co-association networks

404 The two co-association analyses with threshold of $d < 1$ showed a single large module. With threshold $d <$
405 0.5, SNPs from both quadrants I-III and II-IV showed a single module together with three/four SNPs that
406 were isolated or forming a small cluster. With this threshold at least three and two sub-modules could be
407 observed in quadrants I-III and quadrants II-IV, respectively. Analyses with threshold $d < 0.1$ mainly
408 resulted in singletons together with multiple small modules (Fig. 7).

409 We did not find any significantly enriched GO term in the largest module from quadrants I-III at $d < 0.1$.
410 However, we found two significantly enriched GO terms (FDR < 0.05) in the largest module from quadrants
411 II-IV at the same threshold: GO:0055114 (oxidation-reduction process) and GO:0016491 (oxidoreductase
412 activity).

413

414 An improved gene annotation for identifying adaptive gene functions in *Lolium* 415 *perenne*

416 Previous gene space completeness analysis (Veeckman *et al.*, 2016) showed that the gene space was well
417 represented in the *L. perenne* genome assembly (previously published set of 28,182 annotated genes), but
418 that gene prediction was incomplete, as compared to BUSCO (81.6%) and PLAZA 2.5 monocots core gene
419 families (CoreGF, 76.9%). Our additional gene annotation resulted in 39,967 consensus gene models.
420 Gene space completeness was estimated at 92.6% (single: 89.0%, duplicated: 3.6%, fragmented: 2.5%,
421 missing: 4.9%, no. of genes: 1440) using BUSCO and 89.4% using the PLAZA 2.5 CoreGF. This
422 corresponded to an overall increase of completeness of more than 10% compared to the previously
423 published gene annotation set. Functional annotation resulted in GO, InterPro and AnnoMine annotations
424 for 23,879 *L. perenne* genes (59.8%). This final gene set was better suited for checking whether outlier loci
425 from the CANCOR test matched candidate regions or were located in the proximity of a known gene, as it
426 was more complete and more informative thanks to the improved functional annotation. Using the initial
427 gene annotation set, 306 out of the 633 CANCOR outlier loci were positioned within or close to a gene, the

428 average distance to the closest gene was 16 kb and 93 loci were positioned on scaffolds without a gene.
429 Using the new gene annotation set resulted in 374 CANCOR outlier loci positioned within or close to a
430 gene, the average distance to the closest gene dropped to 9 kb and only 30 loci were positioned on
431 scaffolds without a gene.

432

433 **Discussion**

434 A novel approach to detect genomic and phenotypic adaptive diversity and to 435 identify environmental factors imposing selection

436 In our study, the combined GEA-GWAS approach was less effective than the CANCOR test in
437 simultaneously detecting the environmental variable and phenotypic trait associated with a putative
438 adaptive locus, even if the FDR thresholds used with GEA and GWAS were more liberal than the one used
439 with CANCOR (GEA-GWAS: 49 outliers at FDR = 0.2 with 34 showing significant direct environment-
440 phenotype correlation at p -value < 0.05; CANCOR: 633 outliers at FDR = 0.1). Certain climate-genotype-
441 phenotype associations found with the GEA-GWAS approach were also found with CANCOR (4 and 10
442 outlier SNPs found with both GEA-GWAS and CANCOR depending on FDR thresholds used for GEA-
443 GWAS, see Supporting Information, Results S1). But in general different associations were found with the
444 two methods. The GEA-GWAS approach detected interesting soil-genotype-phenotype associations that
445 were not detected by the CANCOR test, probably because the soil variables had little contribution in the
446 first two canonical dimensions used for CANCOR outlier detection. GEA-GWAS outliers associated with
447 soil variables were also associated with phenotypic traits describing the morphology of plants, investment in
448 sexual reproduction, phenology or plant growth. These set of outliers could be of interest for eventual
449 breeding programs that would aim to improve adaptation to soil features.

450 In most cases, a large part of the phenotypic variance remains unexplained by loci detected by GWAS
451 (Maher, 2008), a problem that was notably encountered in perennial ryegrass (Harper et al., 2019). GWAS
452 models control for false-positive associations due to population structure or genetic relatedness and
453 inference statistics are corrected for multiple tests (Yu et al., 2006). But because of these corrections, they
454 are prone to miss causal loci with small effect involved in polygenic adaptations (Josephs et al., 2019) or
455 other adaptive loci whose allelic distribution is confounded with population structure (Atwell et al., 2010), a
456 trend that is particularly common in natural populations (Barton, Hermisson, & Nordborg, 2019; Gienapp et
457 al., 2017; Storz, 2005). When compared with CANCOR, the GEA-GWAS method is likely more well suited
458 to identification of major effect loci affected by a single environmental variable and a phenotypic response
459 dominated by a single trait. On the other hand, CANCOR is not directly based on extreme associations of
460 genotyped loci with single environmental or phenotypic variables. And it can be expected as more powerful
461 to detect groups of co-varying small effect loci involved in response to multivariate environment and
462 associated with multivariate phenotypic responses.

463 Despite the high dimensionality of the genotype and the environment, univariate GEA methods have been
464 the most popular approach to identify adaptive loci (Coop, Witonsky, Di Rienzo, & Pritchard, 2010; Frichot
465 et al., 2013; e.g. Joost et al., 2007; Lasky, Forester, & Reimherr, 2017; Stucki et al., 2017; Yoder et al.,
466 2014). Meanwhile, it has been claimed that multivariate methods are far more effective at detecting weak
467 polygenic adaptation, as these methods can analyze the covariation between groups of loci and multiple
468 environmental predictors (Forester et al., 2018; Rellstab, Gugerli, Eckert, Hancock, & Holderegger, 2015).
469 Many adaptive processes are indeed expected to be driven by weak polygenic effects as a result of recent
470 selection on standing genetic variation that has not yet led to allele fixation or conditional neutrality (Berg &
471 Coop, 2014; Le Corre & Kremer, 2012; Savolainen et al., 2013; Tiffin & Ross-Ibarra, 2014). Several
472 multivariate ordination techniques have recently been proposed to identify adaptive loci (Capblancq et al.,
473 2018; Forester, Jones, Joost, Landguth, & Lasky, 2015; Grivet, Sork, Westfall, & Davis, 2008; Luu et al.,
474 2017). Although the need of integrating phenotypic data in adaptation studies has recurrently been
475 stressed (Barrett & Hoekstra, 2011; Berg & Coop, 2014; Exposito-Alonso et al., 2018; Fournier-Level et al.,
476 2011; Lasky et al., 2017; Steane et al., 2014), few studies investigating environmental adaptation have
477 combined phenotypic and environmental data so far. Here, we introduced a CANCOR test to integrate both
478 types of data in a single genome scan.

479 Multivariate analyses such CANCOR are not expected to be biased by collinearity. Keeping all available
480 variables, as we did, showed that our CANCOR approach can be used in an exploratory manner and make
481 possible to avoid the variable selection step. Environmental and phenotypic variables whose regression
482 slopes are best correlated to the canonical variables revealed adaptive trends consistent with functional
483 ecology expectations (Fig. 5).

484 The CANCOR analysis using the loci as observations revealed roughly the same patterns of association
485 between environment and phenotype as the CANCOR analysis using populations as observations.
486 However canonical correlations were larger with the former analysis suggesting that adaptive trends are
487 better revealed at the genomic level than at the population level (Fig. 3b-c and Fig. 3e-f).

488

489 Adaptation to winter temperature

490 The CANCOR test detected 36 to 169 outlier loci highly associated ($|r| > 0.5$) with traits reporting for spring
491 growth in a cold winter environment (*CH300h_po16* and *CH400h_po16*) and 370 with winter damage
492 during a cold winter (*WID_po16*). This result points to phenotypic adaptations to cold winter conditions
493 (quadrant I, Fig. 5) that are under highly polygenic determinism (Fig. 4b and Supporting Information, Table
494 S8). The CANCOR analysis indicated that perennial ryegrass populations from areas with low minimum
495 winter temperatures (low *tnn_wi*) are more resistant to winter damages (*WID_po16*) than others when
496 grown in the cold winter conditions of Northern Europe (Fig. 6a). Previous research on perennial ryegrass
497 also showed that populations from southern Europe were the most susceptible to cold stress (Lorenzetti,
498 Tyler, Cooper, & Breese, 1971). The CANCOR test detected only five outlier loci associated with spike

499 emergence date (*HEA_avg*), a trait also involved in adaptation to winter temperature (Fig. 4b and
500 Supporting Information, Table S8). The proportion of phenotypic variance explained by these outlier loci (r^2)
501 ranged from 0.25 to 0.33 (univariate linear model phenotype ~ outlier locus, results not shown). This is in
502 accordance with previous results which found a few major genes involved in the determinism of spike
503 emergence date in perennial ryegrass (Armstead et al., 2004, 2008; Keep et al., 2020; Skøt et al., 2005,
504 2011). These previous studies identified a major QTL explaining 70% of the trait variation in a F_2 mapping
505 family. This QTL showed a high degree of synteny with the *Hd3* spike emergence date QTL region of rice
506 LG6 that codes for the *Flowering locus T*. The gene prediction analysis found that the spike emergence
507 date loci pointed out by our CANCOR test included three loci located within the *Flowering locus T* (*LpFT3*
508 gene in the *L. perenne* genome) (Skøt et al., 2011; Veeckman, Vandepoele, Asp, Roldán-Ruiz, & Ruttink,
509 2016) and one locus located in its close proximity (at 272 bp downstream of the gene; Supporting
510 Information, Table S8). Our results are thus in agreement with these previous findings and evidence that
511 spike emergence date loci in perennial ryegrass evolved naturally along a winter temperature gradient (Fig.
512 6a).

513 The functional ecology theory tells that adaptation to climatic stresses can be provided by escape,
514 avoidance and tolerance strategies (Levitt, 1962). A tolerance strategy notably involves a strong reduction
515 or cessation of growth during the stress period (Gillespie & Volaire, 2017). In perennial ryegrass, the peak
516 of spring vegetative growth occurs during a 15 days period preceding spike emergence (Roschanski et al.,
517 2018). Late spike emergence of perennial ryegrass populations from areas with low minimum winter
518 temperature, and likely with long winter period, corresponds to an escape strategy in which the peak of
519 vegetative spring growth is scheduled to escape the latest period of cold stress. Our results confirm that
520 this important adaptive feature is determined by a small number of genes. On the other hand, small winter
521 damage in the northernmost experimental garden for populations from low minimum temperature areas
522 indicates additional tolerance mechanisms under highly polygenic determinism, which in turn favor a strong
523 spring vegetative growth after a cold winter.

524

525 Adaptation to summer length

526 The CANCOR test detected 26 and 46 outlier loci associated ($|r| > 0.5$) with aftermath heading (*AHD_po17*
527 and *AHD_lu16*), 3, 20 and 21 associated with spike density (*DST_avg*, *DST_lu17* and *resDST_lu17*) and
528 33 with growing rate in a dry summer (*SGR_lu16*). These results revealed phenotypic adaptations to long
529 summer duration (quadrant IV, Fig. 5) that are under polygenic determinism (Fig. 4b and Supporting
530 Information, Table S8). Sixty-one outliers in total were associated with aftermath heading or spike density
531 and thus with investment in sexual reproduction.

532 Our results are consistent with previous reports indicating that perennial ryegrass populations from dry
533 habitats recover from drought more rapidly than those from moist habitats (Norris & Thomas, 1982). The
534 CANCOR analysis evidenced that perennial ryegrass populations from areas with long summer season

535 (high *su_an*), and thus with high probability of exposure to drought stress, use several functional strategies
536 to adapt to this climatic constraint as described by Volaire (2018). A dehydration escape strategy is likely
537 provided by investment in sexual reproduction with high aftermath heading and spike density (Berger,
538 Palta, & Vadez, 2016). Better growing rate in a long and dry summer could be due to the growth of
539 numerous elongating stems from aftermath heading in relation with the escape strategy, but it can also be
540 due to some features enabling stress avoidance such as root architecture optimizing soil water extraction
541 (Voltaire, 2018).

542 The CANCOR test detected only two outlier loci associated ($|r| > 0.5$) with lignin content (one associated
543 with *ADL_10_me17* and another one with *ADL_10_me17* and *ADL_avg*) (Fig. 4b and Supporting
544 Information, Table S8) with an r^2 ranging from 0.26 to 0.27 (univariate linear model phenotype \sim outlier
545 locus, results not shown). This suggests that the genetic determinism of lignin content involves some large-
546 effect genes. According to our gene prediction analysis, one of these outlier loci is located in the coding
547 region of the *Anti-sigma-I factor RsgI6* gene which binds to and hydrolyses insoluble and soluble xylan
548 substrates (Bahari et al., 2011), a group of hemicelluloses that is found in all cell walls of grasses
549 (Mellerowicz & Gorshkova, 2011). High lignin content in vegetative biomass provides a high density of leaf
550 tissues (high Leaf Dry Matter Content or LDMC), which has been reported to contribute to resistance to
551 water stress (Garnier, Shipley, Roumet, & Laurent, 2001; Wilson, Thompson, & Hodgson, 1999).

552 The adaptive features of populations from long summer areas have however counterparts in less summer-
553 stressful climates. A trade-off between summer growth in the southernmost experimental garden
554 (*SGR_lu16*) and investment in sexual reproduction (*AHD_po17*, *AHD_lu16*, *DST_avg*, *DST_lu17* and
555 *resDST_lu17*) on the one hand, and summer growth (*SMH_po17* and *SGR_po17*), autumn growth
556 (*AMH_po17* and *AGR_po17*) and winter persistency (*SCD_wi1617_po*) in the northernmost experimental
557 garden on the other hand, was evidenced along the summer length gradient (Fig. 6b). The outlier loci
558 correlated to these two kinds of phenotypic features were common to some extent (Fig. 4b), suggesting
559 that this trade-off could be partly due to antagonistic pleiotropy (Exposito-Alonso, Burbano, Bossdorf,
560 Nielsen, & Weigel, 2019; Savolainen et al., 2013). However, the number of outlier loci found for autumn
561 growth in the northernmost experimental garden (*AGR_po17*) (289, 45,66% of all outliers) was notably
562 higher than the number found for summer growth in the southernmost experimental garden (*SGR_lu16*)
563 (44, 6,95% of all outliers) (Fig. 4b).

564 From an ecophysiological point of view, a strong investment in seed production may have a negative
565 impact on winter survival of vegetative tillers (low *SCD_wi1617_po*) (Barre et al., 2017). Balfourier &
566 Charmet (1991) also found correlations between aftermath heading in perennial ryegrass natural
567 populations and the latitude, temperature and aridity factors of their sites of origin. They observed that
568 populations from hot and dry regions tended to invest more in seed production, while populations from cool
569 and wet areas had more vigorous vegetative growth (higher spring and autumn growth and persistence)
570 and less aftermath heading. This trade-off between vegetative and reproductive investments was also

571 pointed out as a major lever of adaptation to warming conditions in other perennial grasses (Voltaire,
572 Barkaoui, & Norton, 2014).

573

574 Lessons for perennial ryegrass breeding in the context of climate change

575 During the 50 past years, perennial ryegrass has been subjected to intense breeding to create cultivars for
576 sowing meadows. Breeding efforts in Europe have been successful to strongly reduce aftermath heading
577 and this has resulted in a correlative improvement of autumn growth and persistency in cool climate areas
578 of Europe. A correlative increase in forage quality was also obtained including a lower fiber (notably lignin)
579 content in vegetative biomass (Sampoux et al., 2011).

580 In the next decades, longer and drier summers are foreseen to occur in a large part of Europe due to
581 anthropogenic climate change (Ergon et al., 2018). Although the length of the winter cold period is
582 expected to shorten with milder average temperature, low temperatures events may still occur even quite
583 late in the season (Dalmannsdottir et al., 2017; Ergon et al., 2018). Therefore, new efforts in breeding
584 programs aiming to adapt perennial ryegrass to longer and drier summers should not be at the expense of
585 adaptation to winter cold stresses. Our results showed that sets of different loci are involved in adaptation
586 to long summer climate and adaptation to low winter temperatures. It should thus be possible to combine to
587 a large extent these two kinds of adaptive features by genetic recombination.

588 The co-association network analysis revealed modules for both analyses of SNPs from quadrants I-III and
589 quadrants II-IV (at $d < 0.1$, see Fig. 7). The observation of these modules point to the presence of relatively
590 independent groups of SNPs with homogeneous trends of variation under the environmental conditions
591 defined by these quadrants. Some of these modules are likely linked to functional genes that collectively
592 serve a similar role of adaptation to cold winters (quadrants I-III) or long summers (quadrants II-IV). Indeed,
593 we observed significantly enriched GO terms in the largest module of quadrants II-IV at $d < 0.1$. These GO
594 terms were associated with redox regulation: oxidation-reduction processes (GO:0055114) and
595 oxidoreductase activity (GO:0016491), which play an essential role in the acclimation of plants to abiotic
596 stresses (Suzuki, Koussevitzky, Mittler, & Miller, 2012). This and other modules could be interpreted as
597 reporting for adaptive genes with either, very close additive effects on phenotypes or with true interaction
598 (epistasis), the latter meaning that adaptive alleles need to covary to have an effect on adaptation. All in all,
599 co-association networks revealed a potential utility of CANCOR for investigating the interaction of adaptive
600 loci involved in polygenic adaptations. Further experimental studies, higher density of sequencing and new
601 progress in functional gene annotation would however be required to better understand the specific roles of
602 adaptive genes and their interactions, and in general, the genomic architecture of environmental adaptation
603 in perennial ryegrass.

604

605 Concluding remarks

606 Our pool-Seq GBS and HiPlex genotyping led to the identification of around 60.000 GBS tags of 86 bp per
607 population (max. 80.000 GBS tags), in a genome of about 2.2 Gbp. Thus, only about 0.23% of the total
608 genome size was effectively sequenced and markers from neighboring GBS tags were on average spaced
609 about 40.000 bp apart. Given this fairly low GBS tag density and the expected short LD within outcrossing
610 *L. perenne* natural populations (Blackmore et al., 2016; Keep et al., 2020), adaptive loci not in LD with GBS
611 tags may have gone undetected. Whole genome re-sequencing would enhance the chance to detect more
612 adaptive loci but would obviously require a higher cost for sequencing and computational power.
613 Nevertheless, it is noteworthy that a significant part of the adaptive genetic variability has been detected
614 with markers covering only 0.23% of the total genome size.

615 A statistical relationship between environment, genotype and phenotype does not constitute the
616 unequivocal identification of adaptive loci. The identification of putatively adaptive loci should be confirmed
617 by the implementation of empirical selection experiments testing the fitness consequences of specific
618 alleles or of their combination (Hoban et al., 2016; Pardo-Diaz, Salazar, & Jiggins, 2015). Despite its
619 possible limitations, our approach is distinctive at simultaneously analyzing multivariate environment,
620 genotype and phenotype data. Because environment, genotype and phenotype are in essence mutually
621 correlated and multi-dimensional, the ordination-based CANCOR test is a straightforward and efficient way
622 to detect adaptive loci while at the same time identifying environmental gradients imposing selection and
623 phenotypic traits responsible for adaptation.

624

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642

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Data Accessibility

The DNA data is available in the NCBI Sequence Read Archive (BioProject PRJNA445949, Accessions SRR10243777 to SRR10244245). Supplemental data is available at <https://doi.org/10.5061/dryad.0p2ngf1xk>. Supplemental data includes: 1) Table S1 - Accessions from the natural diversity of perennial ryegrass used in the study; 2) Table S2 - Re-sequenced genomic regions in candidate genes putatively involved in climatic adaptation using Highly Multiplex Amplicon Sequencing (HiPlex). The table includes gene descriptions, primers and amplicons in GFF format, and primer sequences and amplicons in BED format (region between primers); 3) Table S3 - Description and values of variables reporting for the environment at sites of origin of studied populations from the natural diversity of perennial ryegrass; 4) Table S4 - Seasonal climatic conditions at the three experimental gardens (LU, ME, PO) over the duration of the experiments; 5) Table S5 - Description and values of phenotypic traits recorded on studied populations from the natural diversity of perennial ryegrass in three experimental gardens; 6) Table S6 - Number of outlier loci per environmental variable according to the GEA and GWAS univariate mixed models; 7) Table S7 - Outlier loci detected as strongly associated with environmental variables in GEA linear mixed models (FDR = 0.2) and with phenotypic traits in GWAS mixed models (FDR = 0.2); 8) Table S8 - CANCOR outlier SNP loci: Associated environmental variables and phenotypic traits and closest known gene including position, distance to outlier SNP, InterPro domain, gene ontology and functional annotation derived from gene prediction analysis; and 9) Data S1 - Genomic data: allele frequencies of 189,968 SNP loci in the 469 natural populations of *perennial ryegrass*. The remaining information that supports the findings of this study has been uploaded as a Supporting Information file: 1) Methods S1: HiPlex SNP set; 2) Methods S2: Environmental variables (climate-related variables and soil variables); 3) Methods S3: High throughput phenotyping; 4) Methods S4: GEA linear mixed models; 5) Methods S5: GWAS linear mixed models; 6) Methods S6: CANCOR test; 7) Results S1: Outlier loci detected by the CANCOR and the GEA-GWAS approaches. Code for running the CANCOR test and data files to replicate the analysis are available at <https://doi.org/10.5281/zenodo.3992813>.

Author Contributions

Author contributions: J.L.B.P., P.B., S.M. and J.P.S. designed research; T.K. performed the GWAS analysis, T.R., E.V. and K.V. performed the gene prediction and annotation analyses, J.L.B.P. performed all other analyses; T.K., T.L., A.E.G., A.M.R., E.W., K.J.D., M.H., H.M., T.R., E.V., I.R.R., K.V. and J.P.S. collected data; J.L.B.P., P.B., T.K., S.M. and J.P.S. interpreted results; J.L.B.P. and J.P.S. wrote the manuscript with feedback from P.B., T.K., T.L., A.E.G., K.J.D., H.M., T.R., I.R.R. and S.M.

Tables

Table 1. Environmental variables found as associated with SNP loci of perennial ryegrass by either the GEA-GWAS or CANCOR methods (FDR = 0.1 with both methods). Additional information about environmental variables is available in Supplemental Information Methods S2 and Table S3.

Environmental variable	Type	Description	Unit	Method†
<i>bd_subsoil</i>	Soil data	Subsoil bulk density	g cm ⁻³	GEA-GWAS
<i>bio.ad.20</i>	BIOCLIM derived variables	Precipitation - evapotranspiration of wettest quarter	mm	CANCOR
<i>bio.ad.24</i>	BIOCLIM derived variables	Evapotranspiration of wettest quarter	mm	CANCOR
<i>bio.ad.27</i>	BIOCLIM derived variables	Evapotranspiration of coldest quarter	mm	GEA-GWAS
<i>bio2</i>	BIOCLIM derived variables	Mean diurnal range	°C	CANCOR
<i>bio3</i>	BIOCLIM derived variables	Isothermality (<i>bio2</i> / <i>bio7</i> × 100)	%	CANCOR
<i>bio4</i>	BIOCLIM derived variables	Temperature seasonality (standard deviation of average daily mean temperature per year-slice x100)	°C × 100	CANCOR
<i>bio6</i>	BIOCLIM derived variables	Average daily minimum temperature (<i>tasmin</i>) of coldest 14/15 days period	°C	CANCOR
<i>bio7</i>	BIOCLIM derived variables	Temperature Annual Range	°C	CANCOR
<i>bio10</i>	BIOCLIM derived variables	Mean temperature of warmest quarter	°C	GEA-GWAS
<i>dtr_au</i>	ETCCDI derived indices	Average daily temperature range for autumn period	°C	CANCOR
<i>dtr_wi</i>	ETCCDI derived indices	Average daily temperature range for winter period	°C	CANCOR
<i>lmts</i>	Ecophysiological indices	Length of the heat stress period	number of days	GEA-GWAS
<i>oc_topsoil</i>	Soil data	Topsoil organic carbon content	%	GEA-GWAS
<i>pet_wi</i>	Seasonal climate descriptors	Cumulated evapotranspiration for winter period	mm	GEA-GWAS
<i>r01mm_au</i>	ETCCDI derived indices	Count of days when precipitation ≥ 1mm for autumn period	count of days	CANCOR
<i>r01mm_wi</i>	ETCCDI derived indices	Count of days when precipitation ≥ 1mm for winter period	count of days	CANCOR
<i>rx1day_au</i>	ETCCDI derived indices	Maximum 1-day precipitation for autumn period	mm	CANCOR
<i>sdii_au</i>	ETCCDI derived indices	Simple precipitation intensity index for autumn period	mm	CANCOR
<i>sdii_sp</i>	ETCCDI derived indices	Simple precipitation intensity index for spring period	mm	CANCOR
<i>sdii_wi</i>	ETCCDI derived indices	Simple precipitation intensity index for winter period	mm	CANCOR

<i>sis_wi</i>	Seasonal climate descriptors	Average surface incident shortwave solar radiation per day for winter period	W m ⁻²	GEA-GWAS and CANCOR
<i>su_an</i>	ETCCDI derived indices	Number of summer days during the year	count of days	CANCOR
<i>tasmax_wi</i>	Seasonal climate descriptors	Average daily maximum temperature for winter period	°C	CANCOR
<i>tasmin_wi</i>	Seasonal climate descriptors	Average daily minimum temperature for winter period	°C	CANCOR
<i>tawc_soil</i>	Soil data	Total available water content from Pedo-Transfer-Function	mm	GEA-GWAS
<i>tnn_wi</i>	ETCCDI derived indices	Minimum value of daily minimum temperature for winter period	°C	CANCOR
<i>tr_an</i>	ETCCDI derived indices	Number of tropical nights during the year	count of nights	GEA-GWAS
<i>txx_wi</i>	ETCCDI derived indices	Maximum value of daily maximum temperature for winter period	°C	CANCOR

†GEA-GWAS: variables found significantly associated with GEA-GWAS outlier loci (FDR = 0.1 with GEA and GWAS). Only variables with strongest association with outlier SNPs are shown. CANCOR: variables with norm of regression slope projection on the first environmental canonical plane greater than 0.95 and highly correlated ($|r| > 0.5$) to some CANCOR outlier loci (FDR = 0.1 with the CANCOR test).

Table 2. Phenotypic traits found as potentially adaptive in natural populations of perennial ryegrass by either the GEA-GWAS or CANCOR methods (FDR = 0.1). Additional information about phenotypic traits is available in Supplemental Information Methods S3 and Table S5.

Phenotypic trait	Exp. garden(s)	Record year(s)	Description	Unit	Method†
<i>ADL_10_me17</i>	ME	2017	Acid Detergent Lignin content in aerial biomass dry matter	% dry matter	CANCOR
<i>ADL_avg</i>	ME	2017	Acid Detergent Lignin content in aerial biomass dry matter (average over record dates)	% dry matter	CANCOR
<i>AGR_po17</i>	PO	2017	Autumn growth rate	mm / growing-degree-days	CANCOR
<i>AHD_lu16</i>	LU	2016	Aftermath heading	1 (no fertile stem) to 9 (100% plants with fertile stems)	CANCOR

AHD_me16	ME	2016	Aftermath heading	1 (no fertile stem) to 9 (100% plants with fertile stems)	GEA-GWAS
AHD_po17	PO	2017	Aftermath heading	1 (no fertile stem) to 9 (100% plants with fertile stems)	CANCOR
AMH_po17	PO	2017	Autumn maximum height	mm	GEA-GWAS and CANCOR
CH300h_po16	PO	2016	Canopy height 300 degree days before spike emergence	mm	CANCOR
CH400h_po16	PO	2016	Canopy height 400 degree days before spike emergence	mm	GEA-GWAS and CANCOR
CHs500_me17	ME	2017	Canopy height 500 degree days after start of spring growth	mm	GEA-GWAS
DST_avg	LU, PO	2017	Spike density (average over exp. gardens)	1 (no fertile stem) to 9 (maximum density)	CANCOR
DST_lu17	LU	2017	Spike density	1 (no fertile stem) to 9 (maximum density)	CANCOR
HEA_avg	LU, PO	2016, 2017	Spike emergence (heading) date (average over exp. gardens and record years)	Growing-degree-days from start of spring growth (see Methods S6)	CANCOR‡
HFY_lu15	LU	2015	Proportion of plants heading during sowing year	1 (no fertile stem) to 9 (100% plants with fertile stems)	GEA-GWAS
HFY_po15	PO	2015	Proportion of plants heading during sowing year	1 (no fertile stem) to 9 (100% plants with fertile stems)	GEA-GWAS
HST_lu17	LU	2017	Straw height	cm	GEA-GWAS
resDST_lu17	LU	2017	Residual of regression of DST_lu17 on HEA_avg	1 (no fertile stem) to 9 (maximum density)	CANCOR
SCD_wi1516_po	PO	2015 to 2016	Soil coverage loss throughout winter 2015-2016 at PO	Difference between late and early scores, each recorded on a 1 (no living plants) to 9 (best soil coverage) scale	GEA-GWAS
SCD_wi1617_po	PO	2016 to 2017	Soil coverage loss throughout winter 2016-2017 at PO	Difference between late and early scores, each recorded on a 1 to 9 (best soil coverage) scale	CANCOR
SGR_lu16	LU	2016	Summer growth rate	mm / growing-degree-days	CANCOR

SGR_po17	PO	2017	Summer growth rate	mm / growing-degree-days	CANCOR
SMH_me16	ME	2016	Summer maximum height	mm	CANCOR
SMH_po17	PO	2017	Summer maximum height	mm	CANCOR
VAC_avg	LU, PO	2016, 2017	Vigor after cutting (average over exp. gardens and record dates)	1 (no regrowth) to 9 (strongest regrowth)	GEA-GWAS
VAC_lu17	LU	2017	Vigor after cutting (average after two cutting dates at LU in 2017)	1 (no regrowth) to 9 (strongest regrowth)	GEA-GWAS
WID_po16	PO	2016	Winter damage	1 (no damage) to 9	CANCOR

†GEA-GWAS: traits found significantly associated with GEA-GWAS outlier loci (FDR = 0.1 with GEA and GWAS). Only variables with strongest association with outlier SNPs are shown. CANCOR: Traits with norm of regression slope projection on the first phenotypic canonical plane greater than 0.90 and highly correlated ($|r| > 0.5$) to some CANCOR outlier loci (FDR = 0.1 with the CANCOR test).

‡ Trait included in the CANCOR results but with norm of regression slope smaller than 0.95.

Figures

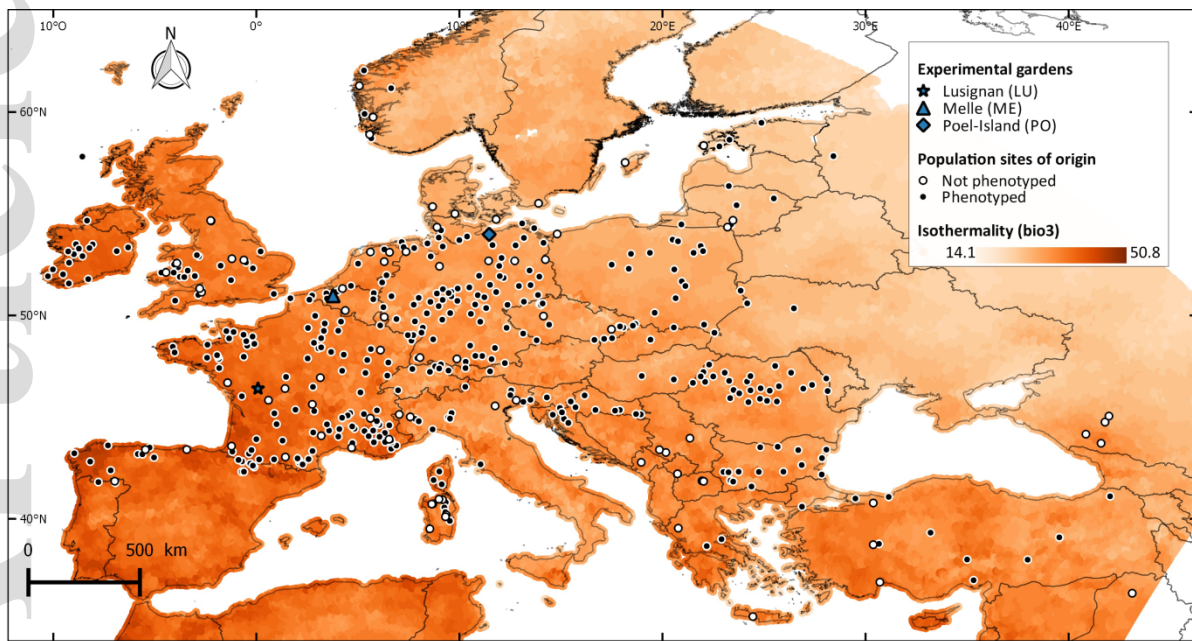


Fig. 1 – Spatial distribution of the 469 perennial ryegrass populations studied and locations of experimental gardens used for phenotyping. Isothermality values are displayed in background as an indicator of climatic variability across Europe.

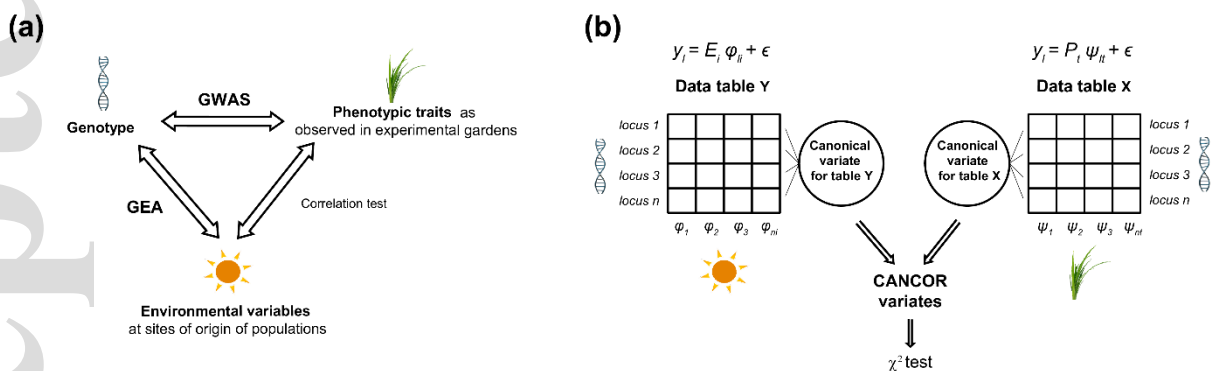


Fig. 2 – Two approaches used to detect adaptive loci. a) The GEA-GWAS approach: a locus is inferred as highly associated with both the environmental variable (GEA) and the phenotypic trait (GWAS). The environmental variable and the phenotypic trait should also be significantly correlated. b) The additive fixed effects (univariate regression slopes) of environmental variables and phenotypic traits on population alternative allele frequencies (AAFs) (y_i) of genotyped loci make up Tables Y and X, respectively. The CANCOR analysis is performed using columns of Y and X as input variables and loci as observations (see further details in Supporting Information, Methods S6). After determining the number of canonical

dimensions reporting for selection gradients (Supporting Information, Fig. S1), a χ^2 test on Mahalanobis distances is implemented to detect outlier loci (Fig. 3a and Supporting Information, Fig. S2).

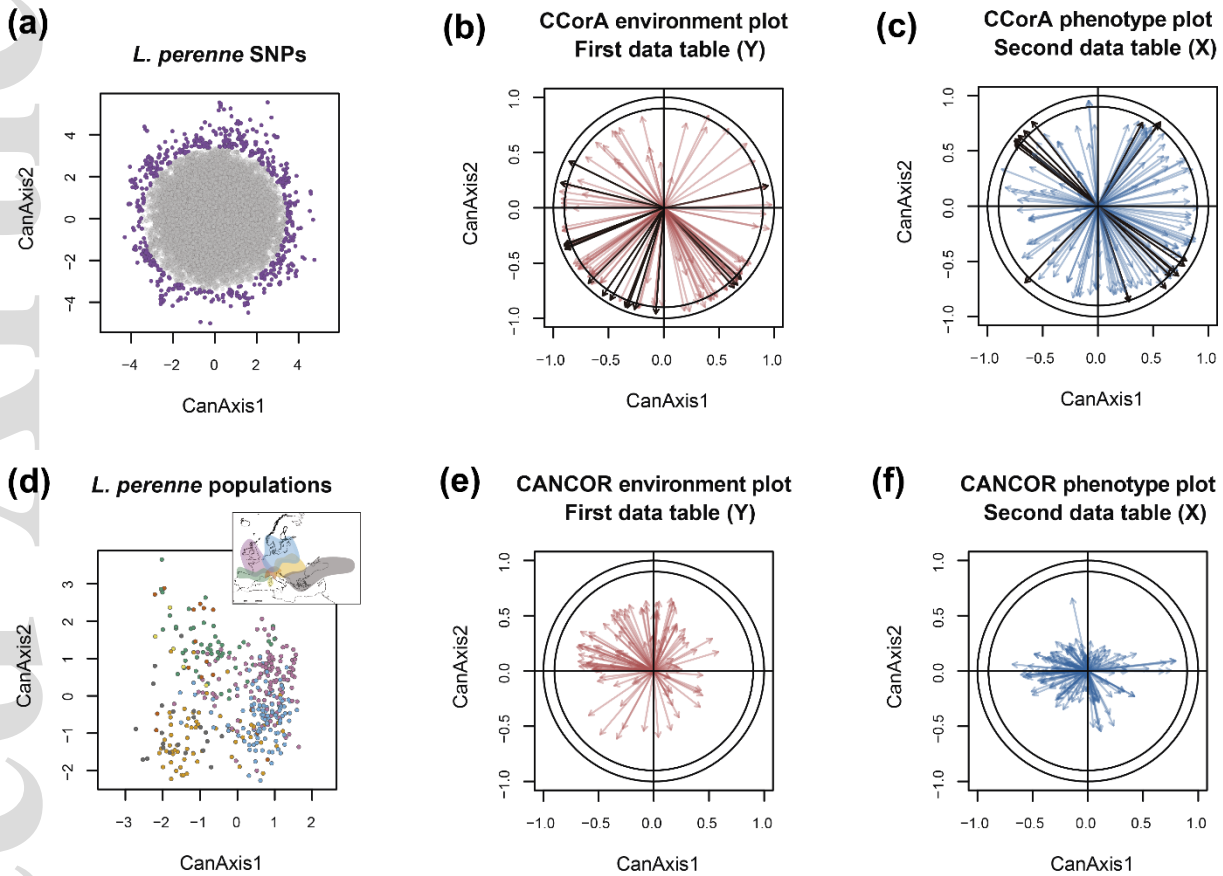


Fig. 3 – The CANCOR analyses. (a-c) Analysis using loci as observations. (d-f) Analysis using populations as observations. (b-c) Projections of slopes of univariate regressions of SNP alternative allele frequencies (AAFs) on environmental variables at sites of origin of populations (Y, see Fig. 2b) and on population mean values of phenotypic traits (X, see Fig. 2b) in the first environmental (b) and phenotypic (c) canonical planes, respectively. (e-f) Projections of environmental and phenotypic variables in the first environmental (e) and phenotypic (f) canonical planes, respectively. In (b) and (c), projections of Y and X input variables are displayed in black if their norm is greater than 0.95 and 0.9, respectively and if the correlation of the corresponding environmental or phenotypic variable with the population AAF of at least one outlier locus is such as $|r| > 0.5$. The projection of the regression slope of the environmental variable *HEA_avg* is also displayed in black although its norm equals 0.83. In (b-c) and (e-f), inner and outer circles mark 0.9 and 1 projection norm values respectively. Dots in (a) represent the coordinates of loci in the X (phenotypic) biplot of the first two canonical axes. Loci detected as significant by the CANCOR selection signal test (at FDR = 0.1) are displayed in purple. Dots in (d) represent the coordinates of populations in the X (phenotypic) biplot

of the first two canonical axes and dot colors represent neutral genetic clusters (as per Blanco-Pastor et al., 2019). Environmental and phenotypic variables whose regression slope projections are displayed in black in (b) and (c), respectively, are described in Table 1, Table 2 and Fig. 5. Detailed information about these variables is provided in Supporting Information, Methods S2 and S3.



Fig. 4 – Outlier loci revealed by the CANCOR test whose alternative allele frequency is highly correlated ($|r| > 0.5$) with environmental variables or phenotypic traits well represented in the first environmental and phenotypic canonical planes (projection norms of corresponding input variables > 0.95 and 0.90 ,

respectively) or with *HEA_avg*. (a) Loci are plotted in the Y biplot representing the first two environmental canonical axes. (b) Loci are plotted in the X biplot representing the first two phenotypic canonical axes. Note that loci positions are computed on the basis of alternative allele frequencies. Purple and green colors indicate positive and negative correlations, respectively, between the locus alternative allele frequency and the environmental (a) or phenotypic (b) variable. See description of variables in Table 1 and Table 2. See detailed information on these variables in Supporting Information, Table S3, Table S5, Methods S2 and Methods S3.

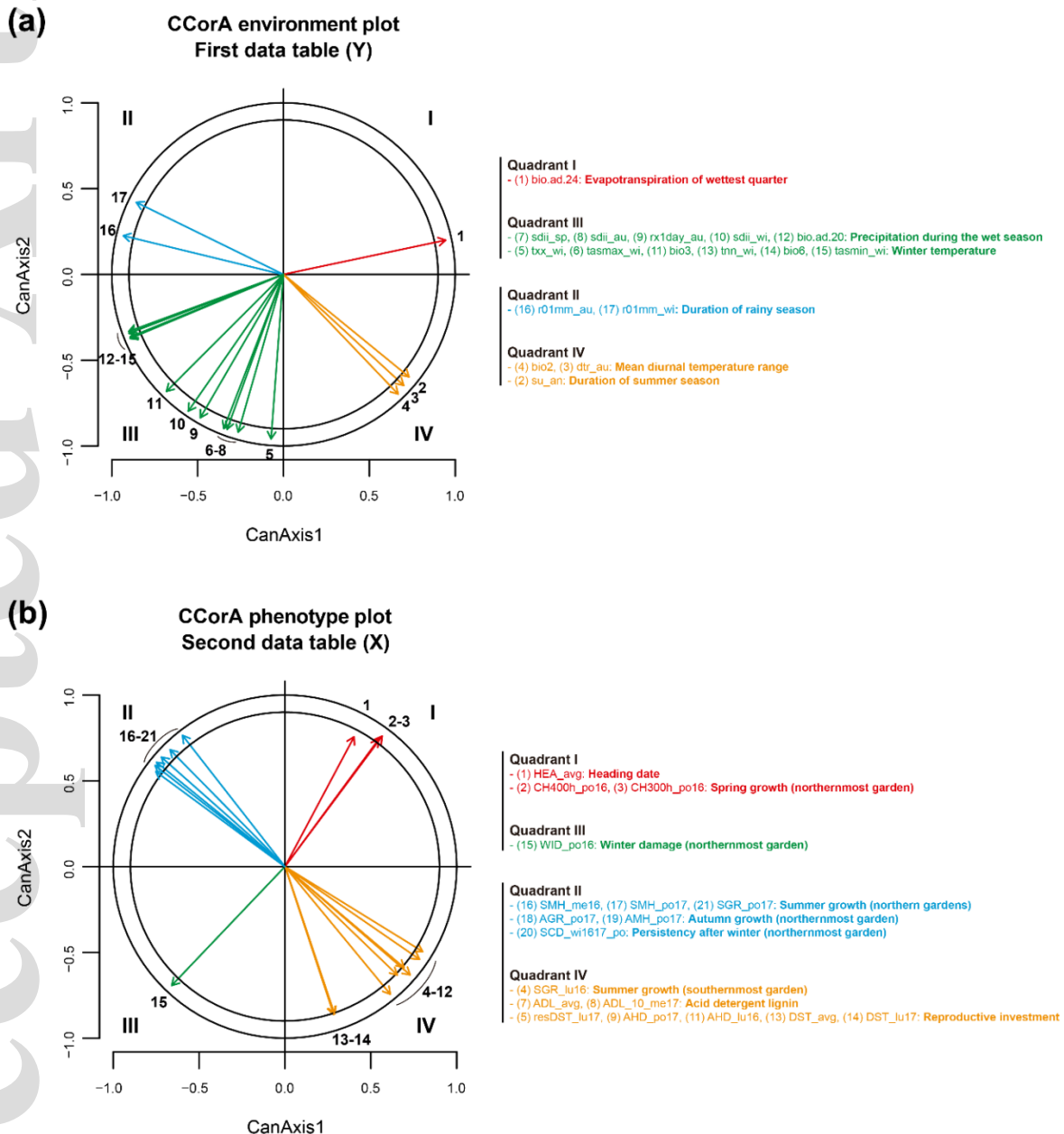
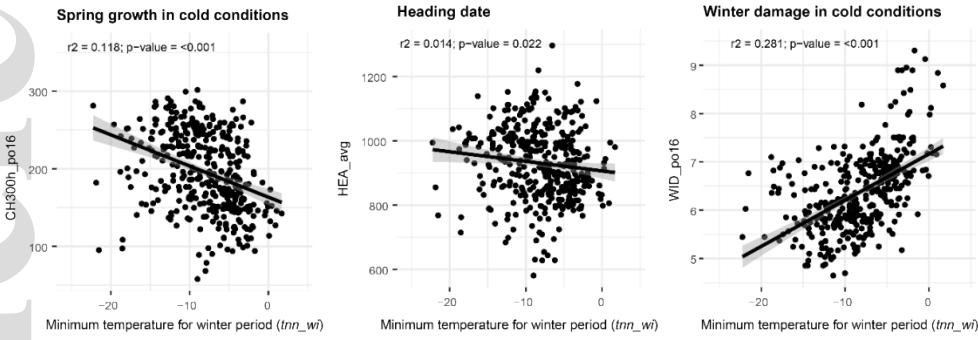


Fig. 5 – Synthetic representation of main climatic adaptations in perennial ryegrass natural populations. (a) and (b) represent the first environmental (Y) and phenotypic (X) canonical planes, respectively, of the CANCOR analysis using loci as observations. Projections of input environmental and phenotypic variables (regression slopes) are displayed if their norm is larger than 0.95 and 0.90, respectively. In addition, the corresponding environmental and phenotypic variables should be highly correlated to the population alternative allele frequency (AAF) of at least one outlier locus ($|r| > 0.5$). The projection of the regression slope of the environmental variable *HEA_avg* is also displayed although its norm equals 0.83. Colors and roman numbers I, II, III and IV indicate quadrants in the CANCOR canonical planes and groups of associated climate and phenotypic variables. Note that arrow positions are computed on the basis of the correlation between the variable and SNP alternative allele frequencies. Also note that the diagonal from quadrant I (red) to III (green) represents a cold-dry to mild-wet winter gradient whereas the diagonal from quadrant II (blue) to IV (orange) represents a long rainy season to long summer gradient.

a) Adaptations to winter temperature



b) Adaptations to summer length

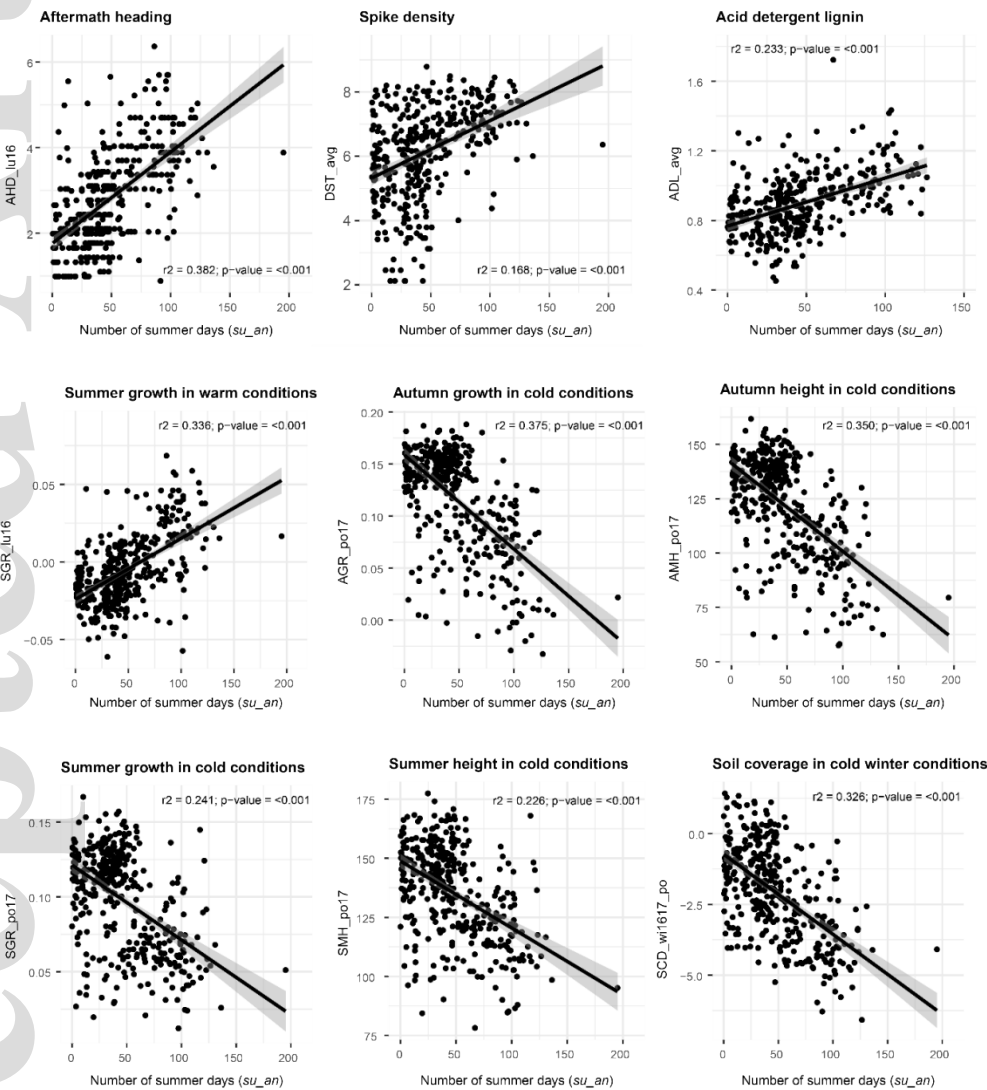


Fig. 6 – Relationships between two main selective climatic gradients represented by minimum value of daily minimum temperature in winter (tnn_wi) and number of summer days in the year (su_an) at sites of origin of populations and key phenotypic responses (mean values of populations) depicted by scatter plots.

Results of linear regressions of phenotypic traits on climatic variables are also displayed (r^2 , p-values and trend lines).

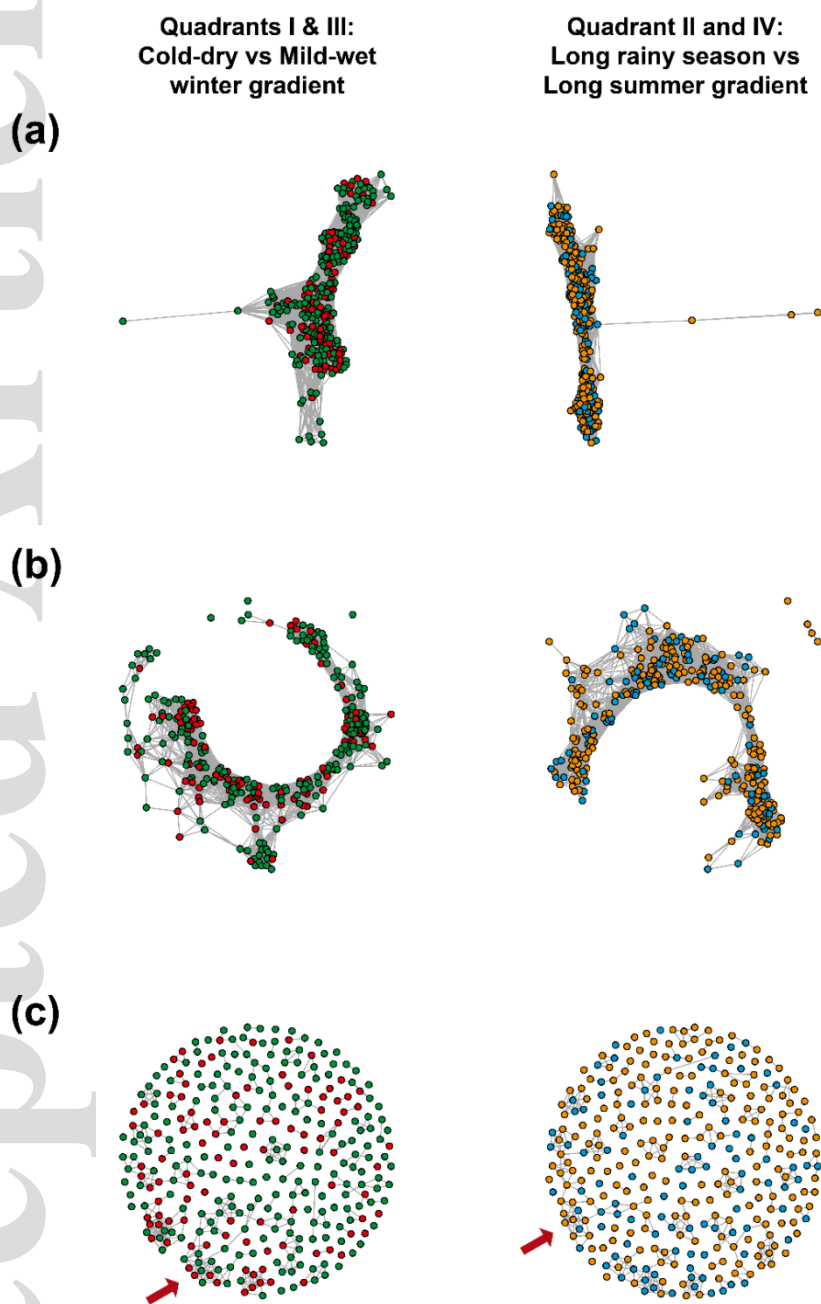


Fig. 7 – Co-association modules for the outlier SNPs identified by the CANCOR test. Each co-association network represents a distinct module. Colors schemes are according to the four quadrants of the CANCOR analysis (Fig. 5) and are displayed on the basis of alternative allele frequencies (SNPs from the same module can display different colors because one color represents the alternative allele as adaptive and the

other color represents the reference allele as adaptive). Climatic gradients corresponding to environmental variables with highest scores in each quadrant are indicated. Phenotypic traits associated with these quadrants are displayed in Fig. 5b. (a), (b) and (c) show alternative networks obtained with three different thresholds of pairwise Euclidean distances (< 1 , < 0.5 and < 0.1 , respectively). Red arrows point to the modules used for the Gene Ontology enrichment analyses.