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1 **Resequencing *Vrs1* gene in Spanish barley landraces revealed reversion of six-rowed
2 to two-rowed spike**

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5 **Concise title:** Natural variation in Vrs1

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19 **Abstract**

20 *Six-rowed spike 1* (*Vrs1*) is a gene of major importance for barley breeding and germplasm
21 management as is the main gene determining spike row-type (2-rowed vs 6-rowed). This is a
22 widely used DUS trait, and has been often associated to phenotypic traits beyond spike type.
23 Comprehensive re-sequencing *Vrs1* revealed three two-rowed alleles (*Vrs1.b2*; *Vrs1.b3*;
24 *Vrs1.t1*) and four six-rowed (*vrs1.a1*; *vrs1.a2*; *vrs1.a3*; *vrs1.a4*) in the natural population.
25 However, the current knowledge about *Vrs1* alleles and its distribution among Spanish barley
26 subpopulations is still underexploited. We analyzed the gene in a panel of 215 genotypes,
27 made of Spanish landraces and European cultivars. Among 143 six-rowed accessions, 57 had
28 the *vrs1.a1* allele, 83 were *vrs1.a2* and three showed the *vrs1.a3* allele. *Vrs1.b3* was found in
29 most two-rowed accessions, and a new allele was observed in 7 out of 50 two-rowed Spanish
30 landraces. This allele, named *Vrs1.b5*, contains a 'T' insertion in exon 2, originally proposed as
31 the causal mutation giving rise to the six-row *vrs1.a2* allele, but has an additional upstream
32 deletion that results in the change of 15 amino acids and a potentially functional protein. We
33 conclude that eight *Vrs1* alleles (*Vrs1.b2*, *Vrs1.b3*, *Vrs1.b5*, *Vrs1.t1*, *vrs1.a1*, *vrs1.a2*, *vrs1.a3*,
34 *vrs1.a4*) discriminate two and six-rowed barleys. The markers described will be useful for DUS
35 identification, plant breeders, and other crop scientists.
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37
38 **Keywords**: barley, landraces, *Vrs1*, SNP
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A feature relevant to describe the history of the barley crop *Hordeum vulgare* subsp. *vulgare* is the spike row-type, two- and six-rowed, according to the fertility of the lateral spikelets of each triplet sitting at each rachis node. *H. vulgare* subsp. *spontaneum*, the wild ancestor of barley, is two-rowed, as explained at length in Komatsuda et al. (2007). So far, five genes determining row-type (*vrs1*, *vrs2*, *vrs3*, *vrs4* and *vrs5*) have been cloned. *Vrs1* (syn. *HvHoX1*) encodes a homeodomain-leucine zipper class I (HD-ZIP I) transcription factor that inhibits the development of lateral spikelets (Komatsuda et al. 2007). *Vrs2* encodes a homolog of *SHORT INTERNODES* (Youssef et al. 2017a). *Vrs3* encodes a putative Jumonji histone demethylase (Bull et al. 2017; van Esse et al. 2017). *Vrs4* encodes an orthologue of the maize *RAMOSA 2* gene (Koppolu et al. 2013). The last gene, *Vrs5* (syn. *Int-c*) encodes a homologue of maize *TEOSINTE BRANCHED 1* (Ramsay et al. 2011). However, only two of them, *Vrs1* and *Vrs5*, affect spike row-type in natural populations (Komatsuda et al. 2007; Saisho et al. 2009; Ramsay et al. 2011; Youssef et al. 2017b).

Several possible mutations at *Vrs1* convert the sterile lateral spikelets into fertile ones. These mutations occur naturally and may have been favored by farmers that interpreted increased fertility as a yield-increasing trait, even though biomass productivity differs little between the two forms (Evans and Wardlaw 1976). The six-rowed trait has appeared independently in several occasions during the history of the crop, acting as a driver of germplasm differentiation (von Bothmer et al. 2003; Komatsuda et al. 2007). Actually, the distinction between two-rowed and six-rowed types is one of the main divides in barley germplasm, as breeders tend to maintain their stocks apart, to avoid the cumbersome process of recovery of pure spike types.

In addition to two two-rowed alleles (*Vrs1.b2* and *Vrs1.b3*), at least three independent mutations in *Vrs1* (*vrs1.a1*, *vrs1.a2* and *vrs1.a3*, all six-rowed) are found among current barleys (Komatsuda et al. 2007). These last alleles are caused by a deletion (*vrs1.a1*) or insertion (*vrs1.a2*) in the coding sequence, resulting in frame shifts; whereas *vrs1.a3* is due to an amino acid change in the homeodomain region. Different surveys resequencing the gene across wild and cultivated barleys (Saisho et al. 2009; Cuesta-Marcos et al. 2010; Ramsay et al. 2011; Youssef et al. 2012, 2017b), ancient DNA from historic landraces (Leino and Hagenblad 2010), or through the use of specific KASP markers in herbarium specimens (Lister et al. 2013), also found the previous alleles and identified another two, *Vrs1.t* (*deficiens*), which is caused by a single amino acid change in the C-terminal region of the protein (Sakuma et al. 2017) and *vrs1.a4* (six-rowed, without any apparent change in the open reading frame (ORF) of *Vrs1.b* alleles).

74 The *Vrs1* region is associated not just to row-type. In fact, it turns up in many barley
75 association scans, for a wide variety of agronomic traits (Cuesta-Marcos et al. 2010; Muñoz-
76 Amatriaín et al. 2014; Alqudah et al. 2016). Recently, some studies have provided functional
77 proof of the gene involvement in several phenotypic traits. Liller et al. (2015) found an effect
78 of *Vrs1* on tillering, while Thirolugachandar et al. (2017) reported increased leaf width, vein
79 number, leaf nitrogen content, and grain number associated to the six-rowed allele.

80 Spain is at the end of the routes of distribution that brought barley and other crops to Europe,
81 starting from the Neolithic and ending probably in the Middle Ages (Fischbeck 2003;
82 Komatsuda et al. 2007), receiving crops with adaptations to environmental factors
83 encountered along different routes (Banks et al. 2013; Müller 2015). Therefore, Spanish
84 landraces actually summarize the evolution of the crop in, at least, Southern Europe.
85 Additionally, Spain is one of the few European countries in which cereal landraces were
86 collected and kept in germplasm banks before they disappeared from cultivation, because they
87 were cultivated up to the second half of the 20th century (Igartua et al. 1998; Pujol-Andreu
88 2011).

89 Before cloning *Vrs1*, polymorphisms in the linked *Chloroplast Elongation Factor G* gene
90 (cMWG699), closely linked to the *Vrs1* locus, were used as a surrogate for its row-type
91 characterization (Komatsuda et al. 1998; Tanno et al. 1999, 2002). Cuesta-Marcos et al. (2010)
92 developed four SNP markers within the *Vrs1* gene that have been widely used by the scientific
93 and plant breeding community as diagnostic for row-type. However, the current knowledge
94 about *Vrs1* alleles and its distribution among barley subpopulations is still underexploited. In
95 this work, we carry out a comprehensive survey of haplotypes at this locus by analyzing
96 Spanish barley landraces, which are complementary of barley accessions analyzed in other
97 studies (Saisho et al. 2009; Cuesta-Marcos et al. 2010; Ramsay et al. 2011; Sakuma et al. 2017;
98 Youssef et al. 2017b), with a predominance of six-rowed over two-rowed accessions.

99

100 **Materials and Methods**

101 **Plant materials**

102 This study involved 176 Spanish barley landraces (50 two-rowed and 126 six-rowed), most of
103 them collected before 1954 (Supplementary Table 1). Of these landraces, 137 (126 six-rowed
104 and 11 two-rowed) belonged to the Spanish Barley Core Collection (SBCC, Igartua et al. 1998)

105 and 39 come from the set assembled by Moralejo et al. (1994). Thirty-six additional cultivars
106 originated from other countries were studied for comparative purposes: 8 landraces from
107 Morocco (4 two-rowed and 4 six-rowed), 7 of them obtained from the USDA World collection,
108 and 28 widely diverse cultivars (15 two-rowed and 13 six-rowed) that represented the
109 cultivated gene pool from the world. Finally, we included 3 wild barley accessions from
110 Morocco (Molina-Cano et al. 1982) donated by J.L. Molina-Cano (Table 1).

111 **SNPs genotyping**

112 Four SNPs within the *Vrs1* gene (HORVU2Hr1G092290 in the new barley genome sequence
113 (Mascher et al. 2017)) were genotyped as a part of the 9k Infinium iSelect SNP chip (Comadran
114 et al. 2012). Two of those SNPs (12_30897 and 12_30901) were included in the Barley SNP
115 Panel from Eureka Genomics Corporation (Hercules, CA). The CAPs marker cMWG699/*Taq*I
116 (Komatsuda et al. 1998; Tanno et al. 1999), corresponding to HORVU2Hr1G092180, was
117 evaluated in all the samples as previously described (Casas et al. 2005). The plant materials
118 tested with each system are detailed in Table 1.

119 **Sanger sequencing**

120 Sanger-sequencing of *Vrs1* and *Int-c* (HORVU4Hr1G007040) of selected accessions were
121 carried out as described by Ramsay et al. (2011).

122 **Exome sequencing**

123 Exome capture was performed according to the methods described by Mascher et al. (2013).
124 DNA sequencing, made at CNAG (Centro Nacional de Análisis Genómico, Barcelona), and data
125 analysis were performed as described in Cantalapiedra et al. (2016). Briefly, mapping of paired-
126 end reads (2x101 bp) to the Morex WGS assembly was carried out with BWA MEM (Li and
127 Durbin 2009). Variant calling was done by combining SAMtools (Li et al. 2009) and GATK
128 (McKenna et al. 2010). In addition, snpEff (Cingolani et al. 2012) was used to estimate the
129 effect of polymorphisms on coding sequences. Data for *Vrs1* and *Int-c* were retrieved by
130 inspecting the corresponding Morex WGS contigs (contig_135757, *Vrs1*, and contig_5747, *Int-*
131 *c*), as identified by BLASTN alignment at http://webblast.ipk-
132 gatersleben.de/barley_ibsc/viroblast.php.

133 **RNA extraction and reverse-transcription PCR**

134 Total RNA was extracted from immature spikes, leaf blades, leaf sheaths, nodes and
135 internodes at awn primordium stage using TRIzol (Invitrogen). RNA was quantified using a
136 NanoDrop 2000 (Thermo Fisher Scientific). To remove genomic DNA contamination, RNA was
137 treated with RNase-free DNase I (Roche). First-strand cDNA was synthesized with SuperScript
138 III (Invitrogen) and first-strand cDNA derived from 20 ng RNA was used as PCR template.
139 Primers used for RT-PCR are listed in Supplementary Table 2. Barley *Actin* gene was used as
140 positive control.

141 **Phylogenetic analysis**

142 Over two hundred *Vrs1* nucleotide sequences were retrieved with BLASTN from the NCBI nt
143 database, using the sequence of SBCC153 (1,133 bp) as query. Hits with low similarity or query
144 coverage, as well as redundant ones, were removed. The surviving sequences were trimmed to
145 the length of SBCC153 and renamed using the allele and haplotype names defined in the work
146 of Saisho et al. (2009). Haplotypes "hap13" and "hap3.2" were also filtered out for bearing
147 large deletions. Sequences for barley genotypes widely used as standards for genomic studies,
148 Barke, Bowman, Haruna Nijo and Morex *Vrs1* were included. A multiple alignment was
149 computed with clustal-omega-1.2.1 (Sievers et al. 2011). A parsimony haplotype network was
150 generated with software TCS v1.21 (Clement et al. 2000) and default parameters, which
151 consider gaps as a fifth character. The resulting phylogenetic tree was optimally plotted with
152 http://cibio.up.pt/software/tcsBU (dos Santos et al. 2016).

153

154 **Results**

155 **SNPs genotypes of *Vrs1***

156 This study investigates 215 accessions that offer a good perspective of the diversity of the
157 crop, with particular focus on the western Mediterranean region (Table 1). *Vrs1* alleles were
158 initially inferred combining spike row-type and the four SNPs within *Vrs1* presented by the 9k
159 Illumina Infinium assay (Cuesta-Marcos et al. 2010). The SNP markers provided information to
160 discriminate among genotypes (Table 2). BOPA marker 12_30897 (G/A) differentiates *Vrs1.b3*
161 (two-rowed) and *vrs1.a3* (six-rowed) from the other alleles. Marker 12_30900 (C/G) is specific
162 to the *vrs1.a3* allele. Marker 12_30896 (G/A), although it does not contribute to function of
163 *Vrs1*, can be used to identify six-rowed barleys with the *vrs1.a1* allele from the rest. The last
164 marker 12_30901 (G/A) separates one of the major branches of the phylogenetic tree of *Vrs1*

165 (with alleles *Vrs1.b2*, *vrs1.a2* and *Vrs1.t*) from the other two major branches of cultivated
166 barley (with alleles *vrs1.a1*, *Vrs1.b3* and *vrs1.a3*). The BOPA scores of the 215 accessions are
167 shown in Supplementary Table 1.

168

169 **Sequence validation of *Vrs1* alleles**

170 Sanger sequencing of 28 accessions were also carried out (Supplementary Table 1). The
171 sequence data discriminated between some non-committal genotypes and concluded all the
172 allele calls by SNP genotyping. As a whole, nine polymorphisms differentiated seven *Vrs1*
173 alleles (Table 2). The sequences of lines identified as *vrs1.a1*, *vrs1.a2*, *Vrs1.b2*, *Vrs1.b3*, and
174 *Vrs1.t* were identical to the sequences downloaded from NCBI. A new allele, named *Vrs1.b5*,
175 was found in seven two-rowed Spanish accessions. Exome sequencing of 73 genotypes (mostly
176 Spanish landraces) independently confirmed the polymorphisms identified (Supplementary
177 Table 3), providing full allelic discrimination. Thus, 26 lines were correctly classified as *vrs1.a1*,
178 37 lines were *vrs1.a2*, 2 lines had the *vrs1.a3* allele, and 7 carried the *Vrs1.b3* allele and 1 as
179 *Vrs1.b5*.

180

181 **Discovery of a novel allele *Vrs1.b5***

182 The new *Vrs1* allele was identified in seven two-rowed Spanish landraces (Fig. 1A). This allele
183 has been named *Vrs1.b5*, the number being the next in sequence available for this gene. The
184 *Vrs1.b5* allele, presents the distinctive thymine ('T') insertion in exon 2, typical of *vrs1.a2* but,
185 on top of it, there was a single base deletion G/_ in the same exon, 45 bp upstream of this
186 insertion. The coupled deletion/insertion results in a frameshift of a stretch of 15 amino acids
187 (Fig. 1B) and restored two-rowed spike from the six-rowed spike (Fig. 1C). The frameshift was
188 located outside of the homeodomain, in a region apparently not relevant for the function of
189 the DNA-binding domain (Fig. 1B). The spike phenotype of the lines carrying the new allele is
190 definitely two-rowed (Fig. 1C), indicating that the 15 substituted amino acids were not
191 essential for the function of VRS1 in terms of suppressing the development of lateral florets.
192 This change, a single base deletion, has not been observed in any other sequence reported for
193 this gene. A molecular phylogeny analysis with 47 unique sequences from both domesticated
194 and wild barleys, positioned the new *Vrs1.b5* allele only one-step apart from the six-row
195 *vrs1.a2* allele, both sharing *Vrs1.b2* as common ancestor (Fig. 2).

196

197 **Expression of *Vrs1.b5***

198 Transcript of *Vrs1* was detected in the two accessions (SBCC153 and SBCC155) carrying *Vrs1.b5*
199 (Fig. 3). *Vrs1* was predominantly expressed in the immature spikes, as previously reported
200 (Sakuma et al. 2010, 2013). A six-rowed accession (SBCC039) carrying *vrs1.a2*, the immediate
201 ancestor of *Vrs1.b5* allele, showed the same gene expression pattern with the *Vrs1.b5* carriers.
202 Implication was that the gain of *Vrs1* function in *Vrs1.b5* was effected by the frameshift, not by
203 any change of transcription.

204

205 **Diversity and geographical distribution of *Vrs1* alleles**

206 Using the four SNPs from Cuesta-Marcos et al. 2010, together with the morphological
207 identification of the row number, allows the precise identification of most, but not all, *Vrs1*
208 alleles. To illustrate this, we predicted the *Vrs1* allele for another study involving 138 European
209 winter cultivars (Digel et al. 2016, Supplementary Table 4). Marker-based assignment of *Vrs1*
210 alleles allowed unequivocal identification of 60 *Vrs1.b3* alleles, 4 *vrs1.a1*, and 30 *vrs1.a3*. For
211 the rest, marker information complemented with spike row-type allowed assigning allele
212 *vrs1.a2* to 39 cultivars*,* and five cultivars (all two-rowed) were still inconclusive. Scoring the
213 rest of polymorphisms in Table 2 would allow further differentiation of the *Vrs1.b2*, *Vrs1.b5*
214 and *Vrs1*.t alleles.

215 We could also predict correctly row type and specific allele for 109 of the 126 landraces
216 studied by Russell et al. (2016). We retrieved the data for SNPs and indels within *Vrs1* (Morex
217 WGS contig_135757) from that study, identifying four polymorphic sites of the nine presented
218 in Table 2 (Supplementary Table 5). Most of the 72 six-rowed accessions carried the *vrs1.a1*
219 allele (52), originating from Asia and in Africa; *vrs1.a3* was present in 13 accessions, mainly
220 from Eastern Europe, and *vrs1.a2* was only found in 4 landraces from Spain and the French
221 Pyrenees. Regarding two-rowed accessions, 39 were identified as having the *Vrs1.b3* allele.
222 Further 13 accessions were identified as carrying one of the *Vrs1.b2* alleles, and four could not
223 be determined due to missing data.

224

225 **Association of *Vrs1* and *EF-G* alleles**

226 Previously, variation in the *EF-G* locus, closely linked to *Vrs1*, was used to infer different origins
227 within cultivated barley (Tanno et al. 1999, 2002; Casas et al. 2005), and allows further
228 differentiating the *Vrs1.t* allele (A-type) from the *Vrs1.b2* and *vrs1.a2* lineage (D-type). Most
229 two-rowed accessions analyzed in this study (57 out of 72) had the *Vrs1.b3* allele, associated
230 with the K-type in *EF-G*. Twelve two-rowed lines, (3 wild, 2 landraces from Morocco and 7
231 Spanish landraces), however, showed a D-type, which is typical of six-rowed lines.

232 The wild barleys and cultivated landraces from Morocco all carried the *Vrs1.b2* allele, as
233 expected, as a similar finding was reported previously by Komatsuda et al. (2007).

234 Among 143 six-rowed accessions analyzed, 83 carried *vrs1.a2* allele, and 78 of the *vrs1.a2*
235 accessions carried the D-type of EF-G (*cMWG699*) confirming their tight linkage (Komatsuda et
236 al. 1999) and association (Tanno et al. 2002). The *vrs1.a2* was derived from *Vrs1.b2* (Fig. 3) as
237 described earlier (Komatsuda et al. 2007) and all the *Vrs1.b2* carriers were D-type carriers
238 (Table 2, Supplementary Table 1).

239 All the accessions carrying *Vrs1.b5* and most of the six-rowed accessions (78 out of 83) with
240 *vrs1.a2* also carried the D-type at the *EF-G* locus, indicating that both alleles belong to the
241 same lineage, an implication of the restoration of gene function in *Vrs1.b5* from *vrs1.a2* due to
242 the deletion. The *Int-c* gene that intervenes in the size of the lateral spikelets was sequenced in
243 several lines (Supplementary Table 1). Using Sanger and exome sequence data available for 75
244 accessions, six-rowed lines carried the *Int-c.a* allele, as expected, whereas seven two-rowed
245 lines, including two with the *Vrs1.b5* allele, were *int-c.b1*, typical of two-rowed cultivated lines
246 (Ramsay et al. 2011).

247

248 **Discussion**

249 The present study adds a new two-rowed allele *Vrs1.b5* to the catalogue of *Vrs1* diversity. The
250 new allele was created by a restoration of gene function in the ancestral recessive allele by a
251 single nucleotide deletion. This sort of mutation, a kind of gain-of function, is unusual in
252 nature. The direction of mutation from recessive *vrs1* to dominant *Vrs1* was opposite to the
253 normal direction so far discovered (Komatsuda et al. 2007). In the present study, we identified
254 7 two-rowed lines with the *Vrs1.b5* allele, out of 50 two-rowed Spanish lines analyzed, i.e.,
255 14% of two-rowed Spanish barleys carry the new allele. The phenotype of these lines is
256 definitely two-rowed, and two accessions surveyed for *int.c* carry the allele typically found in

257 two-rowed lines (Ramsay et al. 2011). A maximum parsimony phylogenetic analysis suggests
258 that *Vrs1.b5* was a reversion of *vrs1.a2* to the two-rowed state through a new mutation. The
259 reversion of the six-rowed to two-rowed seems far more uncommon than the opposite in the
260 history of the crop (Komatsuda et al. 2007). Phylogenetically, loss-of-function allele *vrs1.a2*
261 seems to derive from *Vrs1.b2*. Later, a deletion in *vrs1.a2* likely gave rise to *Vrs1.b5*, which
262 restored the ORF and reverted to the two-rowed phenotype. Thirulogachandar et al. (2017)
263 performed a phylogenetic analysis for plants HD-ZIP I proteins, identifying putative motifs
264 evolutionary conserved. Motif 16, 14 amino acids long starting from Lys29, which corresponds
265 to part of the 15 amino acid region changed in the *Vrs1.b5* allele, separated the monocot from
266 the dicot proteins, as reported by those authors. The amino acid change in *Vrs1.b5* allele did
267 not affect functionality of the protein although the motif was predicted to have a nuclear
268 localization signal ('RRRRRRSAR').

269

270 **Origin of *Vrs1.b5***

271 The presence of the *Vrs1.b2* to *vrs1.a2* lineage in the western Mediterranean was previously
272 reported by Komatsuda et al. (2007), who proposed that the *vrs1.a2* allele could be native to
273 the region. This view was supported by studies carried out with the *EF-G* locus (marker
274 *cMWG699*). Several surveys done with this marker concluded that the D-type (associated to
275 *vrs1.a2* and *Vrs1.b2*) was found preferentially in the Mediterranean region (Tanno et al. 1999,
276 2002; Casas et al. 2005; Baba et al. 2011), but it was also present in winter six-rowed cultivars
277 from Germany, France, and other western European countries (Casas et al., 2005). Since both
278 two- and six-rowed barleys carrying the D allele are present in North Africa, Baba et al. (2011)
279 proposed that the origin of the D allele was in Morocco. Our results illustrate that the D allele
280 is profusely present in Spanish six-rowed landraces, most of them with the *vrs1.a2* allele, and
281 in a small group of two-rowed landraces featuring the new *Vrs1.b5* allele. Moreover, even
282 today, a large proportion of six-rowed *vrs1.a2* genotypes can still be found among European
283 modern winter cultivars, as derived from data provided in Digel et al. (2016). Therefore, its
284 geographic origin cannot be indicated with certainty. *Vrs1.b2* probably appeared in the Middle
285 East. Recent sequencing of a 6,000-year-old barley from a cave in Israel revealed that it carried
286 a putative two-rowed *Vrs1.b2* genotype (Mascher et al. 2016). *Vrs1.b2* has also been found in
287 the old landrace Palmella Blue (Komatsuda et al. 2007), collected in Egypt early in the 20th
288 century (https://npgsweb.ars-grin.gov/gringlobal/accessiondetail.aspx?id=1025310). In this
289 study, we have found this allele in two landraces from Morocco which, could represent

290 remains of the same genetic stock, found at the two ends of its geographical distribution after
291 westwards expansion though the Mediterranean during the Neolithic (Zilhao 2011).

292 Considering together the presence of the precursor allele of *Vrs1.b5* (*vrs1.a2*) exclusively in
293 Western Europe and, in lower frequencies, in Morocco (Casas et al. 2005, Baba et al. 2011, and
294 data derived from Digel et al. 2016, and Russell et al. 2016), and the discovery of *Vrs1.b5* in
295 Spanish landraces, we hypothesize that this new allele is native to the western part of the Old
296 World.

297

298 **Application of the SNP markers for germplasm characterization**

299 An updated classification of the *Vrs1* alleles can facilitate the analysis and differentiation of
300 genotypes. Earlier efforts by Cockram et al. (2012), who developed a set of KASP markers for
301 morphological traits assessed to determine distinctiveness, uniformity and stability (DUS) of
302 new plant breeding varieties, including *Vrs1*, did not differentiate among all possible alleles.
303 Similarly, the four SNP markers developed by Cuesta-Marcos et al. (2010) are not fully
304 diagnostic, but at least allow an easy first discriminating step that could be implemented in all
305 datasets based on the 9K and 50K (Bayer et al. 2017) barley chips. We illustrated its application
306 by correctly estimating the *Vrs1* allele for 133 of the 138 cultivars evaluated by Digel et al.
307 (2016), with five inconclusive (Supplementary Table 4). Four of the remaining genotypes
308 shared a common parent with the *Vrs1.t* allele (Intro) in their pedigree, and two of them have
309 recently been identified as *deficiens* (Sakuma et al. 2017).

310 Different studies carried out genome wide association analysis for type of spike or yield related
311 traits and found QTL in the region of the *Vrs1* locus. Even though Cuesta-Marcos et al. (2010)
312 developed the 4 SNP BOPA markers described in Table 2, these authors reported more highly
313 significant associations by the use of 'synthetic markers' which summarized the
314 dominant/recessive nature of the *Vrs1* allele than with any SNP within the gene. In another
315 genome-wide study with six-rowed cultivars and advanced breeding lines, Berger et al. (2013)
316 identified markers in the *vrs1* region associated with QTL for test weight but were not able to
317 differentiate the lines. The authors concluded that either there was a closely linked gene for
318 test weight, or there were two or more *vrs1* alleles segregating in the breeding materials with
319 one of them contributing directly to increased test weight. An examination of the specific *Vrs1*
320 alleles present in their dataset would have shed further light on these hypotheses. In the work

321 by Muñoz-Amatriaín et al. (2014), with the USDA barley core collection, the largest panel
322 tested up to date, the top hit for spike row number corresponded to marker 12_30896. This
323 marker separates six-rowed *vrs1.a1* accessions from the rest, which includes not only 2-rowed
324 accessions, but also other 6-rowed ones with different alleles. Therefore, this marker does not
325 discriminate row type. It was captured by GWAS probably because there was an imbalance of
326 allelic frequencies in the genotypes, with a majority of 6-rowed presenting *vrs1.a1*. In the last
327 study published by Thirulogachandar et al. (2017), the two SNPs associated with variation in
328 leaf area are 12_30896 and 12_30900 (both within *Vrs1* and differentiating six-rowed lines
329 with allele *vrs1.a1* or *vrs1.a3*, respectively). Overall, a comprehensive evaluation of *Vrs1* for
330 the markers differentiating all alleles, as haplotypes instead of considering them
331 independently, would allow an accurate characterization of the alleles present in barley
332 materials as the ones reported in those studies. A definitive allele characterization could
333 provide new insights on association of specific *Vrs1* alleles to the relevant agronomic and
334 morphological traits reportedly related to this gene.

335 This study completes the allelic catalogue of gene *Vrs1*, offers new insights on explanations for
336 their geographic distribution, and provides a full list of SNP markers useful for breeders and
337 germplasm banks to better analyse genetic variation associated to this gene, and facilitate
338 germplasm classification.

339

340 **Data accessibility**

341 Newly reported sequences for *Int-c* and *Vrs1* are accessible at European Nucleotide Archive
342 under references LT727691-LT727723.

343 **Authors' contributions**

344 AMC, EI and TK conceived this work. PG, MM and JMC selected and provided the plant
345 accessions. AMC and SS performed laboratory work. AMC, CPC and BCM analyzed the DNA
346 sequence data. BCM was responsible for the phylogenetic analysis. AMC, BCM, EI and TK
347 drafted the document. All the authors read and approved the manuscript.

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531

532 **Table 1** Plant materials and genotyping platforms used in this study.

Country/ Region	Type of material	Spike row-type	No.	Genotyping	Reference
Morocco	Wild	Two-rowed	3	Eureka Genomics, EF-G, Sanger	Molina-Cano et al. 1982
Morocco	Landraces	Two- and six-rowed	8	Eureka Genomics, EF-G, Sanger (3)	Moralejo et al. 1994, Molina Cano et al. 2005
Spain	Landraces	Two-rowed	39	Eureka Genomics, EF-G, Sanger (5)	Moralejo et al. 1994
Spain	Landraces	Two- and six-rowed	137	9k iSelect, EF-G, exome capture (66), Sanger (14)	Igartua et al. 1998
Europe/ USA	Cultivars	Two- and six-rowed	28	9k iSelect, EF-G, exome capture (7), Sanger (3)	This study

533

534 **Table 2** *Vrs1* alleles detected in this study.

535

	Nucleotide positions referred to Morex WGS contig_135757 (*vrs1.a1* allele)											
	Exon 1	Exon 2				Intron 2	Exon 3		3' UTR	Row	EF-G	No. of
Allele	1067	1240	1246	1288	1393	1608	1725	1818	1961	type	type	accessions
Vrs1.b2	G	G	G	–	C	T	G	A	C	2	D	5
vrs1.a2	G	G	G	T	C	T	G	A	C	6	D	83
Vrs1.b5	G	–	G	T	C	T	G	A	C	2	D	7
vrs1.a1	G	G	G	–	C	C	–	A	T	6	A	57
Vrs1.b3	A	G	T	–	C	C	G	A	C	2	K	57
vrs1.a3	A	G	T	–	G	C	G	A	C	6	A	3
Vrs1.t1	G	G	G	–	C	T	G	G	C	2	A	3
Effect	Gly8>Asp	Glu26>F.S.	Glu26>Asp	Ala40>F.S.	Phe75>Leu		Glu152>F.S.	Ser184>Gly				
BOPA	12_30897				12_30900	12_30901			12_30896			

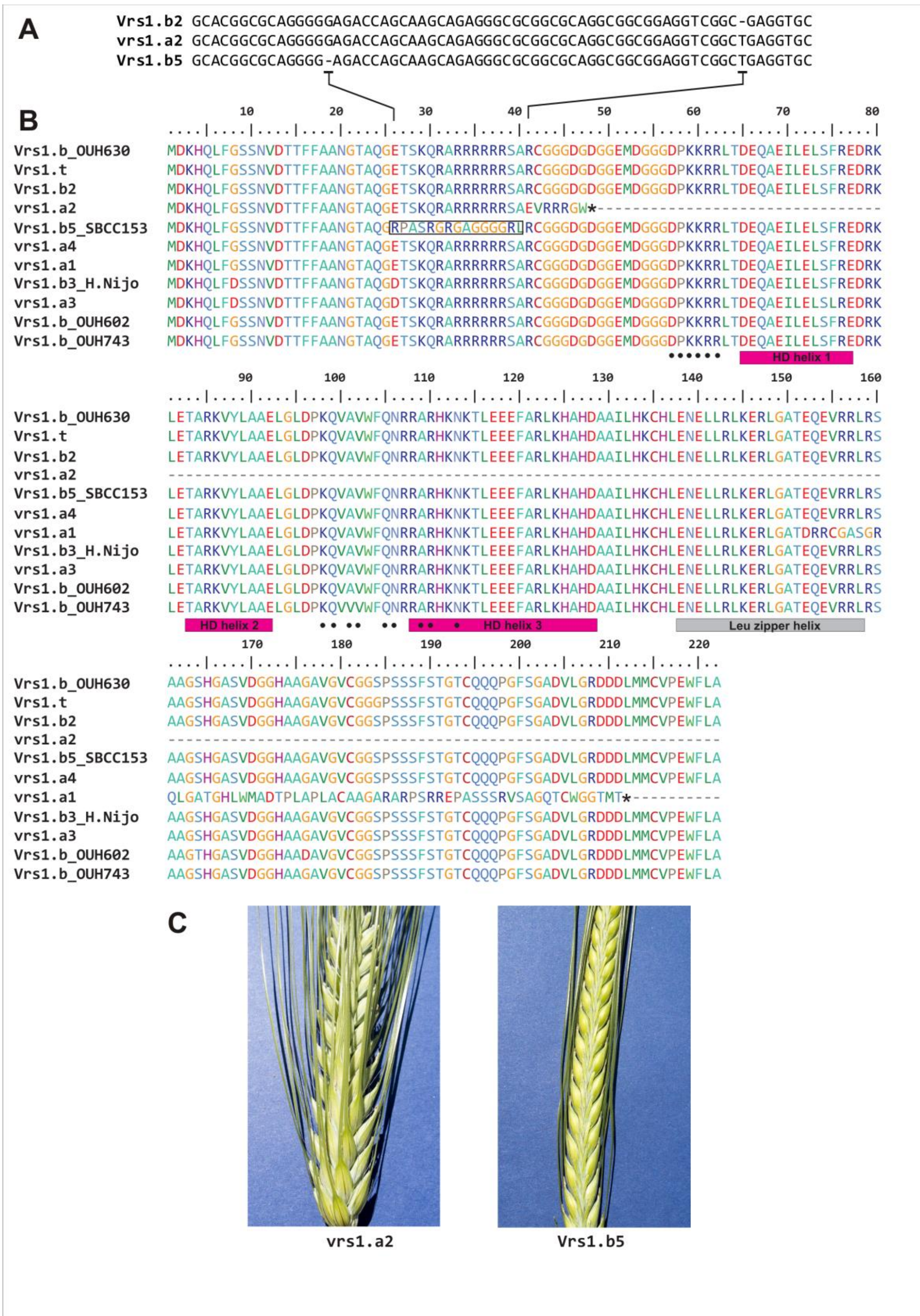
536 BOPA markers 12_30901 and 12_30896 interrogate the complementary strand, therefore are usually reported as A/G and G/A, respectively

537 **Figure legends**

538 **Fig. 1** Discovery of *Vrs1.b5*. (A) Alignment of *Vrs1.b2*, *vrs1.a2* and *Vrs1.b5* DNA partial
539 sequences of exon 2 indicating the creation of *Vrs1.b5* by 1-bp deletion in *vrs1.a2*. (B) Multiple
540 alignment of protein sequences of different alleles of the *Vrs1* gene. Secondary structure
541 elements of the homeodomain (HD) and the leucine zipper dimerization domain (Leu zipper)
542 are shown as color-filled boxes. Predicted protein-DNA interfaces residues are marked with
543 circles. Boxed sequences highlight differences among alleles and asterisks mark premature
544 stop codons. Three wild barley lines (''OUH'' identifiers) used as outgroups in a previous
545 phylogeny (Komatsuda et al. 2007) are also shown. Alignment was computed with clustal-
546 omega-1.2.1. (C) Barley spikes from Spanish landraces SBCC039 (*vrs1.a2*) six-rowed (left) and
547 SBCC155 (*Vrs1.b5*) two-rowed (right).
548

549 **Fig. 2** Sequence analysis of 47 aligned *Vrs1* alleles. Parsimony network where edges represent
550 single-mutation transitions, and nodes correspond to haplotypes. Tiny circles represent
551 intermediate states (with no associated genotypes). Six-rowed barley accessions are displayed
552 as thick circles. The central haplotype corresponds to OUH630. The labeled circles match the
553 haplotypes shown in Figure 1B.
554

555 **Fig. 3** Reverse transcription PCR (RT-PCR) analysis of *Vrs1*
556 *Vrs1* was predominantly expressed in the immature spikes in both six-rowed (SBCC039) and
557 two-rowed barley (SBCC153 and SBCC155). All organs were collected from main tiller at the
558 awn primordium stage. The PCR product of *Vrs1* was directly sequenced to confirm the
559 specificity. *Actin* was used as a control.
560
561



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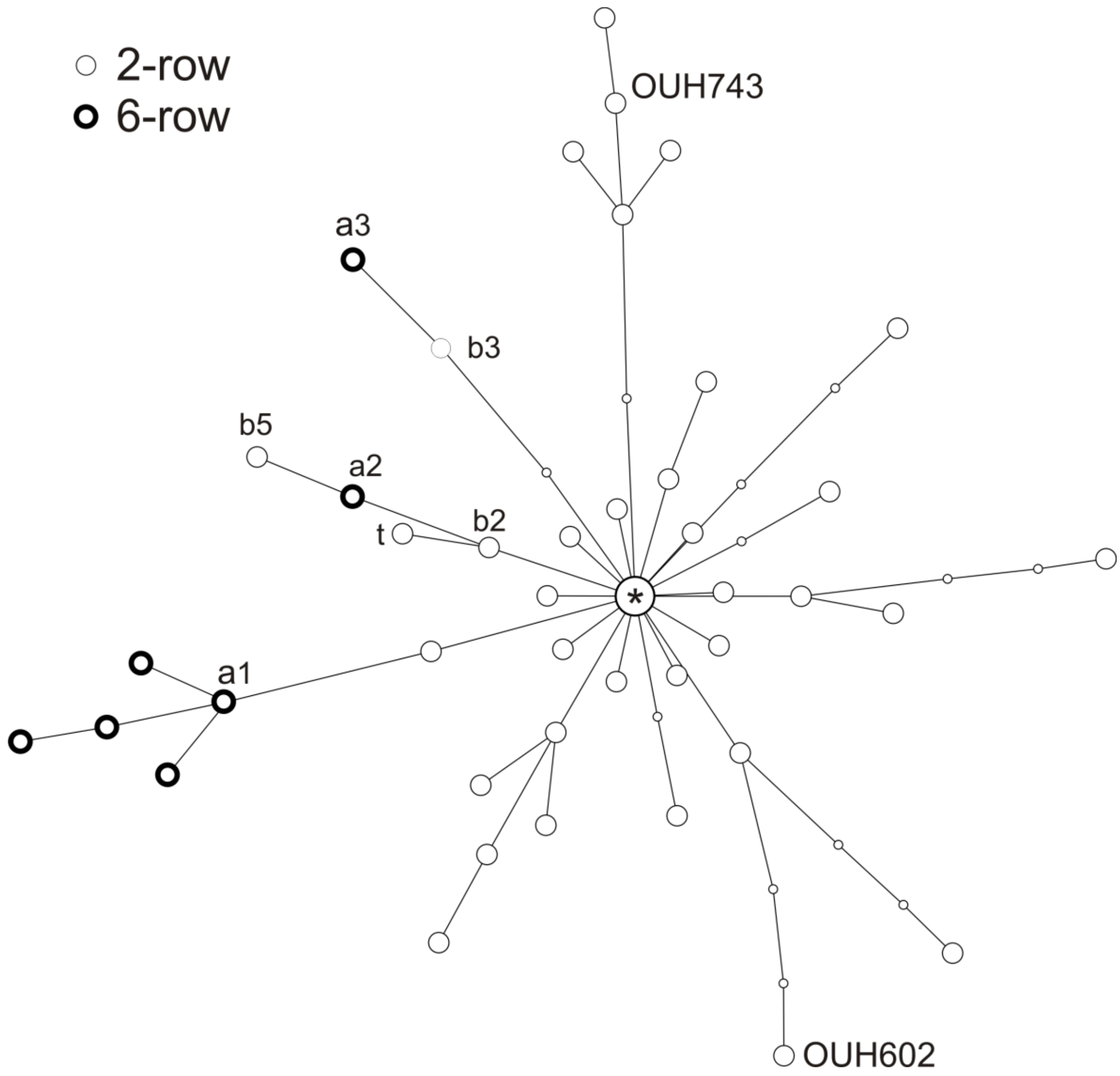

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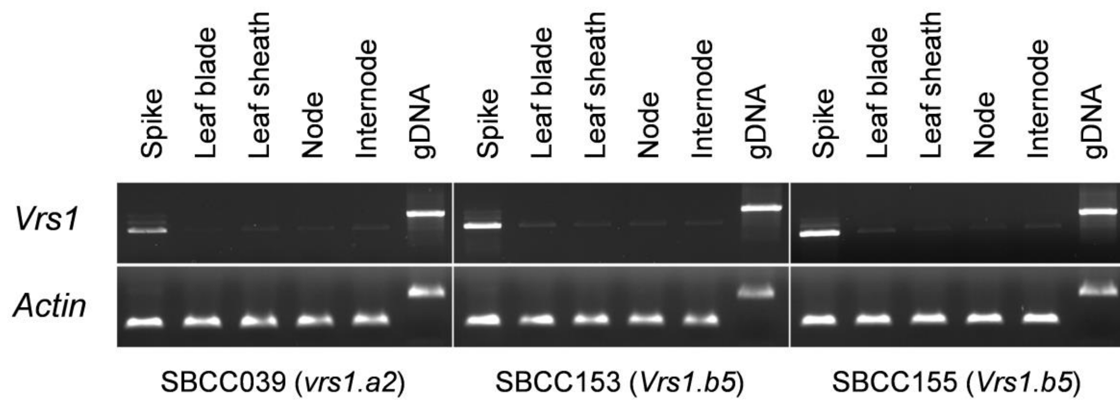

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