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FUNCTIONAL SNP MARKERS FOR THE VERNALIZATION REQUIREMENTS IN BARLEY: AN OPTION 1 APPROACH

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FUNCTIONAL SNP MARKERS FOR THE VERNALIZATION REQUIREMENT IN BARLEY: AN OPTION 1 APPROACH

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INTRODUCTION

1. Cultivated barley can predominantly be classified as possessing a winter or spring seasonal growth habit (SGH), according to the effects of low temperature on flowering time. Winter lines require a prolonged period of vernalization (typically 6 weeks below 10 °C) to promote subsequent flowering, and are normally planted in the autumn for harvesting the following year. Spring varieties progress to flowering without vernalization treatment, and are typically sown and harvested in the same year. Although infrequent, some lines are classified under a third SGH class, and are described as 'alternative' or 'facultative'. Such germplasm lacks unambiguous classification, and has been variously described. For example, von Zitzewitz *et al* (2005) classify facultative as cold tolerant, vernalization unresponsive, while the International Union for the Protection of New Varieties of Plants (UPOV) describe alternative types as displaying an intermediate flowering time to that of winter and spring lines when grown in the absence of vernalization (see document TG/19/10: *http://www.upov.int/en/publications/tg-rom/tg019/tg_19_10.pdf*).

2. The possibility of developing a gene-based molecular marker assay that could be used to assess the vernalization characteristic in barley was investigated in a research project funded by the DEFRA Science Directorate (project VS0137). For DUS purposes, the scoring of this characteristic currently involves planting out significant areas of submitted winter varieties in springtime in order to confirm their seasonal growth type. Winter varieties do not flower at all when planted in the spring and since the material does not produce heads or grains, the trial is not used to score any other characteristics. A molecular test for the vernalization characteristic in barley would serve to demonstrate to the international community the viability and advantages of using a molecular approach to assess functional traits of relevance to DUS. It is also clearly attractive as a direct replacement for the existing field trials on a cost-benefit basis.

3. The previous Defra Science Directorate project characterized sequence diversity at *VRN-H1* and the *VRN-H2* candidate genes in EU barley varieties and assessed the association between polymorphisms at these loci and SGH. A combination of different PCR/agarose-gel based molecular markers were developed that can identify all known spring alleles at the *VRN-H1* locus and major deletion polymorphism at the *VRN-H2* locus. Spring and winter types are easily and reliably identified by the assay. The test can be performed within days of receipt of a candidate variety, saving time and allowing sufficient time for any problems to be verified later by sowing. This is the first example of which we are aware where a DNA test for a DUS characteristic has been successfully developed that may directly replace a field trial.

4. We aim to evolve the qualitative test described above to establish a procedure to detect and quantify the percentage of off-types in an unknown sample allowing an assessment of uniformity to be made. A reference database of *VRN-H1* and *VRN-H2* alleles found in 'common knowledge' varieties will also be needed for DUS purposes. The development of these elements forms the basis of this project. Ideally, a single-well multiplex assay for allelic status at *VRN-H1* is required, to complement the single-well assay for *VRN-H2*. In addition,

we aim to characterize the third, less routinely observed, 'alternative' SGH state. The UPOV Test Guidelines (document TG/19/10) describe alternative types as lines that flower later than spring lines in the absence of vernalizing temperatures and the current field trials utilize the UPOV criteria in order to identify this phenotype. The 'alternative' state was not initially investigated within the Defra Science Directorate project as there were so few varieties of this type available to test at the time of the project. Since that time, two more varieties have been described as 'alternative' in trials during 2006. The issue of accurate classification of 'alternative' types will be greatly advanced by determining the identity and phenotypic effects of alleles at vernalization and possibly photoperiod response loci.

RESULTS TO DATE

5. In order to accurately determine the presence of off-types, it is first necessary to be able to classify each of the different spring, winter and alternative alleles that could potentially be present. This is not an insignificant task. Following our work conducted during the previous Defra project VS0137, we were able to classify all of the spring and winter *VRN-H1* alleles present in a collection of >400 European barley cultivars, identifying three novel forms (Figure 1), and delivering ~200,000 bp of DNA sequence.

Development of a PCR assay diagnostic for allelic status at VRN-H1

6. Using this information we present here a simple and cost effective single-well PCR-based assay that is able to discriminate between all currently known spring and winter *VRN-H1* alleles (Figure 2). Furthermore, this multiplex assay is able to discriminate between four of the nine haplotypes (including both winter alleles), with the remaining five spring alleles all resulting in the amplification of the same size product.

Investigation of VRN-H1 and VRN-H2 genotypes in 'alternative' varieties

7. As outlined in the project brief, the 'alternative' growth habit is ill-defined. With the aim of identifying DNA polymorphisms characteristic of 'alternative' growth habit we have extracted DNA from the seven UK barley varieties shown in Table 1.

VRN-H2

8. Using the PCR assay described by Karsai et al (2005), we genotyped the ZCCT-H genes, diagnostic for allelic state at *VRN-H2*. The results found are shown in Table 1. We found that of the seven 'alternative' varieties for which genotype scores were obtained, three lacked the *ZCCT-H* gene cluster, indicating the presence of a spring *VRN-H2* allele, predicting a spring SGH according to the two-locus epistatic model of vernalization response. The remaining four varieties are predicted to possess winter *VRN-H2* alleles, suggesting that the *VRN-H1* locus could be responsible for the observed phenotype.

VRN-H1

9. As a preliminary investigation into the VRN-H1 alleles present in 'alternative' varieties, we employed the ten genetic markers illustrated in Figure 3 to design preliminary haplotypes, according to the designations described by Cockram *et al* (2007). The observed haplotypes are shown in Table 1. Surprisingly, given the number of varieties previously screened, six of the seven 'alternative' lines genotyped displayed novel haplotypes. 'Damas' was the only variety to display a previously observed haplotype, identical to the predominant winter 1A

haplotype (although this does not preclude a genetic mutation within this allele determining the observed phenotype). The genotype combination observed in 'Novetta' is very similar to the 1A haplotype, differing only in the allele found at the SSR in the 5' UTR.

10. The identification of novel *VRN-H1* haplotypes in seven of the eight 'alternative' varieties has two immediate implications: firstly, 'alternative' varieties are potentially a rich source of novel *VRN-H1* alleles. Secondly, given that just nine *VRN-H1* alleles had previously been identified, the abundance of novel alleles in 'alternative' varieties suggests that the observed growth habit is likely to be due to variation at *VRN-H1*. Accordingly, we have initiated full-length sequencing of *VRN-H1* in all 8 'alternative' varieties. For each 'alternative' line, we have utilized the 22 primer pairs described by Cockram *et al* (2007) to amplify PCR products across the gene. The results of these experiments are shown in Table 2. Where major deletions have occurred, one or more primer pairs internal to the deletion will fail to amplify PCR products. In this way, a preliminary analysis of gene configuration can be determined. All primer pairs attempted to date on the variety 'Damas' (the only variety to possess a previously observed haplotype) resulted in amplification, consistent with it possessing winter haplotype 1A. However, this does not preclude 'Damas' possessing a SNP or small deletion accounting for the observed phenotype. Failure of one or more intron I primer pairs to amplify in all the remaining 'alternative' varieties suggests deletions could account for the observed phenotype. Intron I primer pairs 1 to 7 failed to amplify from 'Gaelic', 'Kiruna' and 2/1788, suggesting they all contain a large deletion of around 6 kb. PCR analysis suggests smaller deletions are present in 'Novetta', 2/1149, 2/1797 and 2/2159, all of which occur within the 4.1 kb region of nucleotide conservation between wheat and barley. The location of putative deletions within this region supports the hypothesis that they contribute to the observed phenotype. Initial attempts to PCR across these putative deletion breakpoints have been successful, indicating they represent real deletions, and not just mutations in the primer sites. The next stage is to sequence the amplicons obtained, leading to complete VRN-H1 allele sequence in all eight 'alternative' varieties.

Development of the reference database

11. In the previous Defra project, we characterized allelic status at VRN-H1 using primer pair 1 (Figure 3). However, we have since shown that this assay is not sufficient to predict growth habit in all *VRN-H1* alleles (haplotypes 2 and 5C). Towards overcoming this, we utilised the single-well multiplex PCR assay described here to genotype all 97 lines from project VS0137. We have also included 16 recent winter and spring entries to the UK National List. The *ZCCT-H* locus was genotyped in all new germplasm. Database entries are presented in Table 3.

Determining assay sensitivity

11. The next step towards detection of off-types is to deploy the single-well multiplex assay described above. However, due to the complexity of primer pairs in this assay, we first chose to determine the sensitivity of allele detection using a simpler PCR reaction. To do this, two primer pairs were initially optimized in separate reactions to establish the optimum PCR cycling conditions for use. Primer pair 1 (*VRN-H1*-F3 / *VRN-H1*-R3) assays for the absence of intron I deletions, as seen in winter haplotype 1A and spring haplotype 2. Primer pair 2 (*VRN-H1*-F1 / *VRN-H1*-R1) assays for the presence of a 5.2 kb intron I deletion found in spring haplotypes 4B and 5A. PCR conditions were tested across an annealing temperature gradient, with and without the addition of GC-rich buffer to the reaction mix. We find that an

annealing temperature of 62 ºC without the inclusion of GC buffer results in robust amplification in primer pairs 1 and 2 (Figure 4).

11. Following the optimization of PCR cycling conditions, primer pairs 1 and 2 were deployed on a DNA dilution series to determine the limits of their ability to detect DNA template in the reaction mix. The sensitivity of the primers used has a direct relation to their ability to detect off-types. DNA from the winter variety 'Flagon' and the spring variety 'Golf' were extracted using a DNAeasy kit (Qiagen). DNA concentration was then determined using the average of three measurements. The mean DNA concentrations for 'Flagon' and 'Golf' are 59.8 and 23.2 ng/µl, respectively. The first PCR reaction was set up using the following protocols: 1 µl DNA, 2 µl Buffer, 2 µl $MgCL_2$, 0.5 µl forward primer (10 µlmol), 0.5 µl reverse primer (10 µlmol), 0.2 µl FastStart *Taq* (Roche), 0.2 µl DNTPs and 4.6 µl H_2O. 1 µl of each of these DNA-containing master-mixes were then used to spike 19 µl of DNA-free master-mix, which was subsequently used to spike the next master-mix, and so on for the remaining five serial dilutions. In this way, a dilution series of eight PCR reactions for each primer pair were created. Two dilution series replicates were conducted for each primer pair. The ability of primer pair 1 to detect DNA template was lost between serial dilution 3 and 4 using 'Flagon' DNA (59.8 ng/µl) and between dilution 2 and 3 using 'Golf' DNA (23.2 ng/µl) (Figure 5). These results show that primer pair 1 is able to detect template DNA concentrations of 0.150 ng/µl, while at 0.007 ng/µl insufficient template is present for amplification to reliably occur.

12. These experiments determine the sensitivity threshold of each primer pair. Accordingly, we can predict the sensitivity of these primers on a bulk DNA extraction made from a sample of seed containing one off-type. The dilution detected at 0.150 ng/µl equates to one off-type in 400 in real terms, and although primer pair 1 is unable to detect levels at 0.007 ng/µl, it is possible that the dilution reliably detectable could be less than one in 400. Further experiments are needed to verify this possibility.

13. Now the sensitivity of the primers is established, the next step will be to test them on an appropriate DNA mixture that recreates the presence of off-types at given concentrations. Subsequently, a protocol will be established for DNA extraction from large bulks of seed spiked with a known number of off-type seed, followed by PCR assays for the detection of off-types.

CONCLUSIONS TO DATE

(i) A multiplex PCR assay has been developed, diagnostic of all spring and winter alleles.

(ii) Progress towards explaining the 'alternative' growth habit has been promising, identifying six novel *VRN-H1* haplotypes in the seven varieties for which results were obtained.

(iii) The positions of major intron I deletions have been identified by PCR analysis in seven of the eight 'alternative' lines examined.

(iv) Towards understanding the mutations that have taken place, full length sequencing of *VRN-H1* in 'alternative' varieties has been initiated, and is ~50 % complete. Once finalized, this will allow deployment of appropriate diagnostic assays in the detection of off-types.

(v) Primers for detection of off-types have been optimized and their sensitivity determined using serial DNA dilutions.

(vi) The reference database of *VRN-H1* and *VRN-H2* alleles has been updated with the results of the single-well multiplex *VRN-H1* assay, as well as the inclusion of genotypes for an additional sixteen varieties.

ACKNOWLEDGEMENTS

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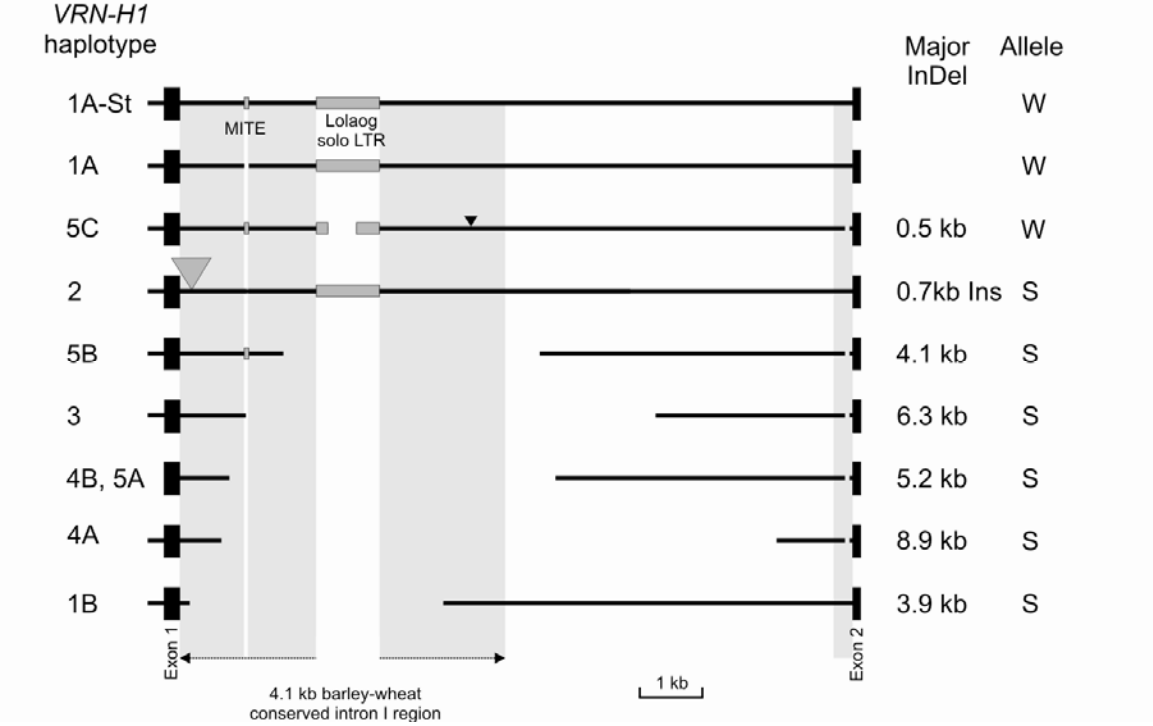

Figure 1. Diagram of intron I configuration in spring (S) and winter (W) VRN-H1 alleles. The sizes of major intron I deletions (haplotypes 1B, 3, 4A, 4B, 5A, 5B, 5C) and insertions (haplotype 2, grey triangle) are indicated. Haplotypes 1B, 2 and 4B represent novel alleles identified during Defra project, VS0137. Regions of intron I nucleotide conservation between barley and T. monococcum are shaded in grey. Pervious studies (von Zitzewitz et al 2005) describe a 0.44 kb region immediately downstream of the 5' breakpoint in haplotype 3 as the most likely to harbour the 'vernalization critical' region. The deletion breakpoint series identified here shows the minimal 'vernalization critical' region could be more diffuse, as spring alleles with an insertion upstream (haplotype 2) and downstream (haplotype 5B) of the 0.44 kb region are observed.

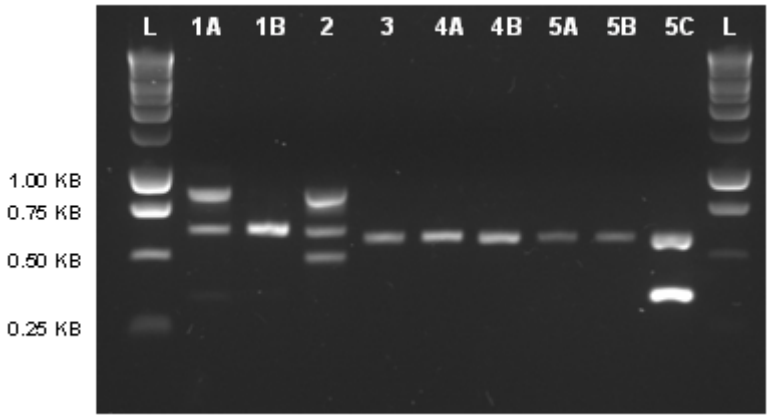

Figure 2. Single-well multiplex VRN-H1 PCR assay. Representative varieties from VRN-H1 haplotypes are as follows: 1A ('Pelican'), 1B ('Etu'), 2('Dram'), 3 ('Beatrice'), 4A ('Triumph'), 4B ('Maris Cannon'), 5A ('Pohto'), 5B ('Pegasus'), 5C ('Express'). Haplotypes 1A and 5C represent winter alleles, the remaining seven haplotypes are spring. L = 1 kb DNA ladder.

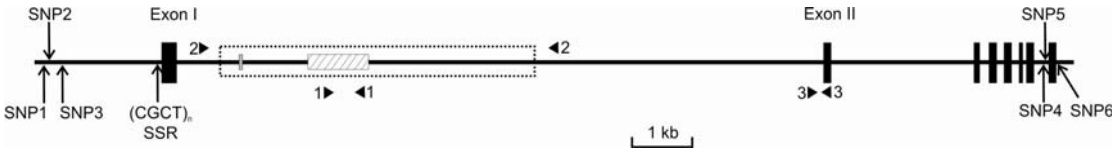

Figure 3. Diagram of the genomic winter vrn-H1 allele from the cultivar 'Strider' (GenBank accession AY750993), indicating the positions of primer pairs 1, 2 and 3, the (CGCT) SSR in the 5'UTR, SNPs 1 to 3 (promoter), SNP4 and SNP5 (intron VII) and SNP6 (3'UTR). Solid black boxes denote exons. The position of the Lolaog solo LTR within intron I is indicated by the hatched box. The 5.2 kb intron I deletion characteristic of haplotype 5A is indicated by the dashed line box.

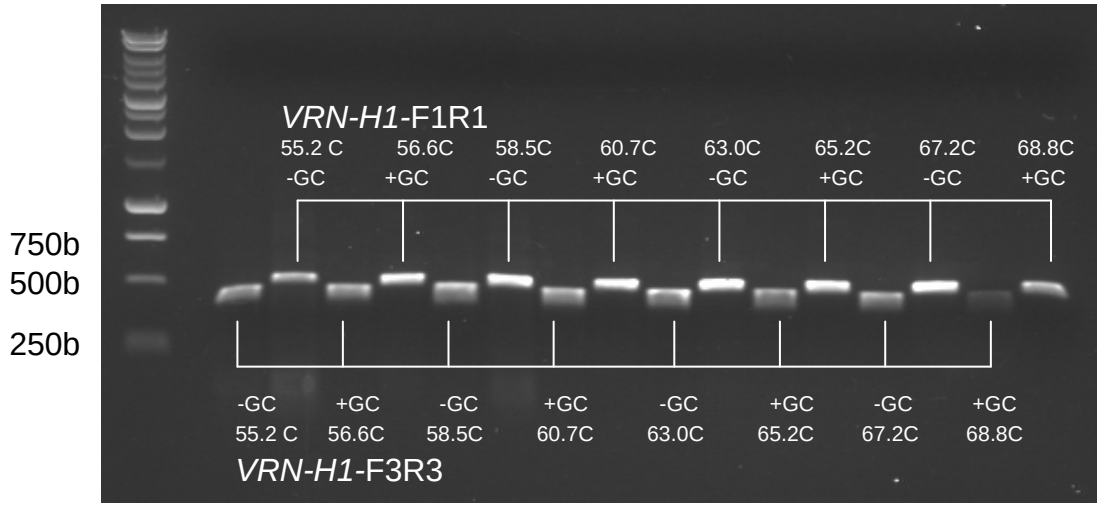

Figure 4. Optimisation of VRN-H1 primer pairs. Annealing temperatures and presence /absence of GC-rich template buffer is indicated.

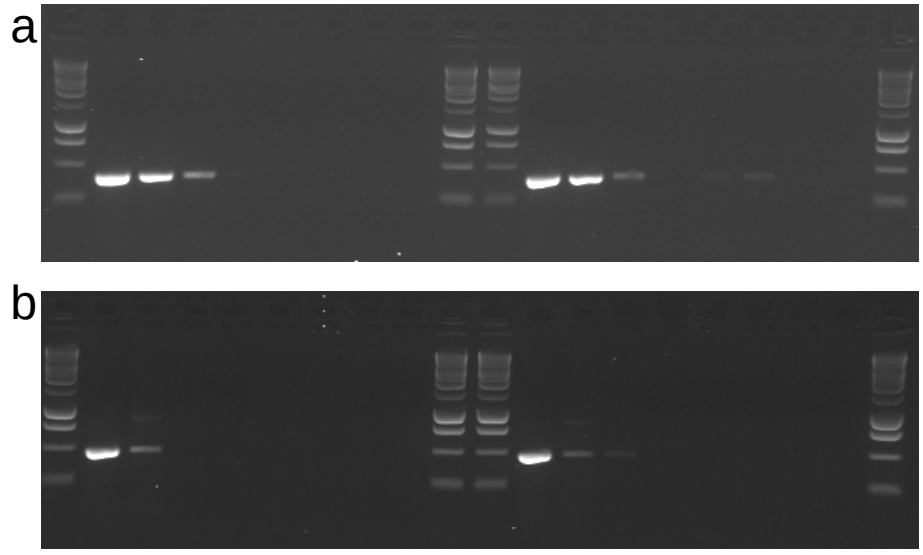

Figure 5. Determining the sensitivity of primer pair VRN-H1-F3 / VRN-H1-R3. A = 'Flagon' (winter); B = 'Golf' (Spring). Lane 1 = No dilution. Subsequent lanes represent serial 1/20 dilutions.

	SNP1	SNP2	SNP3	SSR	PP1	SNP4	SNP5	SNP6	*ZCCT-H*
Strider	T	A	C	2	0	T	G	C	+Z
Noveta	T	A	C	3	0	T	G	C	-Z
2159	T	A	C	2	0.5	T	G	C	-Z
Damas	T	A	C	2	0	T	G	C	+Z
1149	T	A	C	2	U	T	G	C	+Z
1797	T	G	C	3	0	C	A	G	-Z
Gaelic	T	G	C	2	U	C	A	G	+Z

Table 1. Haplotyping of eight "alternative" varieties, according to the protocols described by Cockram et al (2007). SSR coding: 2 = 174 bp; 3 = 177 bp. ZCCT-H (representing the VRN-H2 candidate locus) scores: +Z = presence of ZCCT-Ha, -Hb, -Hc; -Z = absence of ZCCT-Ha, -Hb, -Hc. PP1 = primer pair 1 (deletion size is indicated in kb, U = unknown deletion). The genotype of the winter 'Strider' vrn-H1 allele is shown as a reference. No results were obtained for 2/1788.

	Primer Pair	Damas	Gaelic	Kiruna	Novetta	2/1149	2/1788	2/1797	2/2159
1	Pr-1	Y	Y	Y	Y	Y	Y	Y	Y
2	Pr-2								
3	Ex-1	Y	Y	Y	Y	Y	Y	Y	Y
4	In1-1	Y	N	N	Y	Y	N	Y	Y
5	In1-2	Y	N	N	N	N	N	Y	N
6	In1-3	Y	N	N	Y	Y	N	N	Y
7	In1-4	Y	N	N	Y	Y	N	N	Y
8	In1-5	Y	N	N	Y	Y	N	Y	Y
9	In1-6	Y	N	N	Y	Y	N	Y	Y
10	In1-7	Y	N	N	Y	Y	N	Y	Y
11	In1-8	Y	Y	Y	Y	Y	Y	Y	Y
12	In1-9	Y	Y	Y	Y	Y	Y	Y	Y
13	In1-10	Y	Y	Y	Y	Y	Y	Y	Y
14	In1-11	Y	Y	Y	Y	Y	Y	Y	Y
15	In1-12	Y		Y	Y	N	Y	N	Y
16	In1-13	Y		Y	Y	Y	Y	Y	Y
17	Ex-2	Y		Y	Y	Y	Y	Y	Y
18	In2-1	Y	Y	Y	Y	Y	Y	Y	Y
19	In2-2	Y	Y	Y	Y	Y	Y	Y	Y
20	In2-3								
21	In2-4								
22	E3-8	Y	Y	Y	Y	Y	Y	Y	Y

Table 2. VRN-H1 PCR amplicons in the eight 'alternative' varieties studied. Y = amplification achieved ; N = no amplification; blank = amplification not yet attempted. Failure of consecutive primer pairs to amplify indicates the position and size of the putative deletions.

	AFP	Variety	VRN-H1	VRN-H2
1	235	G. Promise	S	0
2	278	Triumph	S	0
3	421	Golf	S	0
4	674	Dandy	S	0
5	688	Blenheim	S	0
6	750	Tyne	S	1
7	904	Alexis	S	0
8	1011	Derkado	S	1
9	1029	Graphic	S	0
10	1031	Chariot	S	0
11	1091	Felicie	S	0
12	1133	Delibes	S	0
13	1146	Cooper	S	0
14	1147	Brewster	S	0
15	1187	Cork	S	0
16	1188	Optic	S	0
17	1203	Riviera	S	1
18	1238	Tankard	S	1
19	1246	Clarity	S	0
20	1291	Quartet	S	0
21	1293	Landlord	S	0
22	1350	Ricarda	S	0
23	1354	Chalice	S	0
24	1409	Aspen	S	0
25	1410	Century	S	0
26	1411	Decanter	S	0
27	1415	Linden	S	0
28	1427	Static	S	0
29	1485	Otira	S	1
30	1492	Potter	S	0
31	1501	Saloon	S	0
32	1555	Akita	S	0
33	1566	Prestige	S	0
34	1567	Protégé	S	0
35	1572	County	S	0
36	1574	Cellar	S	0
37	1575	Pewter	S	0
38	1576	Brise	S	1
39	1579	Viskosa	S	1
40	1580	Neruda	S	0
41	1589	Agenda	S	0
42	1668	Spire	S	0
43	1755	troon	S	0
44	1769	Cocktail	S	0
45	1862	Carafe	S	0
46	1865	Doyen	S	0
47	1871	Rebecca	S	0
48	1933	Power	S	0
49	1939	Westminster	S	0
50	1940	Oxbridge	S	0
51	1963	Waggon	S	0
52	1966	NFC Tipple	S	0
53	1968	Wicket	S	0
54	2030	appaloosa	S	0
55	2119	publican	S	0
56	2121	quench	S	0

	AFP	Variety	VRN-H1	VRN-H2
57	514	Halcyon	1A	1
58	578	Pipkin	1A	1
59	806	Puffin	1A	1
60	816	Pastoral	1A	1
61	899	Clarine	1A	1
62	947	Fighter	1A	1
63	951	Manitou	1A	1
64	1002	Blanche	1A	1
65	1071	Intro	1A	1
66	1160	Melanie	1A	1
67	1162	Sunrise	1A	1
68	1171	Fanfare	1A	1
69	1204	Regina	1A	1
70	1214	Muscat	1A	1
71	1262	Volley	1A	1
72	1264	Cobalt	1A	1
73	1266	Tiffany	1A	1
74	1276	Antigua	1A	1
75	1281	Emelia	1A	1
76	1285	Spice	1A	1
77	1318	Pearl	1A	1
78	1320	Jewel	1A	1
79	1334	Spirit	1A	1
80	1397	Angela	5C	1
81	1402	Heligan	1A	1
82	1435	Opal	1A	1
83	1457	Rounder	1A	1
84	1463	Siberia	5C	1
85	1468	Artist	1A	1
86	1528	Haka	1A	1
87	1534	Sarah	1A	1
88	1535	Carola	1A	1
89	1606	Carat	1A	1
90	1626	Pict	1A	1
91	1628	Scylla	1A	1
92	1700	Cannock	1A	1
93	1717	Sequel	5C	1
94	994	Bronze	1A	1
95	1825	Camion	1A	1
96	1880	Saffron	1A	1
97	1886	Amarena	1A	1
98	1263	Rifle	1A	1
99	232	M. Otter	1A	1
100	1221	Prelude	1A	1
101	1471	Leonie	1A	1
102	1604	Diamond	1A	1
103	1705	Kestrel	1A	1
104	1924	Spectrum	1A	1
105	2003	Colibri	5C	1
106	2058	Cassata	1A	1
107	2065	Retriever	1A	1
108	2077	Pelican	1A	1
109	2083	Suzuka	1A	1
110	1910	Flagon	1A	1
111		Accure	1A	1

Table 3. Database of VRN-H1 and candidate VRN-H2 genotypes in UK barley varieties. Spring (yellow), alternative (green) and winter (blue) varieties are colour coded. S = spring haplotype; 1A/5C = winter haplotype. 0 = ZCCT-H gene cluster absent; 1 = ZCCT-H gene cluster present.

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