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FUNCTIONAL SNP MARKERS FOR THE VERNALIZATION REQUIREMENT 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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1. INTRODUCTION

1. This project aimed to develop previous work from a Defra Science Directorate funded research project in which vernalization genes in barley were characterized (see document BMT/11/17). The qualitative test predicting seasonal growth habit that was developed in the previous project has been further refined to allow detection of off-types in a sample. A reference database of *VRN-H1* and *VRN-H2* alleles was built up and additional work carried out on the characterization of "alternative" types.

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 spring-time in order to confirm their seasonal growth habit (SGH). Spring varieties are not currently grown and information from the official Technical Questionnaire (TQ) is used to exclude these varieties from the growing trial. Winter varieties do not flower at all and since the material does not produce heads or grains, the trial for seasonal growth habit is not used to score any other characteristics.

3. A molecular test for the vernalization characteristic in barley would serve to demonstrate 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.

4. The previous Defra Science Directorate project characterized sequence diversity in the *VRN-H1* gene in European Union barley varieties and assessed the association between polymorphisms at *VRN-H1* and *VRN-H2* loci and seasonal growth habit. Specific haplotype combinations at the *VRN-H1* and *VRN-H2* loci were tested to see if they could be accurately identified using low-cost, single reaction PCR assay that could be used to predict seasonal growth type. A PCR test now exists 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, leaving enough time for any problems to be verified later by sowing.

5. In order to utilize the qualitative test developed so far to predict seasonal growth habit in a DUS context, it was necessary to establish a procedure to detect and quantify the percentage of off-types in an unknown sample (i.e. its uniformity). A reference database of *VRN-H1* and *VRN-H2* alleles found in 'common knowledge' varieties was also set up for DUS purposes. In addition, some further work was incorporated to sufficiently resolve the handling of the third, less routinely observed, "alternative" state described in international protocols such as the UPOV Test Guidelines and CPVO technical protocols.

6. The "alternative" growth habit is poorly described in genetic terms, and has had varying definitions in published literature. For example, von Zitzewitz *et al.* (2005) describe these varieties as containing a winter *Vrn-H1* allele combined with a spring *vrn-H2* allele. The resulting plant would retain the frost tolerance loci linked to *VRN-H1* on the short arm of chromosome 5H, while displaying a spring (vernalization non-responsive) phenotype conferred by alleles at *VRN-H2*. Similarly, Szűchs *et al.* (2007) describe alternative varieties as carrying "only a modest vernalization response", delaying flowering by only a few days, which can again be predicted by known allele combinations at *VRN-H1* and *VRN-H2*.

7. The UPOV Test Guidelines (document TG/19/10) describe alternative types as types that flower later than spring types in the absence of vernalizing temperatures and the current field trials utilize the UPOV criteria in order to identify this phenotype.

8. 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 seasonal growth type in trials during 2006 and further work carried out at NIAB identified a novel winter allele, which looked likely to help in the identification of alternative type varieties. This project further investigated the genetic basis of the "alternative" type and tested the predictive power of the work to date.

2. OBJECTIVES

9. A qualitative molecular method for barley has been successfully developed that can detect winter and spring barley types. The work here aimed to refine the test to be suitable for potential use in DUS testing as an alternative approach to the current costly field trials, thereby reducing costs and delivering earlier results in the future.

10. The objective was to develop a scientifically verified molecular testing method for assessment of the "vernalization characteristic" in barley for DUS purposes suitable as a replacement of the field trials approach currently used. This falls into the UPOV Option 1a) category of approaches to the use of molecular markers in DUS testing. Option 1a) is described as "Use of molecular markers which are directly linked to traditional characteristics (gene specific markers)" (see documents TC/38/14-CAJ/45/5 and TC/38/14 Add. – CAJ/45/5 Add).

3. RESULTS

(All Figures are shown in the Annex to this report.)

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

11. Following our work conducted during 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 varieties, identifying three novel forms (Figure 1), and delivering ~200 kb of DNA sequence. 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.

Development of the reference database

12. In the previous Defra project, we characterized allelic status at *VRN-H1* in 100 United Kingdom varieties using primer pair 1, as illustrated in Figure 3. However, we have since shown that this assay is not sufficient to predict growth habit in all *VRN-H1* alleles (spring haplotype 2 and winter haplotype 5C). Towards overcoming this, we utilized the single-well multiplex PCR assay described here to genotype 396 United Kingdom varieties (213 winter and 175 spring varieties) for variation of alleles at *VRN-H1* in United Kingdom barley varieties. In addition to this, we genotyped a further three major flowering-time loci (the vernalization response locus *VRN-H2*, and the photoperiod response loci *PPD-H1* and *PPD-H2*) across all 396 varieties.

13. The construction of a database containing the genotyping information from all these varieties aims to define the alleles and allelic combinations deployed in United Kingdom barley varieties. This should help confirm the predictive power of genotypic identification of winter and spring varieties, and also help identification of genotype combinations that could define the "alternative" SGH. Database entries are presented in Table 1. The SGH of all winter and spring varieties successfully genotyped were predicted by the *VRN-H1* and *VRN-H2* multi-locus genotypes. All winter varieties

possessed winter alleles at both *VRN-H1* and *VRN-H2*. All spring varieties have a spring *Vrn-H1* allele. Although the majority of spring varieties also have a spring allele at *VRN-H2*, a minority (18 %) have a winter allele. Both of these allelic combinations are predicted to result in spring SGH, according to the epistatic interactions between the two loci (where the presence of a spring allele at either locus is predicted to result in a spring variety). In addition, we show that with the exception of three varieties ('Gaulois', 'Tempo' and 'Adonis'), winter United Kingdom barley varieties are characterized by the absence of the *HvFT3* gene, diagnostic for short-day responsive alleles at *PPD-H2*. All but one of the spring varieties ('Rebecca') contain the *HvFT3* gene, and are therefore predicted to almost exclusively carry the short-day non-responsive allele at *PPD-H2*. All of the spring varieties successfully genotyped carry the non-responsive *PPD-H1* allele, while of the 181 winter varieties genotyped, 122 and 59 carry the responsive and non-responsive *PPD-H1* alleles, respectively.

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

14. With the aim of identifying DNA polymorphisms characteristic of "alternative" growth habit we have extracted DNA from eight coded barley varieties which had been previously classified as alternative by their phenotype: the varieties are coded 1 to 8.

VRN-H2

15. Using the PCR assay described by Karsai *et al.* (2005), we genotyped the three *ZCCT-H* genes, diagnostic for allelic state at the *VRN-H2* vernalization response locus (Figure 4). We found that three of the eight "alternative" varieties (4, 7 and 8) lacked the *ZCCT-H* gene cluster, diagnostic for spring alleles at *VRN-H2*. Given that all of the 213 United Kingdom winter varieties for which we obtained genotype information contain the *ZCCT-H* cluster, their absence in these three "alternative" varieties strongly indicates *VRN-H2* as a relevant marker by which their lack of a full vernalization requirement can be recognized. The remaining five "alternative" varieties (1,2,3,5 and 6) possess all three *ZCCT-H* genes, indicative of a winter *Vrn-H2* allele, suggesting that the *VRN-H1* locus could be responsible for the observed phenotype in these varieties.

VRN-H1

16. 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 three single nucleotide polymorphisms (SNPs) from the *VRN-H1* promoter region (designated SNP1, SNP2 and SNP3) and 3' end of the gene (SNP4, SNP5, SNP6) were identified by direct sequencing in each "alternative" variety, returning a total of 22.9 kb of sequence. Their positions, along with those of all other polymorphisms identified in these fragments, are shown in Figures 5 and 6. The simple sequence repeat (SSR) found in the 5'UTR was genotyped using fluorescently labelled primers visualized on the ABI 3730 platform (Figure 7). All currently known spring *Vrn-H1* alleles possess large deletions/insertions within intron 1, relative to winter alleles. The presence or absence of such deletions/insertions was first assayed using the multiplex assay described in Figure 2. In addition, with the aim of identifying any novel intron 1 rearrangements that may be present, all of the "alternative" varieties were further investigated by amplification of a series of 23 overlapping PCR products which together span *VRN-H1*, as described by Cockram *et al.* (2007). Where possible, PCR amplicons spanning any major deletions identified were directly sequenced.

17. Analysis of the polymorphisms (according to the haplotype designations described by Cockram *et al.*. 2007) showed all varieties belonged to one of three previously identified *VRN-H1* haplotypes (Table 2). Nineteen polymorphic features were identified in the 1.1 kb sequenced region of the *VRN-H1* promoter, while eleven polymorphisms were found in the 1.7kb region spanning *VRN-H1* exon 3 to the 3'UTR. "Alternative" varieties 3 and 6 were found to possess the previously characterized spring *VRN-H1* haplotype 5A, with sequencing across the intron 1 breakpoint confirming the major deletion detected is identical to the 5.2 kb deletion found in the spring haplotype 5A variety, 'Golf' (Figure 8). "Alternative" varieties 1 and 7 have genotype configurations identical to that of the previously identified haplotype 5C winter *vrn-H1* allele found in

'Express'. Sequencing the intron 1 breakpoint in these "alternative" varieties showed the location of the 0.5 kb deletion to be exactly the same as that previously identified in 'Express' (Figure 9).

18. The remaining four "alternative" varieties (2, 4, 5 and 8) displayed the winter 1A haplotype. Although the *VRN-H1* multiplex deployed in this study did not detect an intron 1 deletion, it is possible that previously un-described deletions are present in these varieties. Accordingly, we amplified a series of 23 PCR amplicons that span the ~17 kb winter *vrn-H1* allele in all eight "alternative" varieties, (Table 3). Where major deletions have occurred, one or more adjacent primer pairs internal to the deletion will fail to amplify. In this way, a preliminary analysis of gene configuration can be determined. PCR analysis of intron 1 in the four "alternative" varieties which we found possessed previously defined breakpoints confirmed that only one major deletion was present: 1(5C), 7(5C), 3 (5A), 6 (5A). PCR analysis of the remaining varieties predicted to possess an intron 1 configuration characteristic of winter *VRN-H1* haplotype 1A found that three (4, 5 and 8) failed to amplify a 1kb (approximately) fragment in intron 1 (amplicon In1-2). This amplicon spans the "vernalization critical" region, within which deletions are thought to result in spring alleles (von Zitzewitz *et al.* 2005). Therefore, failure to amplify this region suggests it contains a novel insertion/deletion event that disrupts the "vernalization critical" region. However, despite repeated attempts, we were not able to PCR amplify across the predicted insertion/deletion, and so this hypothesis cannot be confirmed. Finally, PCR analysis of intron 1 in the "alternative" variety 2 did not identify any major deletions. Sequencing is not finalised (13.6 kb out of 17 kb completed), but so far *VRN-H1*sequence in variety 2 is identical to the winter haplotype 1A allele. Significantly, despite repeated attempts, we have not been able to amplify and sequence a 1kb (approximately) promoter region in any of the "alternative" varieties. As this region contains conserved predicted regulatory elements thought to control SGH in the related grass species, *Triticum monococcum* (Yan *et al.* 2004), analysis of the potential effect of sequence variation at *VRN-H1* on SGH cannot be conclusive until this region has been successfully sequenced.

19. Interestingly, both varieties 4 and 8 possess the 1A-Z multilocus *VRN-H1*/*VRN-H2* haplotype that predicts a spring SGH (winter *vrn-H1* allele; spring *vrn-H2* allele). Although the epistatic interactions between the *VRN-H1* and *VRN-H2* loci predict that spring varieties can possess a spring allele at either of the two loci, none of the spring varieties in our comprehensive survey of United Kingdom barley germplasm possess a winter *Vrn-H2* and spring *vrn-H1* allelic combination (haplotype 1A-Z) (Table 1). However, during a previous survey of approximately 400 European barley varieties, we identified three varieties ('Perga, 'Urania'and 'Vixen') that possess a 1A-Z haplotype. Interestingly, passport data for these varieties recorded them as winter varieties, but phenotyping under long-day photoperiods in the absence of vernalization found them less delayed in flowering compared to a winter control variety, and so were designated as spring. However, the phenotype was not further investigated by sowing alongside spring varieties, so the conflict between recorded and observed phenotype could be explained by 1A-Z haplotypes resulting in "alternative" SGH. The discovery that three of the eight "alternative" varieties also possess a 1A-Z haplotype suggests that this may be one of the allelic combinations that predict "alternative" seasonal growth habit.

20. Finally, three "alternative" varieties contain *VRN-H1*/*VRN-H2* multi-locus haplotypes diagnostic for winter SGH: varieties 1 (5C+Z), 2 (1A+Z) and 5 (1A+Z). To investigate whether additional sequence variation within *VRN-H1* in "alternative" varieties could be identified, we have initiated full-length sequencing of *VRN-H1* in all 8 "alternative" varieties, using the PCR amplicons listed in Table 3. Full-length sequencing is incomplete, but to date no polymorphisms have been identified.

Genotyping the additional flowering time genes PPD-H1 and PPD-H2

21. As well as the response to cold temperatures (vernalization), flowering in barley is also responsive to day-length, and is predominantly controlled by two major loci. Wild-type alleles at the first of these (*PPD-H1*) promote flowering time in response to long-day photoperiods (approximately 16 hours light), while the non-responsive allele at *PPD-H1* permits flowering to be initiated in the

absence of long-day photoperiods. Winter-associated alleles at the second major photoperiod locus, *PPD-H2*, inhibit flowering under short-day conditions, and so work along with winter alleles at the *VRN* loci to inhibit premature initiation of floral structures in autumn-sown barley. To determine if alleles or allelic combinations at these loci may explain the "alternative" SGH, both *PPD-H1* and the *PPD-H2* candidate gene (*HvFT3*) were genotyped in all eight "alternative" varieties. Sequencing a 829bp *PPD-H1* amplicon spanning the diagnostic G (responsive) → T (non-responsive) SNP in exon 8 identified nine SNPs and one SSR (Figure 10). "Alternative" varieties 1, 2, 4 and 7 were all predicted to possess photoperiod responsive alleles at *PPD-H1* based on the presence of a G nucleotide, while the remaining five varieties are all predicted to carry photoperiod non-responsive alleles (Table 2). Genotyping the 396 United Kingdom barley varieties in this project shows that winter varieties can carry either allele at *PPD-H1*. The occurrence of both alleles in the nine "alternative" varieties studied here suggests that it is not critical in the differentiation of "alternative" SGH. Faure *et al.*(2007) reported that allelic status at *PPD-H2* can be predicted by the presence or absence of the candidate gene *HvFT3*, a barley orthologue of known flowering-time genes in rice and *Arabidopsis*. Here, we modified the published PCR/agarose gel based assay for *HvFT3* by the inclusion of a positive internal control and applied it to the eight "alternative" varieties. We find that all "alternative" varieties lack *HvFT3* (Table 2). Screening of *HvFT3* in the wider collection of 396 spring and winter United Kingdom barley varieties shows that, for those varieties for which we obtained genotypic information, all winter barley varieties are characterized by the absence of *HvFT3*, while *HvFT3* is present in spring varieties (with the exception of 'Rebecca'). This is an important finding as the ability to delay flowering time independently of vernalization under short day (i.e. winter) conditions through having a deleted *HvFT3* allele of *PPD-H2* is predicted to be a characteristic feature of an alternative growth habit and gives an important means by which the alternative varieties tested here which carried spring alleles at *VRN-H1* or *VRN-H2* can be distinguished from true spring varieties.

Summary of VRN-H1 and VRN-H2 genotyping

22. Of the eight "alternative" varieties investigated, no common polymorphism was identified in *VRN-H1* or *VRN-H2* that could uniquely predict SGH. However, the multi-locus genotypes of five of these do predict a non-winter phenotype: Varieties 4 and 8 both possess haplotype 1A-Z, while variety 7 is haplotype 5C-Z. Both of these haplotypes predict lack of a vernalization requirement (ability to flower in spring sowing), due to the combination of winter *vrn-H1* and spring *vrn-H2* alleles. However, this allelic combination is not observed in any of the 396 United Kingdom varieties databased here, and could represent a combination resulting in "late" spring phenotype analogous to the "alternative" designation. Varieties 3 and 6 possess spring alleles at *VRN-H1* and *VRN-H2* (5A-Z), also predictive of lack of vernalization requirement. As such, genotyping would immediately pick them out from all winter submissions as candidates likely to flower in the spring-sown vernalization trial. Of the remaining three, varieties 5 and 8 are predicted to contain an as yet un-sequenced insertion/deletion within the vernalization critical region of *VRN-H1* intron 1, which would putatively abolish its vernalization requirement and identify it as belonging to the "alternative" type. This leaves variety 2 as the only "alternative" variety in which no candidate polymorphism has yet been identified to explain the observed SGH.

Detection of off-types

23. 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 previous Defra project VS0137, we were able to classify all of the spring and winter *VRN-H1* alleles present in a collection of more than 400 European barley varieties, identifying three novel forms (Figure 1), and delivering approximately 200 kb of DNA sequence.

24. As we have not been able to identify a set of polymorphisms that specifically identified all "alternative" varieties, at this stage the detection of off-types would not be able to perfectly distinguish between all three SGH classes. However, it is still possible to investigate the detection of off-types using the known allelic range at *VRN-H1*. An obvious step towards detection of off-types would be to

deploy the single-well multiplex assay described above. Due to the complexity of primer pairs in this assay, we chose to determine the sensitivity of allele detection using primer pairs in separate PCR reactions that individually detect known spring alleles at *VRN-H1*.

25. To date, seven major intron 1 spring rearrangements are recognized (Figure 1): deletions of 0.5 kb (winter haplotype 5C), 3.9 kb (spring haplotype 1B), 4.1 kb (spring haplotype 5B), 5.2 kb (spring haplotypes 4B and 5A), 6.3 kb (spring haplotype 3) an 8.9 kb deletion (haplotype 4A), as well as a 0.7 kb insertion (spring haplotype 2). Primer pairs specific to each deletion/insertion were designed, and their PCR conditions optimized (Table 4). Once achieved, the sensitivity of each primer pair to detect the target allele was tested on 40ng DNA from the winter variety 'Flagon' (haplotype 1A) spiked with a series of serial 1 in 10 dilutions made from a variety containing the intron 1 deletion/insertion to be assayed (variety names listed in Table 4). DNA spikes ranged from neat (i.e. no dilution) to 1 in 10,000 dilutions. Six PCR reactions were assayed, all in duplicate: (1) winter variety (40 ng ±2ng) (2) spring variety (40 ng ±2ng) (3) winter plus 40 ng ±2ng spike (4) winter plus 4ng spike (5) winter plus 0.4 ng spike (6) winter plus 0.04 ng spike (7) winter plus 0.004 ng spike. A negative control lacking genomic DNA template was also included. DNA concentrations were determined using the average of three measurements, and were standardised to 40ng/ul ± 2ng. PCR reactions were set up using the following volumes: 1 µl DNA, 1 µl Buffer, 1 µl $MgCL_2$, 0.5 µl forward primer (10 µlmol), 0.5 µl reverse primer (10 µlmol), 0.1 µl FastStart *Taq* (Roche), 0.1 µl DNTPs and 5.8 µl H_2O. Results are shown in Figure 11. All primer pairs were able to reliably detect spikes of 0.4 ng within a DNA sample containing 40ng of non-target DNA from the winter barley variety 'Flagon'. Five of the seven primer pairs were able to detect spiked DNA at concentrations 0.04ng (haplotypes 1B, 2, 3, 4A and 5C), corresponding to spikes diluted to 1 in 1,000.

26. Although we have shown that it is possible to detect spiked off-type DNA at very low concentrations, the ability to detect in real-world DNA samples depends on the efficiency with which DNA is extracted from the sample material. For ease of application, DNA extraction from dry (submitted) seed would be preferable. The threshold for off-types in UPOV Test Guidelines is 5 plants in 2,000. The uniformity standard of 5 in 2,000 corresponds to a Population Standard of 0.1% and an Acceptance Probability ≥95%. Therefore, in a sample of 400 plants, the number of off-type plants allowed would be 2. We performed two bulk DNA extractions from ground dry seed; (1) a 1 in 400 off-type, containing 399 'Prelude' seeds (winter haplotype 1A) plus 1 'Golf' seed (spring haplotype 5A). (2) a 5 in 400 off-type, containing 395 'Prelude' seeds plus 5 'Golf' seeds. Seeds were ground to flour using a coffee-grinder, and DNA extracted using a chloroform method (*http://www.diversityarrays.com/samplesub.html*), modified as follows: 10 mls flour incubated for 2hrs with 20 mls extraction buffer. 20 mls chloroform added. Re-suspend in 5 mls of buffer. The DNA extractions performed resulted in very large volumes (5mls) of carbohydrates contaminating the DNA and resulting in large jelly-like pellets. PCR amplification using primers specific to the 'Prelude' winter *VRN-H1* allele and the spiked 'Golf' spring *VRN-H1* allele were performed. Due to the apparent impurity of the DNA extraction from ground seed, PCRs were performed using neat DNA, and 1/10 and 1/100 DNA dilutions, in case of inhibition of the PCR reaction due to the presence of co-extracted inhibitors. No amplification was achieved using neat DNA. However, amplification of the background winter 'Prelude' *VRN-H1* allele and the spiked 'Golf' spring allele were achieved in 1/10 dilutions of both the 5 ('Golf')/400 ('Flagon') and 1/400 DNA extractions (Figure 12). It should be noted that PCR amplification was not consistent between replicates, even when assaying for the presence of the winter allele, which was present at up to 400x the concentration of the spring allele (data not shown). However, amplification was achieved in a portion of 1/10 dilutions of the original spiked DNA extractions, from which it can be inferred that detection of the off-type was possible at a level of magnitude lower than that specified by UPOV Test Guidelines. These results show that the sensitivity of the assays employed should be able to routinely detect off-types at a frequency of 1 in 400. However, to ensure this assay is robust in real-world application, a DNA extraction method from seed would need to be developed that excludes the massive volumes of polysaccharide contamination that inhibit PCR detection of off-types. We propose that either exploration of different DNA extraction chemistries more robust to the presence of large amounts of complex carbohydrates from

the endosperm are tested or alternatively, that high quality DNA extractions could easily be made from the young germinated seedlings.

4. CONCLUSIONS

27. (i) A single-well multiplex PCR assay diagnostic of all known spring and winter alleles at the major flowering-time locus *VRN-H1* has been developed;

(ii) Genotyping of the flowering-time genes *VRN-H1, VRN-H2, PPD-H1* and *PPD-H2* in a panel of 396 United Kingdom barley varieties has been completed;

(iii) *VRN-H1, VRN-H2, PPD-H1* and *PPD-H2* have been genotyped in the eight "alternative" varieties identified;

(iv) Although no set of polymorphisms perfectly diagnostic for "alternative" seasonal growth habit has been identified, analysis of multi-locus *VRN-H1/VRN-H2* haplotypes allows six of the eight "alternative" varieties to be predicted to lack a full vernalization requirement, and would suggest that these be targeted for field testing in a spring vernalization trial;

(v) Assays diagnostic for all known spring *Vrn-H1* alleles have been designed, optimized and found to have a sensitivity of at least 0.04ng spiked target DNA; and

(vi) Detection of off-types to the standards in the UPOV Test Guidelines has been demonstrated using DNA extracted from ground seed.

28. Clearly there is no problem in identifying winter (vernalization-requiring) and spring (vernalization-independent) types of barley from the work in this project, however the uniqueness and lack of homogeneity of the alternative types poses a problem with regards to accurate identification and absolute classification with a molecular test. In practice, though, all except one "alternative" variety can be tentatively classified as "alternative" because they possess either *VRN-H1* or *VRN-H2* alleles which are known to partly or completely abolish the vernalization requirement. Of the varieties classified as alternative, two (varieties 4 and 8) are 1A-Z (winter at *VRN-H1*, spring at *VRN-H2*). Formally this is a spring classification, as there is a spring allele present at one of the two loci, but this combination is not seen in any of the 396 spring and winter United Kingdom barley varieties assayed, making these varieties unique. Therefore, it could be suggested that this is one of the allelic combinations that predicts alternative SGH, and the varieties could be grown out in the field after the results of the molecular test. These varieties also carry an unclassified novel deletion/insertion which with further work could lead to a diagnostic assay. One variety (7) is 5C-Z. This is the same situation as above, but with the other winter *VRN-H1* allele (5C). Variety 5 has a novel intron 1 deletion (breakpoint not yet sequenced), so this could distinguish it as alternative. Varieties 4 and 8 above also carry this deletion. This would highlight these varieties for growing out in the field for morphological observation.

29. Varieties 3 and 6 are 5A+Z (spring *VRN-H1*, winter *VRN-H2*) and are predicted to be spring types. We have found no obvious reason why they are alternative, but the presence of spring alleles at both loci in a variety described as winter on the TQ would highlight the need for growing out in the field.

30. Varieties 1 (5C+Z) and 2 (1A+Z) are predicted to have a functional vernalization requirement as they carry winter alleles at both loci. There is no obvious reason why they are classified as "alternative" (although we have suggested previously that the 5C winter *VRN-H1* haplotype is weaker than the predominant 1A haplotype) and indeed they are known and marketed as winter varieties so there is a possibility that due to the fact that both originated from non-United Kingdom testing centres, their classification may be subject to re-appraisal under United Kingdom conditions and that their classification as putative winter varieties by molecular markers is correct.

31. We did find one possible explanation why the six varieties 3, 4, 5, 6, 7 and 8 are predicted to lack a vernalization requirement might flower later than spring varieties in spring sowings. That is, in contrast to all 175 spring varieties except 'Rebecca', these six alternative varieties possessed the deleted winter-associated form of the *HvFT3* (*PPD-H2*) locus predicted to result in a delay in flowering under short-day conditions. What the effect of this allele in a non-vernalization requiring background would be is not sure, particularly as a late spring sowing (as manifested in the vernalization trial) largely avoids what are classically regarded as short days (day length is 13.5 hours at 50° latitude on April 15), therefore this speculative theory would require formal investigation.

32. "Alternative" varieties represent less than 1% of all barley varieties and for distinctness purposes are not considered to be distinct from either winter varieties or spring varieties. For distinctness in the United Kingdom DUS testing system, varieties must differ by two states to be considered distinct (a winter variety is only distinct from a spring variety and vice versa). Seasonal type is used as a grouping characteristic and is part of the variety description. Using the markers developed from this project it is possible to distinguish all the "alternative" varieties (except 1 and 2) as being different from winter or spring types, although it is not yet possible to accurately classify them as alternative.

33. Currently only winter varieties are assessed in the field for seasonal growth habit and TQ information is used to identify spring types. A pragmatic approach to implementing this molecular assay would be to continue to only test (by molecular markers) those varieties that are submitted as winter varieties. Any varieties that did not fall into the winter category (and are therefore genetically spring or alternative types) would be phenotyped in the field. This is likely to be only a very small number compared to the full number of winter varieties currently grown and the history of the very low number of alternative submissions. Uniformity within this characteristic would also be assessed by molecular markers and any "problem" varieties found would be grown in the field for a visual assessment.

34. This project involved a considerable amount of sequencing and therefore a lot more work than originally anticipated due to the nature of the different haplotypes of the alternative types. Although a robust predictive assay is now available to replace the field test and could be implemented effectively, there is still some sequencing work and identification of breakpoints within the alternative varieties that we were unable to complete within the scope of this project.

5. REFERENCES

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[Annex follows]

ANNEX

SUPPORTING DATA AND ANALYSIS

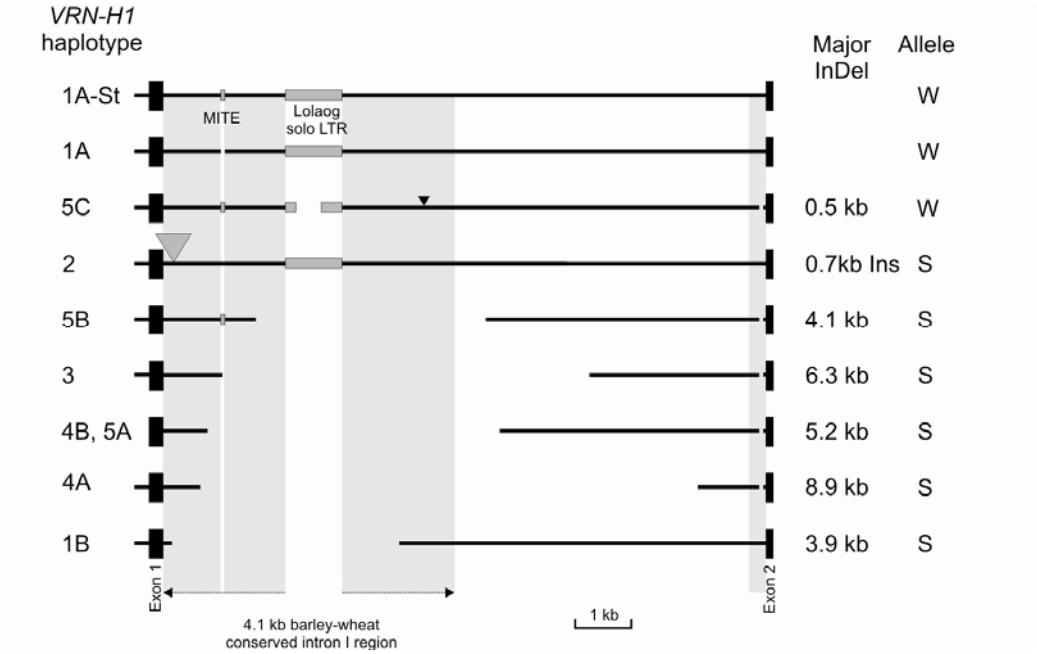


Figure 1. Diagram of intron I configuration in spring (S) and winter (W) *VRN-H1* alleles. The sizes of major intron 1 deletions (haplotypes 1B, 3, 4A, 4B, 5A, 5B, 5C) and insertions (haplotype 2, grey triangle) are indicated. Haplotypes 1B, 2 and 4B represent novel alleles identified during Defra project, VS0137. Regions of intron I nucleotide conservation between barley and the related wheat species *Triticum monococcum* are shaded in grey. Previous 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 harbor 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.

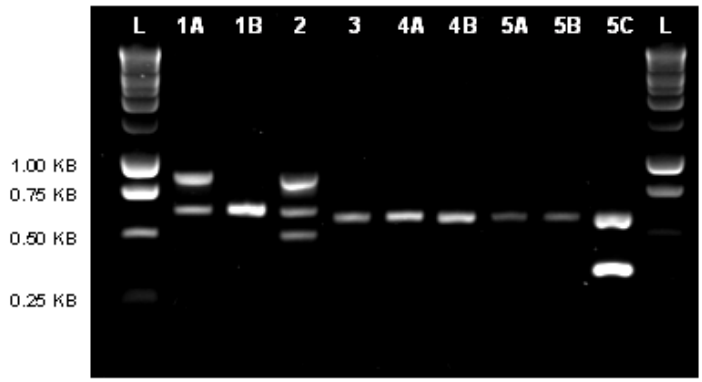


Figure 2. Single-well multiplex *VRN-H1* PCR assay. Representative varieties from *VRN-H1* haplotypes are as follows: 1A ('Pelican'), 1B ('Etu'), 2('Dram'), 3 ('Beatrice'), 4A ('Triumph'), 4B ('Maris Cannon'), 5A ('Pohto'), 5B ('Pegasus'), 5C ('Express'). Haplotypes 1A and 5C represent winter alleles,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

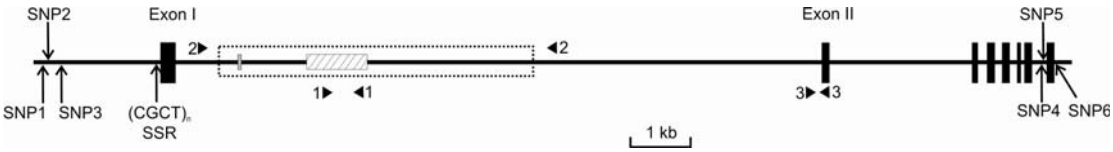


Figure 3. Diagram of the genomic winter *vrn-H1* allele from the variety 'Strider' (GenBank accession AY750993), indicating the positions of primer pairs 1, 2 and 3, the (CGCT) SSR in the 5'UTR, SNPs 1 to 3 (promoter), SNP4 and SNP5 (intron VII) and SNP6 (3'UTR). Solid black boxes denote exons. The position 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.

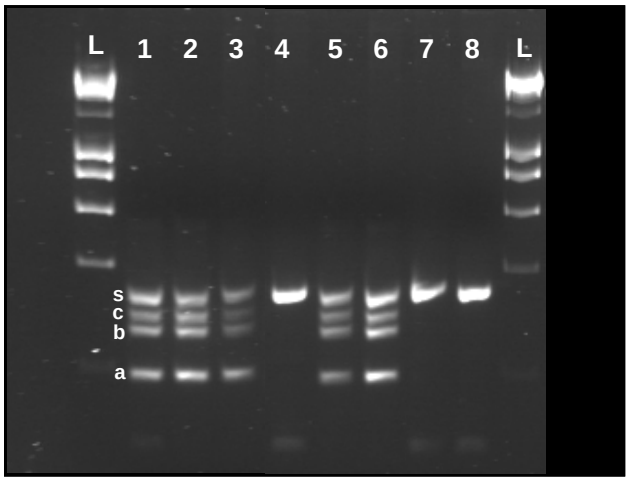
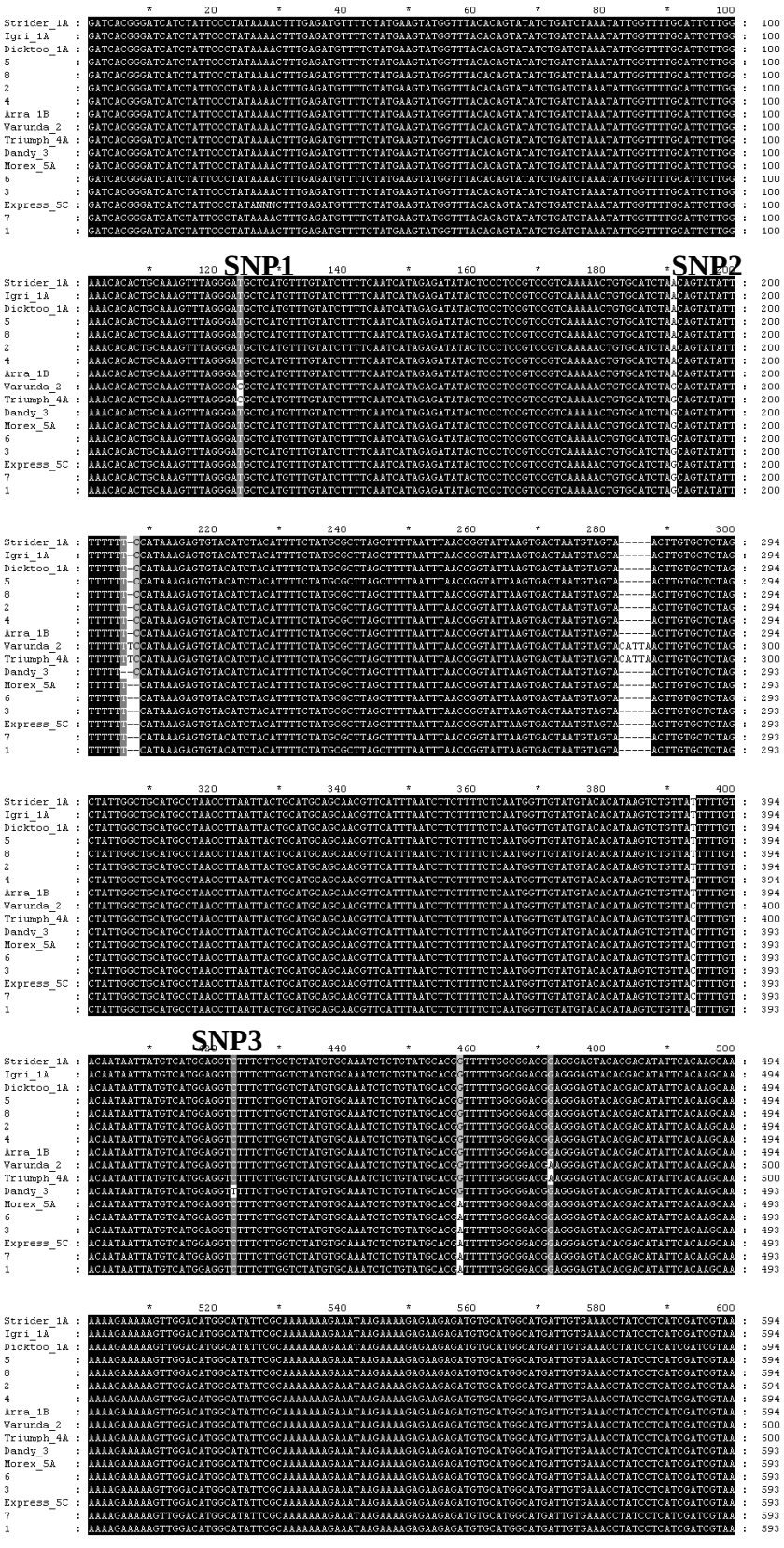


Figure 4. The multiplex PCR/agarose gel assay for allelic status at *VRN-H2*. a = *ZCCT-Ha*; b = *ZCCT-Hb*; c = *ZCCT-Hc*; s = *HvSNF2* (positive control). Presence or absence of all three *ZZCT* genes are diagnostic for winter and spring alleles at *VRN-H2*, respectively. Lane 1 = variety 1; 2 = variety 2; 3 = variety 3; 4 = variety 4; 5 = variety 5; 6 = variety 6; 7 = variety 7; 8 = variety 8. L = 1kb ladder.


SNP1
SNP2
SNP3

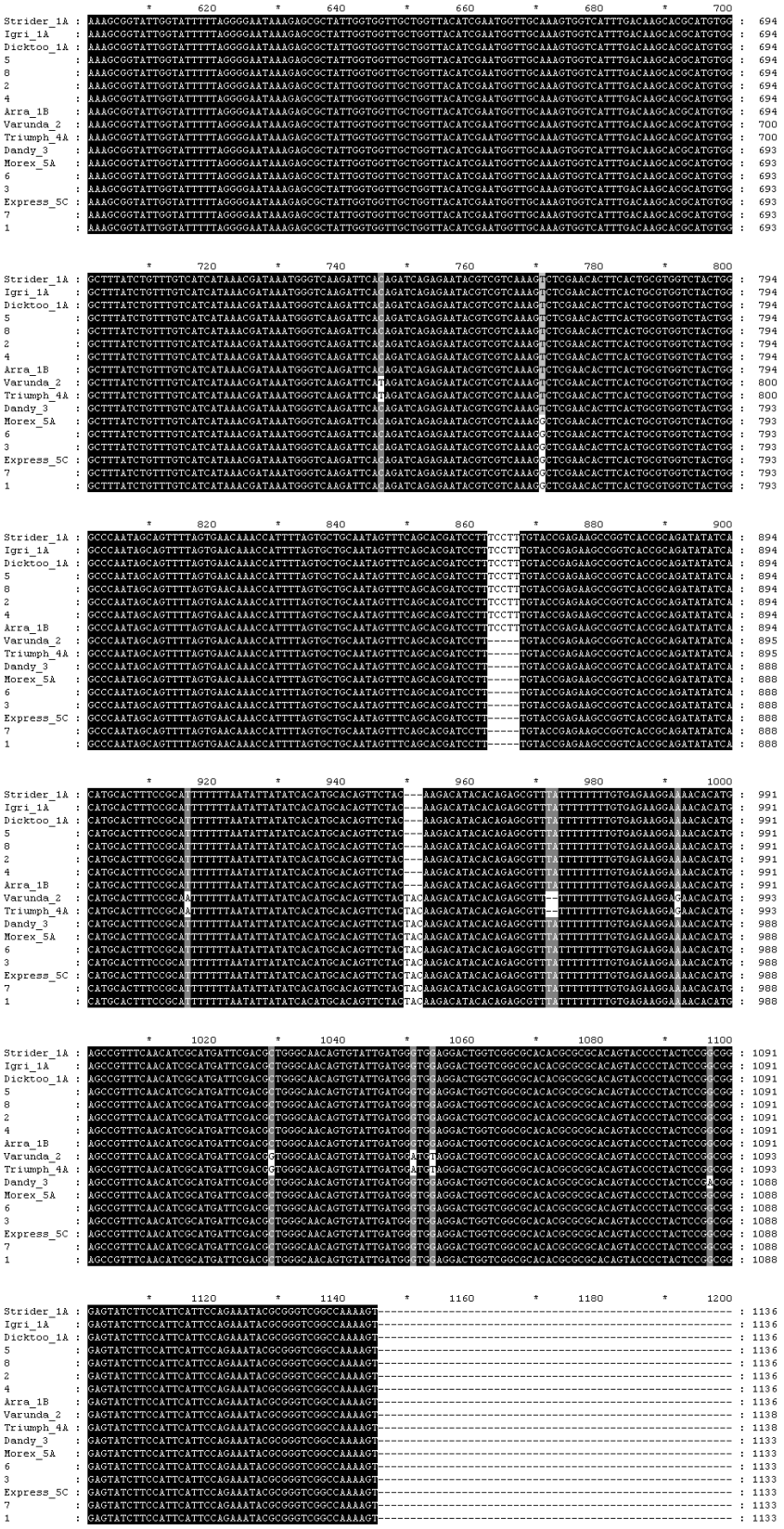


Figure 5. *VRN-H1* promoter sequences of 'alternative' varieties (coded 1 to 8), aligned to representative examples of each of the previously identified *VRN-H1* alleles. *VRN-H1* haplotype designations indicated after variety name. Sequences shown are from -2,071bp to -936bp, relative to variety 'Strider' (AY750993). The three SNPs used by Cockram *et al* 2007 to designate *VRN-H1* haplotypes are highlighted. Amplification and sequencing primers: F = 5'-GCAATGCAGCACCTATGCCA-3', R = 5'-CGTTGCTGCGTGTGGGTTG-3'.


Exon 3
Exon 4
Exon 5
Exon 6
Exon 7

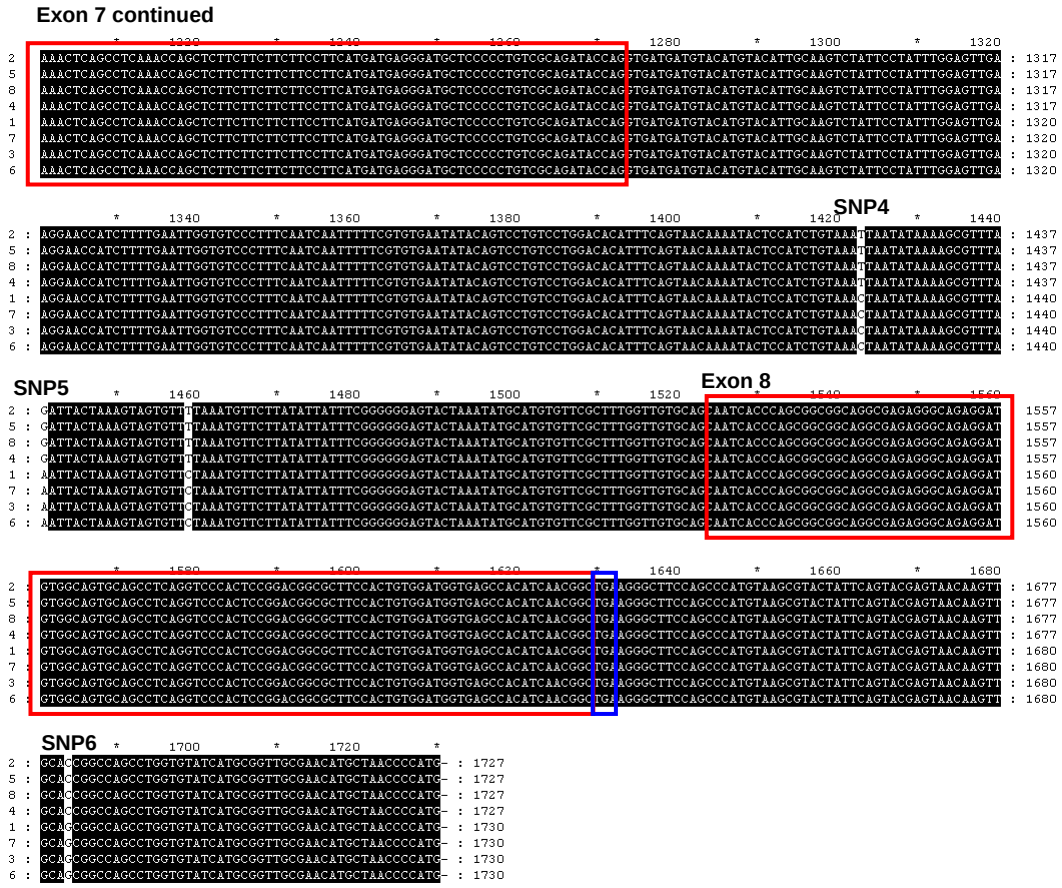


Figure 6. *VRN-H1* sequences of 'alternative' varieties (coded 1 to 8), exon 3 to 3'UTR. Sequences shown are from +13,148 bp to +14,874 bp, relative to variety 'Strider' (AY750993). The three SNPs used by Cockram *et al* 2007 to designate *VRN-H1* haplotypes are highlighted.

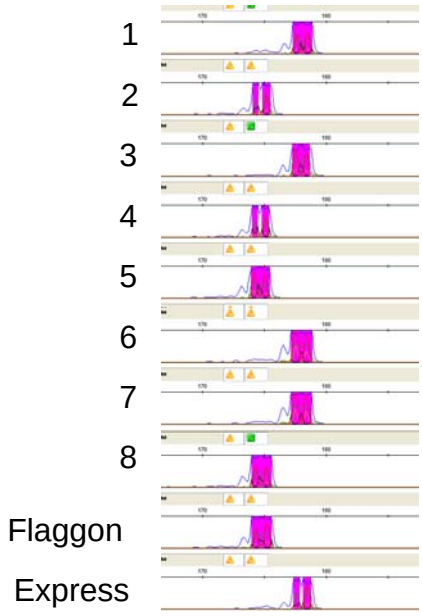


Figure 7. Genotyping the $(CGCT)_n$ SSR in the 5'UTR of *VRN-H1* in 'alternative' varieties (coded 1 to 8). The 174 bp and 178 bp alleles represent SSR repeats of $(CGCT)_4$ and $(CGCT)_5$ respectively.

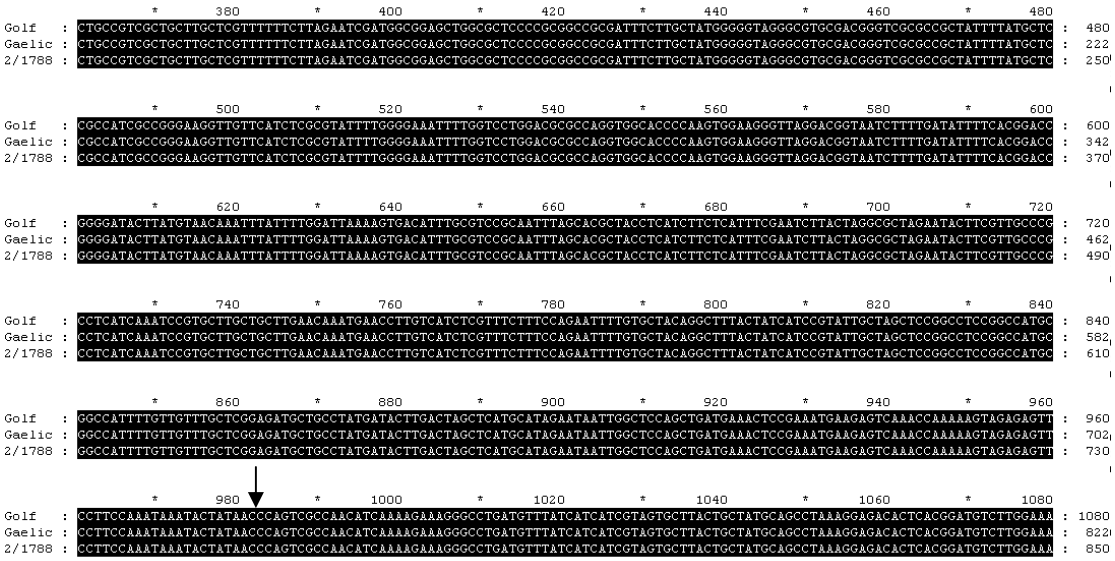


Figure 8. partial alignments of *VRN-H1* intron 1 between the spring variety 'Golf' (haplotype 5A) and two 'alternative' varieties (varieties 3 and 6). The position of the 5.2 kb deletion, relative to the winter *vrn-H1* allele from variety 'Strider', is indicated by the arrow. Amplification and sequencing primers: F = 5'- GTTCTCCACCGAGTCATGGT-3', R = 5'-GGCGGAGTACTGCTACAAC-

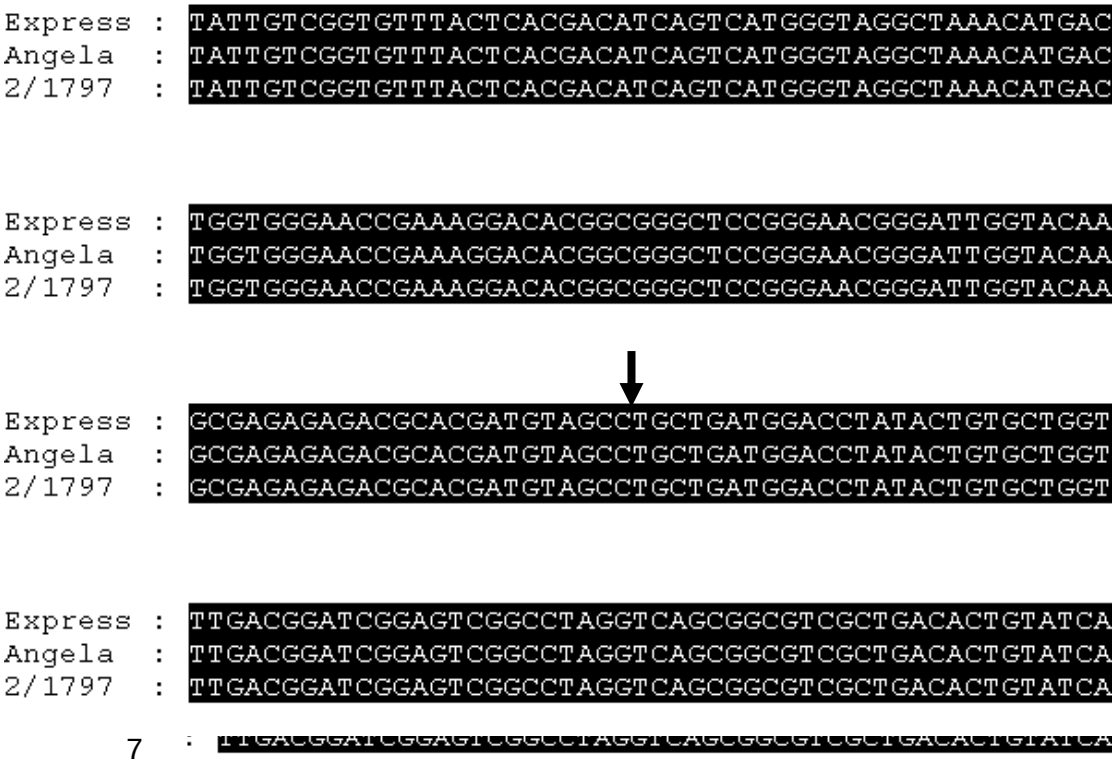


Figure 9. partial alignments of *VRN-H1* intron 1 between the winter variety 'Express' (haplotype 5C) and two 'alternative' varieties (coded 1 and 7). The position of the 0.5 kb deletion, relative to the winter *vrn-H1* allele from variety 'Strider', is indicated by the arrow. Amplification and sequencing primers: F = 5'- CTTGCATGTGTTGTCGGTCT-3', R = 5'-GCTGGGACAAGACTCTACGG-3'.

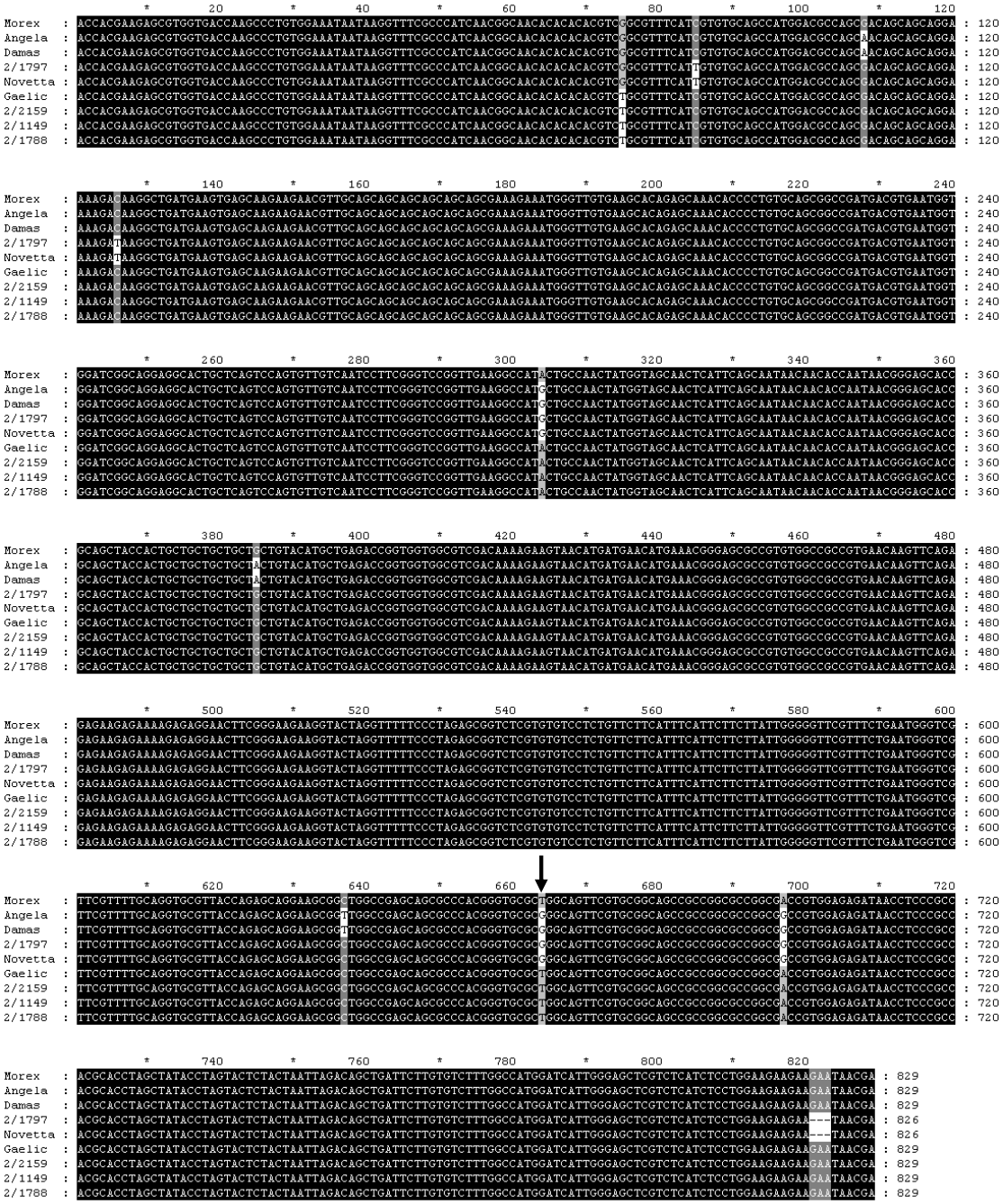


Figure 10. Partial sequencing of the photoperiod response gene, *PPD-H1* in the "alternative" barley varieties. Sequences shown are from +2,418 bp to +3,246 bp, relative to variety 'Morex' (AY943294). The arrow highlights the G → T SNP reported by Turner *et al.* 2005 to determine photoperiod responsive and non-responsive *PPD-H1* alleles, respectively. Amplification and sequencing primers: F = 5'-GATGAAGCAGGGCTCTAACG-3'. R = 5'-CCGGCATGTTCTATGGTAGG-3'.

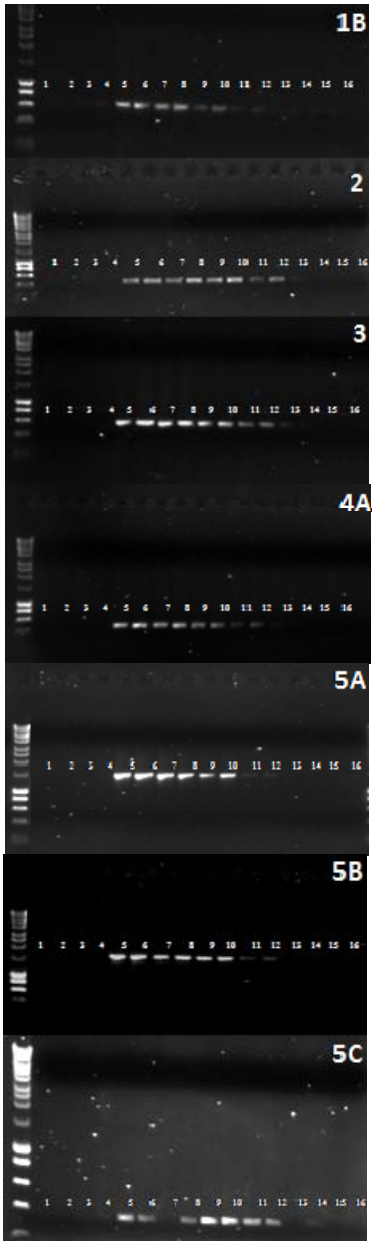


Figure 11. Testing the sensitivity of primer pairs specific to each of the six *VRN-H1* intron 1 deletion/insertions that result in spring alleles (haplotypes 1B, 2, 3, 4A, 5A and 4B), and the winter haplotype 5C (which is distinguished from the 1A haplotype by a 0.5 kb deletion). DNA from the winter variety 'Flagon' (haplotype 1A) were spiked with a series of serial DNA dilutions made from selected spring varieties belonging to each of the six major intron 1 deletion/insertion classes, as well as winter haplotype 5C. Spiked DNAs ranged from no dilution (40ng ±2ng) to a maximum of dilution of 1/10,000 (0.004ng). Lanes 1+2: water negative control; lanes 3+4: 40 ng Flagon DNA, lanes 5+6: spike (40ng ±2ng); lanes 7+8: 40ng Flagon + spike 1 (40ng); lanes 9+10: 40ng Flagon + spike 2 (4ng); lanes 11+12: 40ng Flagon + spike 3 (0. 4ng); lanes 13+14: 40ng Flagon + spike 4 (0.04ng); lanes 15+16: 40ng Flagon + spike 5 (0.004ng). The *VRN-H1* haplotype of each spike is indicated in the top right of each panel. DNA was extracted from the following exemplar varieties: haplotype 1A ('Flagon'), 1B ('Etu'), 2 ('Varunda'), 3 ('Dandy'), 4A ('Blenheim'), 5A ('Golf'), 5B ('Oriol'), 5C ('Express').

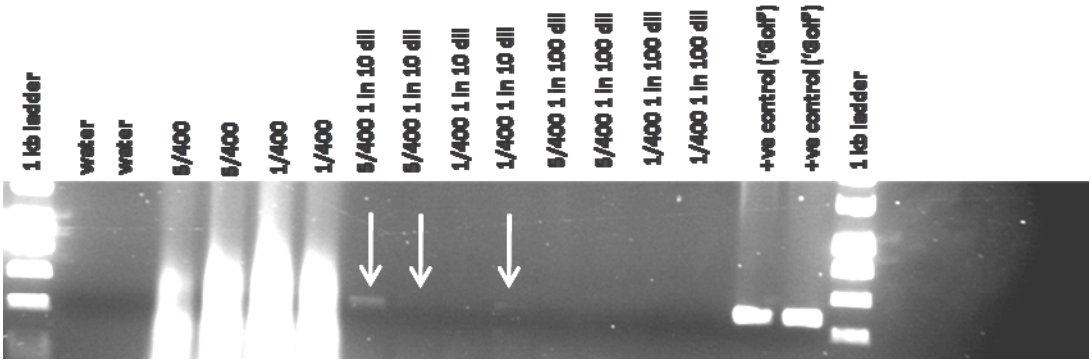


Figure 12. PCR amplification from DNA extracted from ground seed, using primers that assay for the spring haplotype 5A *VRN-H1* allele. 5/400 = 5 seeds of spring variety 'Golf' (*VRN-H1* haplotype 5A) with 395 seeds of the winter variety 'Prelude' (haplotype 1A). 1/400 = 1 seed 'Golf' in 399 seeds 'Prelude'. DNA dilutions as indicated. PCR primers as listed in Table 4. Negative (water) and positive (40ng DNA from spring variety 'Golf') are indicated. At this resolution, the low quality of the DNA extraction obscures any amplification in lanes 3 to 6 (although at lower contrast, it is possible to see that no amplification was achieved using the undiluted template DNA).

Name	AFP	SGH	VRNH1_Mpx	VRN-H2 Mpx	HvFT3_Mpx	PPDH1 T+3081/G	Name	AFP	SGH	VRNH1_Mpx	VRN-H2 Mpx	HvFT3_Mpx	PPDH1 T+3081/G
M OTTER	232	winter	1A	1	0	U	AYANA	1344	winter	1A	1	0	G
HALCYON	574	winter	1A	1	0	U	ASKANOVA	1346	winter	1A	1	0	U
PIPKIN	578	winter	1A	1	0	U	CHINTZ	1377	winter	1A	1	0	T
PUFFIN	806	winter	1A	1	0	U	GOLDRUSH	1381	winter	1A	1	0	G
PASTORAL	816	winter	1A	1	0	U	OLERON	1384	winter	1A	1	0	U
GAULOIS	827	winter	1A	1	1	G	BECKET	1385	winter	1A	1	0	T
GYPSY	884	winter	1A	1	0	T	FLUTE	1386	winter	1A	1	0	G
CLARINE	899	winter	1A	1	0	G	CHORD	1387	winter	1A	1	0	G
FIGHTER	947	winter	1A	1	0	U	MAHOGANY	1388	winter	1A	U	0	G
MANITOU	951	winter	1A	1	0	U	BREEZE	1389	winter	1A	1	0	G
SPRITE	954	winter	1A	1	0	G	MAGNOLIA	1390	winter	1A	1	0	G
BRONZE	994	winter	1A	1	0	G	BISTRO	1392	winter	1A	1	0	G
BLANCHE	1002	winter	1A	1	0	U	MUSETTE	1394	winter	1A	1	0	G
CHESTNUT	1052	winter	1A	1	0	G	SEVILLA	1396	winter	1A	1	0	G
WILLOW	1055	winter	1A	1	0	G	ETHNO	1398	winter	1A	1	0	G
SWIFT	1058	winter	1A	1	0	T	HELIGAN	1402	winter	1A	1	0	G
FIREFLY	1059	winter	1A	1	0	G	MASQUERADE	1403	winter	1A	1	0	G
INTRO	1071	winter	1A	1	0	U	TABETHA	1404	winter	1A	1	0	G
EPIC	1126	winter	1A	1	0	U	MOLLY	1405	winter	1A	1	0	G
LINNET	1127	winter	1A	1	0	T	OPAL	1435	winter	1A	1	0	T
MELANIE	1160	winter	1A	1	0	T	MARINER	1436	winter	1A	1	0	T
DUET	1161	winter	1A	1	0	T	ANTONIA	1438	winter	1A	1	0	G
DUET	1161	winter	1A	1	0	T	HURRICANE	1441	winter	1A	1	0	G
SUNRISE	1162	winter	1A	1	0	G	WIZARD	1442	winter	1A	1	0	G
RHYTHM	1169	winter	1A	1	0	G	STEEPLE	1443	winter	1A	1	0	U
TEMPO	1170	winter	1A	1	1	G	ZULU	1444	winter	1A	1	0	G
FANFARE	1171	winter	1A	1	0	U	ARROW	1445	winter	1A	1	0	T
MADRIGAL	1172	winter	1A	1	0	G	DAMAS	1450	winter	1A	1	0	G
VIVALDI	1173	winter	1A	1	U	U	VILNA	1451	winter	1A	1	0	G
ELECTRON	1179	winter	1A	1	0	T	MILANO	1453	winter	1A	1	0	G
TOSCA	1181	winter	1A	1	0	G	ROUNDER	1457	winter	1A	1	0	G
GAZELLE	1182	winter	1A	1	0	G	SIBERIA	1463	winter	5C	1	0	U
EMERAUDE	1183	winter	1A	1	0	G	HALIFAX	1466	winter	1A	1	0	G
REGINA	1204	winter	1A	1	0	U	ARTIST	1468	winter	1A	1	0	G
TUDOR	1206	winter	1A	1	0	T	MASAI	1469	winter	1A	1	0	G
DRUID	1208	winter	1A	1	0	G	LEONIE	1471	winter	1A	1	0	T
PUNCH	1209	winter	1A	1	0	G	WHISPER	1506	winter	1A	1	0	G
TOKYO	1210	winter	1A	1	0	T	CYNTHIA	1507	winter	1A	1	0	U
MEDOC	1212	winter	1A	1	0	G	ANVIL	1511	winter	1A	1	0	T
JEREZ	1213	winter	1A	1	0	G	GOLDMINE	1527	winter	1A	1	0	G
MUSCAT	1214	winter	1A	1	0	G	HAKA	1528	winter	1A	1	0	U
PACIFIC	1215	winter	1A	1	0	U	HARLAND	1530	winter	1A	1	0	T
MYSTIQUE	1219	winter	1A	1	0	G	CAMPION	1531	winter	1A	1	0	G
PRELUDE	1221	winter	1A	1	0	U	CHAMOMILE	1532	winter	1A	1	0	G
VANILLA	1224	winter	1A	1	0	G	SARAH	1534	winter	1A	1	0	T
GLEAM	1228	winter	1A	1	0	G	CAROLA	1535	winter	1A	1	0	U
KITE	1259	winter	1A	1	0	T	WEAVER	1536	winter	1A	1	0	G
FALCON	1260	winter	1A	1	0	G	VIOLET	1540	winter	1A	1	0	U
VOLLEY	1262	winter	1A	1	0	G	MONTAGE	1541	winter	1A	1	0	G
RIFLE	1263	winter	1A	1	0	T	SUMO	1547	winter	1A	1	0	T
COBALT	1264	winter	1A	1	0	U	BARCELONA	1552	winter	1A	1	0	T
TIFFANY	1266	winter	1A	1	0	T	PIPPA	1599	winter	1A	1	0	T
CREDO	1267	winter	1A	1	0	G	DIAMOND	1604	winter	1A	1	0	T
GLINT	1271	winter	1A	1	0	G	SAPPHIRE	1605	winter	1A	1	0	T
ANTIGUA	1276	winter	1A	1	0	G	CARAT	1606	winter	1A	1	0	G
EMILIA	1281	winter	1A	1	0	U	TUCKER	1608	winter	1A	1	0	G
PILOT	1283	winter	1A	1	0	G	TIPSTER	1610	winter	1A	1	0	G
SPICE	1285	winter	1A	1	0	T	CHICANE	1617	winter	1A	1	0	T
PORTRAIT	1287	winter	1A	1	0	G	MOONSHINE	1619	winter	1A	1	0	G
CRESCENDO	1316	winter	1A	1	0	G	VESUVIUS	1621	winter	1A	1	0	G
RAVEL	1317	winter	1A	1	0	G	GODIVA	1623	winter	1A	1	0	G
PEARL	1318	winter	1A	1	0	U	MILENA	1624	winter	1A	1	0	G
JEWEL	1320	winter	1A	1	0	G	PICT	1626	winter	1A	1	0	G
HONEY	1324	winter	1A	1	0	G	SCYLLA	1628	winter	1A	1	0	T
LARK	1325	winter	1A	1	0	G	CAPTION	1630	winter	1A	1	0	G
BATON	1326	winter	1A	1	0	G	SPINNER	1643	winter	1A	1	0	T
TOFFEE	1327	winter	1A	1	0	G	ADONIS	1666	winter	1A	1	1	T
TURINE	1330	winter	1A	1	0	G	JESSICA	1693	winter	1A	1	0	T
SPIRIT	1334	winter	1A	1	0	T	SUNBEAM	1697	winter	1A	1	0	T
WINNER	1339	winter	1A	1	0	G	CLARA	1698	winter	1A	1	0	T

Name	AFP	SGH	VRNH1_Mpx	VRN-H2 Mpx	HvFT3_Mpx	PPDH1 T+3081/G	Name	AFP	SGH	VRNH1_Mpx	VRN-H2 Mpx	HvFT3_Mpx	PPDH1 T+3081/G
CANNOCK	1700	winter	1A	1	0	T	SEQUEL	1717	winter	5C	1	0	G
SAIGON	1702	winter	1A	1	0	T	PARASOL	1730	winter	5C	1	0	G
KESTREL	1705	winter	1A	1	0	G	COLIBRI	2003	winter	5C	1	0	G
SWALLOW	1706	winter	1A	1	0	G	1		alternative	5C	1	0	G
SOMBRERO	1708	winter	1A	1	0	G	2		alternative	1A	1	0	G
EDEN	1709	winter	1A	1	0	T	3		alternative	5A	1	0	T
PEDIGREE	1715	winter	1A	1	0	G	4		alternative	1A*	0	0	G
SEQUEL	1717	winter	5C	1	0	U	5		alternative	1A*	1	0	T
ANTELOPE	1721	winter	1A	1	0	G	6		alternative	5A	1	0	T
CATHAY	1724	winter	1A	1	0	G	7		alternative	5C	0	0	G
OUTLOOK	1726	winter	1A	1	0	G	8		alternative	1A*	0	0	T
LOMERIT	1727	winter	1A	1	0	G	G PROMISE	235	spring	S	0	1	U
MEAD	1728	winter	1A	1	0	G	TRIUMPH	278	spring	S	0	1	U
CELLINA	1731	winter	1A	1	0	T	GOLF	421	spring	S	0	1	U
SW FARRIER	1733	winter	1A	1	0	G	KLAXON	456	spring	S	1	1	T
LOUISE	1783	winter	1A	1	0	G	DIGGER	597	spring	S	0	1	T
SW HILLARY	1786	winter	1A	1	0	G	DANDY	674	spring	S	0	1	U
CONNOISSEUR	1790	winter	1A	1	0	G	BLENHEIM	688	spring	S	0	1	U
NOCTURNE	1792	winter	1A	1	0	G	TYNE	750	spring	S	1	1	T
WOMBAT	1793	winter	1A	1	0	G	ALEXIS	904	spring	S	0	1	U
WIGWAM	1794	winter	1A	1	0	G	DERKADO	1011	spring	S	1	1	U
KINGSTON	1799	winter	1A	1	0	G	DALLAS	1022	spring	S	0	1	T
LAMBADA	1808	winter	1A	1	0	T	GRAPHIC	1029	spring	S	0	1	U
MARITEM	1814	winter	1A	1	0	T	CHARIOT	1031	spring	S	0	1	U
TALLICA	1820	winter	1A	1	0	G	FELICE	1091	spring	S	0	1	T
MORTIMER	1824	winter	1A	1	0	G	HERON	1107	spring	S	0	1	T
CAMION	1825	winter	1A	1	0	G	DELIBES	1133	spring	S	0	1	T
SAFFRON	1880	winter	1A	1	0	G	COOPER	1146	spring	S	0	1	U
ELMSTEAD	1884	winter	1A	1	0	G	BREWSTER	1147	spring	S	0	1	U
MERODE	1885	winter	1A	1	0	T	CORK	1187	spring	S	0	1	U
AMARENA	1886	winter	1A	1	0	T	OPTIC	1188	winter	S	0	1	U
FARADAY	1892	winter	1A	1	0	G	BRAHMS	1191	spring	S	0	1	T
CORIOLIS	1894	winter	1A	1	0	T	CANASTA	1194	spring	S	0	1	T
NECTARIA	1899	winter	1A	1	0	G	REGGAE	1196	spring	S	1	1	T
REGALIA	1903	winter	1A	1	0	G	JIVE	1201	spring	S	1	1	T
CALLIOPE	1907	winter	1A	1	0	T	GLEN	1202	spring	S	1	1	U
IMOGEN	1908	winter	1A	1	0	G	RIVIERA	1203	spring	S	1	1	T
FLAGON	1910	winter	1A	1	0	U	ONYX	1232	spring	S	U	1	T
RATTLE	1914	winter	1A	1	0	T	LAIRD	1236	spring	S	0	1	T
THALIA	1919	winter	1A	1	0	G	CHIEFTAIN	1237	spring	S	1	1	T
HERMIA	1920	winter	1A	1	0	T	TANKARD	1238	spring	S	1	1	U
CONCEPT	1923	winter	1A	1	0	T	PITCHER	1239	spring	S	0	1	T
SPECTRUM	1924	winter	1A	1	0	G	WREN	1242	spring	S	0	1	T
CHARLESTON	1980	winter	1A	1	0	T	RAGTIME	1244	spring	S	1	1	T
HOUSTON	1981	winter	1A	1	0	T	PRIMERA	1245	spring	S	0	1	U
CELEBRITY	1982	winter	1A	1	0	T	CLARITY	1246	spring	S	0	1	T
CYPRESS	1983	winter	1A	1	0	G	MIKADO	1248	spring	S	0	1	T
CINNAMON	1984	winter	1A	1	0	G	MELITTA	1250	spring	S	0	1	T
DOLPHIN	1985	winter	1A	1	0	G	TARDUS	1253	spring	S	0	1	T
MALWINTA	1987	winter	1A	1	0	T	POLYGENA	1255	spring	S	0	1	T
ARCHIMEDES	1989	winter	1A	1	0	G	TRINIDAD	1256	spring	S	0	1	T
CELSIUS	1990	winter	1A	U	0	T	QUARTET	1291	spring	S	0	1	T
FAHRENHEIT	1991	winter	1A	1	0	G	LANDLORD	1293	spring	S	0	1	T
MOSAIC	1996	winter	1A	1	0	G	HOST	1295	spring	S	0	1	T
SW NORMA	2002	winter	1A	1	0	T	TODDY	1298	spring	S	0	1	T
SILVERSTONE	2017	winter	1A	1	0	T	RAINBOW	1301	spring	S	U	1	T
SURTEES	2021	winter	1A	1	0	T	CHARM	1303	spring	S	0	1	T
CASSATA	2058	winter	1A	1	0	T	TORUP	1311	spring	S	0	1	T
RETRIEVER	2065	winter	1A	1	0	U	RENATA	1312	spring	S	0	1	T
ACCENT	2073	winter	1A	1	0	T	TROSA	1313	spring	S	0	1	T
BLYTHE	2076	winter	1A	1	0	T	LIVET	1349	spring	S	0	1	T
PELICAN	2077	winter	1A	1	0	U	RICARDA	1350	spring	S	0	1	T
SUZUKA	2083	winter	1A	1	0	U	DRAY	1353	spring	S	0	1	T
CEDAR	2088	winter	1A	1	0	G	CHALICE	1354	spring	S	0	1	T
ARCTIC	1211	winter	5C	1	0	G	FERMENT	1356	spring	S	0	1	T
CROWN	1230	winter	5C	1	0	G	ARDILA	1363	spring	S	1	1	T
MADISON	1282	winter	5C	1	0	G	MADRAS	1364	spring	S	0	1	T
CANDY	1345	winter	5C	1	0	G	SPEY	1376	spring	S	0	1	T
SIBERIA	1463	winter	5C	1	0	G	ASPEN	1409	spring	S	0	1	U
TROPIC	1642	winter	5C	1	0	U	CENTURY	1410	spring	S	0	1	U

Name	AFP	SGH	VRNH1_Mpx	VRN-H2 Mpx	HvFT3_Mpx	PPDH1 T+3081/G	Name	AFP	SGH	VRNH1_Mpx	VRN-H2 Mpx	HvFT3_Mpx	PPDH1 T+3081/G
DECANTER	1411	spring	S	0	1	T	VORTEX	1764	spring	S	1	1	T
LINDEN	1415	spring	S	0	1	U	NOVELLO	1768	spring	S	0	1	U
THISTLE	1416	spring	S	0	1	T	COCKTAIL	1769	spring	S	0	1	T
MANDOLIN	1418	spring	S	0	1	T	SEBASTIAN	1770	spring	S	0	1	T
ACAPELLA	1419	spring	S	1	1	T	SW STELLA	1773	spring	S	0	1	T
SPLASH	1420	spring	S	1	1	T	MAYPOLE	1775	spring	S	0	1	T
HORIZON	1423	spring	S	0	1	T	CLASS	1777	spring	S	0	1	T
SPIRAL	1424	spring	S	0	1	T	BRAZIL	1780	spring	S	0	1	T
SABEL	1426	spring	S	0	1	T	PERTH	1834	spring	S	0	1	T
STATIC	1427	spring	S	0	1	T	GRANTA	1835	spring	S	0	1	T
PONGO	1431	spring	S	0	1	T	VELVET	1836	spring	S	0	1	U
BERWICK	1474	spring	S	1	1	T	MACARENA	1837	spring	S	0	1	U
OTIRA	1485	spring	S	1	1	U	AMOURETTE	1842	spring	S	1	1	T
STARLIGHT	1488	spring	S	0	1	T	SKAGEN	1859	spring	S	0	1	T
CINDY	1490	spring	S	0	1	U	CARAFE	1862	spring	S	0	1	U
POTTER	1492	spring	S	0	1	T	ATHENA	1863	spring	S	0	1	T
FOXTROT	1495	spring	S	1	1	T	TOBY	1864	spring	S	0	1	T
ANNABELL	1496	spring	S	U	1	T	DOYEN	1865	spring	S	0	1	U
TAVERN	1498	spring	S	1	1	T	DRUM	1867	spring	S	0	1	T
CHASER	1499	spring	S	0	1	T	REBECCA	1871	spring	S	0	0	U
COLADA	1500	spring	S	0	1	T	INDOLA	1874	spring	S	1	1	T
SALOON	1501	spring	S	1	1	T	FELTWELL	1875	spring	S	0	1	T
THRIFT	1554	spring	S	0	1	T	POWER	1933	spring	S	0	1	T
AKITA	1555	spring	S	0	1	T	HENLEY	1936	spring	S	0	1	U
DEW	1556	spring	S	0	1	T	MINSTREL	1937	spring	S	0	1	T
ALLIOT	1563	spring	S	0	1	T	WESTMINSTER	1939	spring	S	0	1	T
ASTORIA	1565	spring	S	0	1	T	OXFORD	1940	spring	S	0	1	U
PRESTIGE	1566	spring	S	0	1	U	LITHIUM	1946	spring	S	0	1	T
PROTÉGÉ	1567	spring	S	0	1	U	SW SCANIA	1950	spring	S	0	1	T
COUNTY	1572	spring	S	0	1	U	SW MACSENA	1951	spring	S	1	1	T
CELLAR	1574	spring	S	0	1	T	MACAW	1955	spring	S	0	1	T
PEWTER	1575	spring	S	0	1	T	TOUCAN	1957	spring	S	U	1	T
BRISE	1576	spring	S	1	1	T	URSA	1961	spring	S	0	1	T
VISKOSA	1579	spring	S	1	1	T	WAGGON	1963	spring	S	0	1	U
NERUDA	1580	spring	S	0	1	T	NFC TIPPLE	1966	spring	S	0	1	U
AGENDA	1589	spring	S	0	1	U	WICKET	1968	spring	S	0	1	T
AZURE	1591	spring	S	0	1	T	TIMORI	1974	spring	S	0	1	T
FONTANA	1594	spring	S	0	1	T	CORSICA	2025	spring	S	1	1	T
AVEC	1595	spring	S	1	1	T	APPALOOSA	2030	spring	S	0	1	T
ALABAMA	1597	spring	S	0	1	T	HYDRA	2031	spring	S	1	1	T
SACHA	1651	spring	S	0	1	T	RUMMY	2034	spring	S	0	1	T
VEGAS	1659	spring	S	0	1	T	SKITTLE	2035	spring	S	0	1	T
SPIRE	1668	spring	S	0	1	T	POKER	2036	spring	S	0	1	T
SPIKE	1669	spring	S	0	1	T	SILICON	2040	spring	S	0	1	T
CELEBRA	1673	spring	S	1	1	T	KASSIMA	2042	spring	S	0	1	U
HARRIOT	1676	spring	S	0	1	T	TUCSON	2050	spring	S	0	1	T
WIDRE	1679	spring	S	1	1	T	CENTURION	2054	spring	S	0	1	T
WEITOR	1680	spring	S	0	1	T	PARAMOUNT	2055	spring	S	0	1	T
WIKINGETT	1681	spring	S	1	1	T	TARTAN	2092	spring	S	0	1	T
HEATHER	1682	spring	S	0	1	T	MONIKA	2098	spring	S	0	1	T
ANAIS	1687	spring	S	1	1	T	ACROBAT	2102	spring	S	0	1	T
TABORA	1688	spring	S	0	1	T	TOKEN	2103	spring	S	0	1	T
RAKAIA	1741	spring	S	0	1	T	CALICO	2104	spring	S	0	1	T
KIRSTY	1748	spring	S	0	1	T	PUBLICAN	2119	spring	S	0	1	T
TROON	1755	spring	S	0	1	T	QUENCH	2121	spring	S	0	1	T
RANGOON	1758	spring	S	U	1	T	TAPHOUSE	2122	spring	S	0	1	T
GLOBAL	1761	spring	S	0	1	T	PENTHOUSE	2123	spring	S	0	1	T
CAMPALA	1762	spring	S	0	1	T	PRAGUE	2130	spring	S	0	1	T

Table 1. Genotypes of *VRN-H1, VRN-H2, PPD-H1* and *PPD-H2* in 396 United Kingdom barley varieties. *VRN-H1*: S = spring allele, 1A and 5C = winter allele, 1A* - 1A haplotype carrying an as yet uncharacterized Intron I indel polymorphism. *VRN-H2*: 1 = presence of *ZCCT-Ha, -Hb, -Hc*, 0 = absence of *ZCCT-Ha, -Hb, -Hc*. *PPD-H1*: G = long-day photoperiod responsive allele, T = long-day non-responsive allele. *PPD-H2*: 1 = presence of *HvFT3*, 0 = absence of *HvFT3*.

	VRN-H1 Mpx	SNP1	SNP2	SNP3	SSR	Del	SNP4	SNP5	SNP6	VRN-H2	haplotype	predicted pheno	PPD-H1	PPD-H2
Strider	1A	T	A	C	4	0	T	G	C	+Z	1A+Z	Winter		
2	1A	T	A	C	4	0	T	G	C	+Z	1A+Z	Winter	G	0
5	1A	T	A	C	4	0	T	G	C	+Z	1A+Z	Winter	T	0
8	1A	T	A	C	4	0	T	G	C	-Z	1A-Z	weak spring	T	0
4	1A	T	A	C	4	0	T	G	C	-Z	1A-Z	weak spring	G	0
Express	5C	T	G	C	5	5C	C	A	G	+Z	5C+Z	Winter		
1	5C	T	G	C	5	5C	C	A	G	+Z	5C+Z	Winter	G	0
7	5C	T	G	C	5	5C	C	A	G	-Z	5C-Z	weak spring	G	0
3	S	T	G	C	5	5A	C	A	G	+Z	5A+Z	Spring	T	0
6	S	T	G	C	5	5A	C	A	G	+Z	5A+Z	Spring	T	0

Table 2. Haplotyping of eight 'alternative' varieties (coded 1 to 8), according to the protocols described by Cockram *et al.* (2007). *VRN-H1* multiplex assay as described in Figure 2. Major *VRN-H1* intron 1 deletions are coded as follows: 0 = no deletion (winter haplotype 1A); 5C = 0.5kb deletion (winter haplotype 5C); S = deletion of unspecified size (spring *Vrn-H1* allele). $(CGCT)_n$ SSR coding: 4 = $(CGCT)_4$; 5 = $(CGCT)_5$. *ZCCT-H* (diagnostic for allelic status at *VRN-H2*) scores: +Z = presence of *ZCCT-Ha, -Hb, -Hc* (winter allele); -Z = absence of *ZCCT-Ha, -Hb, -Hc* (spring allele). The genotypes of the winter 'Strider' and 'Express' *VRN-H1* alleles are shown for reference. *PPD-H1*, response to long-day photoperiods; G (responsive allele); T (non-responsive allele). *PPD-H2*, response to short-day photoperiods: 0 = responsive (delayed flowering in short-days).

	Primer Pair	1	2	3	4	5	6	7	8
1	Pr-1	Y	Y	Y	Y	Y	Y	Y	Y
2	Pr-2	N	N	N	N	N	N	N	N
3	Ex-1	Y	Y	Y	Y	Y	Y	Y	Y
4	In1-1	Y	Y	N	Y	Y	N	Y	Y
5	In1-2	Y	Y	N	N	N	N	Y	N
6	In1-3	N	Y	N	Y	Y	N	N	Y
7	In1-4	N	Y	N	Y	Y	N	N	Y
8	In1-5	Y	Y	N	Y	Y	N	Y	Y
9	In1-6		Y	N	Y	Y	N	Y	Y
10	In1-7		Y	N	Y	Y	N	Y	Y
11	In1-8		Y	Y	Y	Y	Y	Y	Y
12	In1-9		Y	Y	Y	Y	Y	Y	Y
13	In1-10		Y	Y	Y	Y	Y	Y	Y
14	In1-11		Y	Y	Y	Y	Y	Y	Y
15	In1-12		Y		Y	N	Y	N	Y
16	In1-13		Y		Y	Y	Y	Y	Y
17	Ex-2		Y		Y	Y	Y	Y	Y
18	In2-1		Y	Y	Y	Y	Y	Y	Y
19	In2-2		Y	Y	Y	Y	Y	Y	Y
20	In2-3	Y	Y	Y	Y	Y	Y	Y	Y
21	In2-4	Y	Y	Y	Y	Y	Y	Y	Y
22	E3-8	Y	Y	Y	Y	Y	Y	Y	Y
23	E8-3'UTR	Y	Y	Y	Y	Y	Y	Y	Y

Table 3. Application of the 23 PCR amplicons spanning the (approximate) 17kb winter *vrn-H1* allele to the eight "alternative" varieties. Y = amplification achieved; N = no amplification; blank = amplification not yet attempted. Failure of consecutive primer pairs to amplify indicates the position and approximate size of the putative deletions.

haplotype	Deletion size	Exemplar variety	Forward primer (5' to 3')	Reverse primer (5' to 3')	PCR conditions
1A (W)	0 kb	Flaggon	CTTGCATGTGTTGTCGGTCT	GCTGGGACAAGACTCTACGG	(50'50'50')x36 @ 61C
1B (S)	3.9 kb	Etu	GTTCTCCACCGAGTCATGGT	CCTTGAGTCATAGCCCACCA	(50'50'50')x36 @ 61C
2 (S)	0.7kb insertion	Varunda	CTTGCATGTGTTGTCGGTCT	AGAGATGGAGGCATGGAGCA	(50'50'50')x36 @ 63C +GC buffer
3 (S)	6.4 kb	Dandy	GGCGATGGTTCTTGACAAAG	CGGATGGTTCCTGAGGATATT	(50'50'50')x36 @ 61C
4A (S)	8.9 kb	Blenheim	TAGGCGCTAGAATACTTCGT	CCGTTCAAGAATTTTGTCCA	(50'50'50')x36 @ 61C
4B, 5A (S)	5.2 kb	Golf	GTTCTCCACCGAGTCATGGT	GGGCGGAGTACTGCTACAAC	(50'50'50')x36 @ 61C
5B (S)	4.1 kb	Oriol	GGCGATGGTTCTTGACAAAG	GGGCGGAGTACTGCTACAAC	(50'50'50')x36 @ 61C
5C (W)	0.5 kb	Express	CTTGCATGTGTTGTCGGTCT	GCTGGGACAAGACTCTACGG	(20'20'15')x36 @ 61C

Table 4. Primers and optimal PCR conditions for detection of *VRN-H1* off-types. S = spring allele; W = winter allele.

[End of Annex and of document]