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# Use of genetic markers for the detection of off-types for DUS phenotypic traits in the inbreeding crop, barley

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**Abstract** Detection of crop off-types is of interest for multiple uses, including the assessment of uniformity for new plant variety applications during distinctness, uniformity and stability (DUS) testing for the awarding of plant breeders' rights (PBR). Here, we investigate whether genetic markers, in this case Kompetitive Allele-Specific PCR (KASP), can be used for the identification off-types for phenotypes assessed for DUS in the inbreeding cereal crop, barley (*Hordeum vulgare*). To demonstrate proof of principle, KASP markers diagnostic for phenotypic expression of nine DUS phenotypes, and DNA from two barley varieties ('Pelican' and 'Felicie') carrying contrasting alleles at each marker were used. We found that for the majority of markers, it was possible to robustly call alleles down to template DNA concentrations of 2 ng, but not ≤ 0.2 ng. When used in mixtures of DNA consisting of 'Felicie' DNA spiked with different concentrations of 'Pelican' DNA, robust allele calling was possible in DNA mixtures down to 18 ng:2 ng. Collectively, this demonstrates that where diagnostic markers are available, molecular identification of a single off-type for a given DUS trait within a bulk of ten individuals should be possible. We validated this assumption, with all of the diagnostic genetic markers investigated found to robustly detect DUS off-types at a frequency of 10% in DNA extracted from tissue collected from pools of 10 individuals. Ultimately, this work demonstrates that, where diagnostic polymorphisms are known for DUS traits, KASP markers should be able to robustly detect off-types or cross-contamination within DNA samples from a diploid inbred species down to 10%. While just two varieties that contrasted for the eight DUS targeted were investigated in this study, as the markers used are diagnostic for their relevant phenotype (or a proportion of the variation observed for that phenotype), in theory the approach should be valid for any variety studied— although the introduction of novel alleles via spontaneous mutation or more exotic germplasm pools may mean that marker sets would need to be periodically added to or updated. However, we nevertheless demonstrate the principle that, for a subset of DUS traits, molecular markers can now be robustly used as a tool towards determining all three components of the DUS testing process in barley. These results are relevant for the assessment of varietal uniformity by crop breeders, crop testing authorities and germplasm maintenance, as well as highlighting the potential use of bulk samples rather than individual plant samples for assessment of distinctness by molecular methods.

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## Introduction

The diploid inbreeding crop species barley (*Hordeum
vulgare* L.) is one of the world's most important cereal
crops. The development of new barley varieties with
improved performance takes around 10 years, requiring
considerable investment by crop breeders. The
awarding of Plant Variety Protection (PVP) allows
breeding companies to protect the investment they make
in the development of new crop varieties. The rules
under which PVP is granted are governed by the Inter-
national Union for the Protection of New Varieties of
Plants (UPOV). Currently, seventy-five countries or
intergovernmental agencies are signatories of the UPOV
Convention. These members all acknowledge the
breeders of new varieties of plants by granting them
intellectual property rights, based on the varietal sub-
missions being: 1. New, i.e. not commercialised before.
2. Distinct (D) from existing varieties. 3. Uniform (U),
i.e. sufficiently uniform in the phenotypic traits with
which D is assessed. 4. Stable (S), i.e. the phenotypic
traits assessed remain unchanged after repeated propa-
gation (UPOV Convention Articles 5–9 (UPOV 1991)).
The system used to establish distinctness, uniformity
and stability is known as DUS testing. In line with the
test guidelines agreed by UPOV members (UPOV TG/
19/10), barley DUS testing involves phenotypic evalu-
ation of 28 traits (Supplementary Table 1), undertaken
over two separate growing seasons. Due to the require-
ment of all new varieties to be D, U and S, irrespective
of how well they perform agronomically, breeding com-
panies are interested in using genetic markers to help
ensure their candidate varieties meet the DUS criteria
that are necessary for them to ultimately be released as
commercial varieties protected by PVP. Using 500 bar-
ley varieties, combined with phenotypic data for all 28
barley DUS traits and a 1500 feature single nucleotide
polymorphism (SNP) array, we previously undertook
genome-wide association scans (GWAS) for barley
DUS traits, identifying genetic loci controlling many
of the phenotypes investigated (Cockram et al. 2010).
For a selected trait (the presence or absence of the
pigment anthocyanin), we demonstrated that GWAS

could be used to fine-map genetic loci controlling
DUS traits. Anthocyanin presence/absence was found
to be controlled by the *ANTHOCYANINLESS 2* (*ANT2*)
locus, with GWAS delimiting a physical region that
contained three genes, including a strong candidate gene
encoding a basic helix-loop-helix (bHLH) domain pro-
tein. Population resequencing showed that all varieties
that lacked anthocyanin pigmentation carried a 16 bp
deletion resulting in a truncation of the predicted protein
upstream of the bHLH domain.

The genes and allelic variants controlling three other
barley DUS traits are also known. (1) Two major loci
control the DUS trait 'seasonal type' (UPOV character
29): *VERNALIZATION-H1* (*VRN-H1*) and *VRN-H2*.
Intronic deletions within *VRN-H1* are thought to confer
the mutant vernalisation insensitive 'spring' alleles
(Cockram et al. 2007a, b, 2009; Szűcs et al. 2007;
Hemming et al. 2009; Trevaskis 2010), while deletions
spanning three duplicated genes (*ZCCT-Ha*, *-Hb*, *-Hc*)
confer mutant vernalisation insensitive 'spring' alleles at
*VRN-H2* (Yan et al. 2004; Karsai et al. 2005; Distelfeld
et al. 2009). Barley varieties with the 'alternative' seasonal
type, which can be sown as either winter or spring types,
have characteristic allelic combinations at *VRN-H1*, *VRN-
H2* and a third locus, *PHOTOPERIOD REPSPONSE-H2*
(*PPD-H2*) (Cockram et al. 2015). (2) Two genes control-
ling the trait 'ear: number of rows' (UPOV 13) have been
map-based cloned. Depending on whether the two lateral
spikelets at each floret are infertile (wild type) or fertile
(mutant), barley varieties are described as 2- or 6-rowed,
respectively. Mutations at the *HvHOX1* gene underlying
the *VRS1* locus result in a 6-row phenotype (Komatsuda
et al. 2007), while partial filling of lateral spikelets is
controlled by allelic variation at the *TEOSINTINE
BRANCHED 1* (*TB1*) gene at the *INTERMEDIUM-C*
(*INT-C*) locus (Ramsay et al. 2011). (3) DUS trait 'grain:
disposition of lodicules' (UPOV 27) is controlled by the
*CLYSTOGAMY 1* (*CLY1*) locus, with mutations in the
underlying *HvAP2* transcription factor shown to result in
closed flowering due to the failure of lodicules to expand
(Nair et al. 2010). As well as these cloned genes, four
additional major genetic loci likely control the barley DUS
traits identified by Cockram et al. (2010): *HAIRY LEAF
SHEATH 1* (*HSH1,* Lundqvist et al. 1996) for trait 'lowest
leaves: hairiness of leaf sheaths' (UPOV 2), *SHORT
RACHILLA HAIR* (*SRH,* Franckowiak 1995) for 'grain:
rachilla hair type' (UPOV 22), *TOOTHED LEMMA 1*
(*GTH1*, Lundqvist et al. 1997) for 'grain: speculation of
inner lateral nerves of dorsal side of lemma' (UPOV 25),

and *BLX* (Lundqvist et al. 1996) for 'kernel: colour of aleurone layer' (UPOV 28).

The potential use of molecular markers within the DUS context is a source of ongoing consideration and debate (recently reviewed by Jamali et al. 2019). Diagnostic Kompetitive Allele-Specific PCR (KASP) markers for all of cloned genes controlling DUS traits, along with the major genetic loci identified by GWAS (Cockram et al. 2010), have previously been validated (Cockram et al. 2012). However, while these were shown to be able to discriminate between homozygous alleles (and therefore of use for the assessment of D and S), their ability to detect contrasting alleles in admixed DNA samples (i.e. the assessment of U) has not been determined. Here we investigate the possibility of using molecular approaches for the detection of DUS off-types in an inbreeding diploid crop species, using KASP markers diagnostic for allelic variation diagnostic for, or contributing to the control of, eight barley DUS traits. The DUS KASP markers tested were predominantly found to be sensitive down to concentrations of 2 ng, and all were able to robustly detect single off-types in template DNA extracted from 10 pooled individuals, indicating assessment of uniformity in the DUS context should be possible where the diagnostic polymorphisms underlying the targeted trait are known. The results are discussed with reference to DUS assessment and the release of new crop varieties.

## Methods

### Germplasm and plant growth

The barley varieties 'Felicie' (Application For Protection, AFP, number 2/1091) and 'Pelican' (AFP 2/2077) were sourced (with appropriate permission) from germplasm stocks maintained at NIAB. Seeds were sown in 96-well trays using M2 potting and bedding compost (ICL Levington Advance), and grown in a heated glasshouse, with 16 h light photoperiod maintained using supplemental lighting. DUS phenotypic data for these two varieties was sourced from their publicly available varietal descriptions.

### DNA template preparation

Genomic DNA was extracted from young leaf material following the Tanksley protocol (Fulton et al. 1995),

with the inclusion of 60 μl RNase A (Qiagen) in the microprep buffer. DNA quantity and quality were determined using a Nanodrop 200 spectrophotometer (Thermo Scientific). Starting DNA concentrations was standardised and subsequently used for: (1) serial dilutions, using PCR grade double-distilled water (Sigma-Aldrich), (2) generation of artificially admixed DNA samples, by mixing 'Felicie' and 'Pelican' DNAs in different proportions, to a total concentration of 20 ng/μl. Additionally, bulk DNA extractions were undertaken by sampling leaf material bulked from 10 individual plants (0.3 cm long × 0.5 cm wide per plant), with the proportion of 'Felicie' to 'Pelican' tissue samples in the bulk varying as shown in Supplementary Table 2. The concentration of each bulk DNA sample was standardized to 20 ng/μl using PCR grade double-distilled water.

### Genotyping

SNP genotyping was undertaken using previously described KASP markers (Cockram et al. 2012) that assay genetic loci diagnostic for nine DUS phenotypes, as identified by Cockram et al., (2010): Hv11_11299_GC (trait 'lowest leaves: hairiness of leaf sheaths'), HvOs03g14380_G125A ('kernel: colour of aleurone layer'), HvCly1_A2604G ('grain: disposition of lodicules'), HvANT2_C4289T ('anthocyanin pigmentation'), HvVRNH1_SNP2 ('seasonal habit'), HvPPDH1_A2721G ('long-day photoperiod response'), HvOs02g01490_G607A ('grain: hairiness of ventral furrow'), Hv11_10933_GC ('sterile spikelet attitude') and Hv11_10818_CA ('grain lateral nerve spiculation'). KASP primer sequences are listed in Supplementary Table 3. For each assay, reaction volumes were 2.5 μl KASP V4.0 2× Master Mix (LGC Biosciences), 0.07 μl KASP primer mix (see Supplementary Table 4) and 2.5 μl DNA template (or 2.5 μl PCR-grade water for negative controls). Genotyping was carried out using a ProFlex PCR System Thermocycler (Applied Biosystems) using the following conditions: 1 cycle at 94 °C for 15 min; 10 cycles at 94 °C for 20 s, 65 °C for 60 s with a touchdown of − 0.8 °C/cycle to 57 °C; 35 cycles at 94 °C for 20 s, 57 °C for 60 s; final hold at 10 °C. Fluorescence of VIC and FAM fluorophore 5′ end labelled PCR products were subsequently read using a Scientific QuantStudio™ 12 K Flex real-time PCR System (Thermo Fisher Scientific). ROX was used as a passive fluorescent reference to allow normalisation of variations in signal caused by differences in well-to-

well liquid volume, following the manufacturer's instructions (LGC Genomics). Four technical replicates were assayed for each DNA sample used. Results were visualised using SNP Viewer v2 (LGC Genomics) or with Excel (Microsoft).

## Results

### Barley varieties, DUS trait scores and diagnostic genetic markers

In order to investigate the ability and sensitivity of genetic markers to detect DUS off-types in barley, two UK varieties were selected that differed in their phenotypes for eight DUS traits for which relevant diagnostic or perfect genetic markers were available: 'Felicie' and 'Pelican'. The DUS traits and phenotypic state for each variety, along with details of the relevant KASP genetic markers, are listed in Table 1.

### Limits of diagnostic genetic marker sensitivity

To determine the lower limits of template DNA concentration at which the diagnostic genetic markers selected for study were able to robustly call SNPs, six 1-in-10

serial dilutions of 'Felicie' and 'Pelican' DNAs were made, starting with stock DNA at 20 ng/μl, with dilutions of 2, 0.2, 0.02, 0.002, 0.0002 and 0.00002 ng/μl. These were used as templates for KASP genotyping reactions with the nine diagnostic genetic markers (Fig. 1). Six KASP markers showed robust calling (i.e. clearly distinguishable from the negative water controls) with template concentrations of 2 ng (i.e. 1-in-10 DNA dilutions: Hv11_11299_GC, HvANT2_C4289T, Hv11_10933_GC, Hv11_10818_CA, HvOs02g01490_G607A and HvVRNH1_SNP2; Fig. 1 b, c, e–g, i). For the remaining three markers, robust calls at template concentrations down to 2 ng were only possible for one of the two alleles, as at this template level HvOs03g14380_G125A (relevant to DUS phenotype 'Kernel: colour of aleurone layer') could not clearly call 'Felicie' alleles, while HvPPDH1_A2721G ('Time of ear emergence') and HvCly1_A2604G ('Grain: disposition of lodicules') could not clearly call 'Pelican' alleles (Fig. 1).

### Detection of DUS off-types via in vitro admixture

Having determined the sensitivity limits of the diagnostic genetic markers selected for study to call alleles in the presence of template DNA from a single variety, the

Table 1 Genetic markers used to predict DUS phenotypes, along with information describing phenotypic trait expression and KASP allele for the two barley varieties investigated ('Felicie' and 'Pelican'), which contrast for all of the eight DUS phenotypes assessed via diagnostic genetic markers here. The limits of the assay to reliably call alleles at different template DNA concentrations are indicated. Fluorophores: V = VIC, F = FAM. [*]Allele-specific KASP primers assay base on opposite DNA strand. [†]Strongly. [$]Long day (16 h daylight) photoperiod sensitivity

| DUS trait | CPVO Trait No. | KASP marker | Trait expression Felicie | Felicie allele (fluorophore) | Trait expression Pelican | Pelican allele (fluorophore) | Sensitivity limit of marker (ng DNA) |
|---|---|---|---|---|---|---|---|
| Kernel: colour of aleurone layer | 1 | HvOs03g14380_G125A[*] | Whitish | C (F) | Coloured[†] | T (V) | < 20 > 2 |
| Hairiness of leaf sheaths | 3 | Hv11_11299_GC | Absent | G (V) | Present | C (F) | 2 |
| Auricle anthocyanin colouration | 4 | HvANT2_C4289T | Absent | C (F) | Very strong | T (V) | 2 |
| Time of ear emergence | 7 | HvPPDH1_A2721G | Insensitive[$] | A (F) | Sensitive[$] | G (V) | < 20 > 2 |
| Sterile spikelet: attitude | 20 | Hv11_10933_GC | Divergent | C (V) | Parallel | G (F) | 2 |
| Grain: speculation of lateral nerves | 25 | Hv11_10818_CA | Absent | A (F) | Medium | C (V) | 2 |
| Grain: hairiness of ventral furrow | 26 | HvOs02g01490_G607A | Present | A (V) | Absent | G (F) | 2 |
| Grain: disposition of lodicules | 27 | HvCly1_A2604G | Frontal | G (V) | Clasping | A (F) | < 20 > 2 |
| Seasonal growth type | 28 | HvVRNH1_SNP2 | Spring | G (V) | Winter | A (F) | 2 |
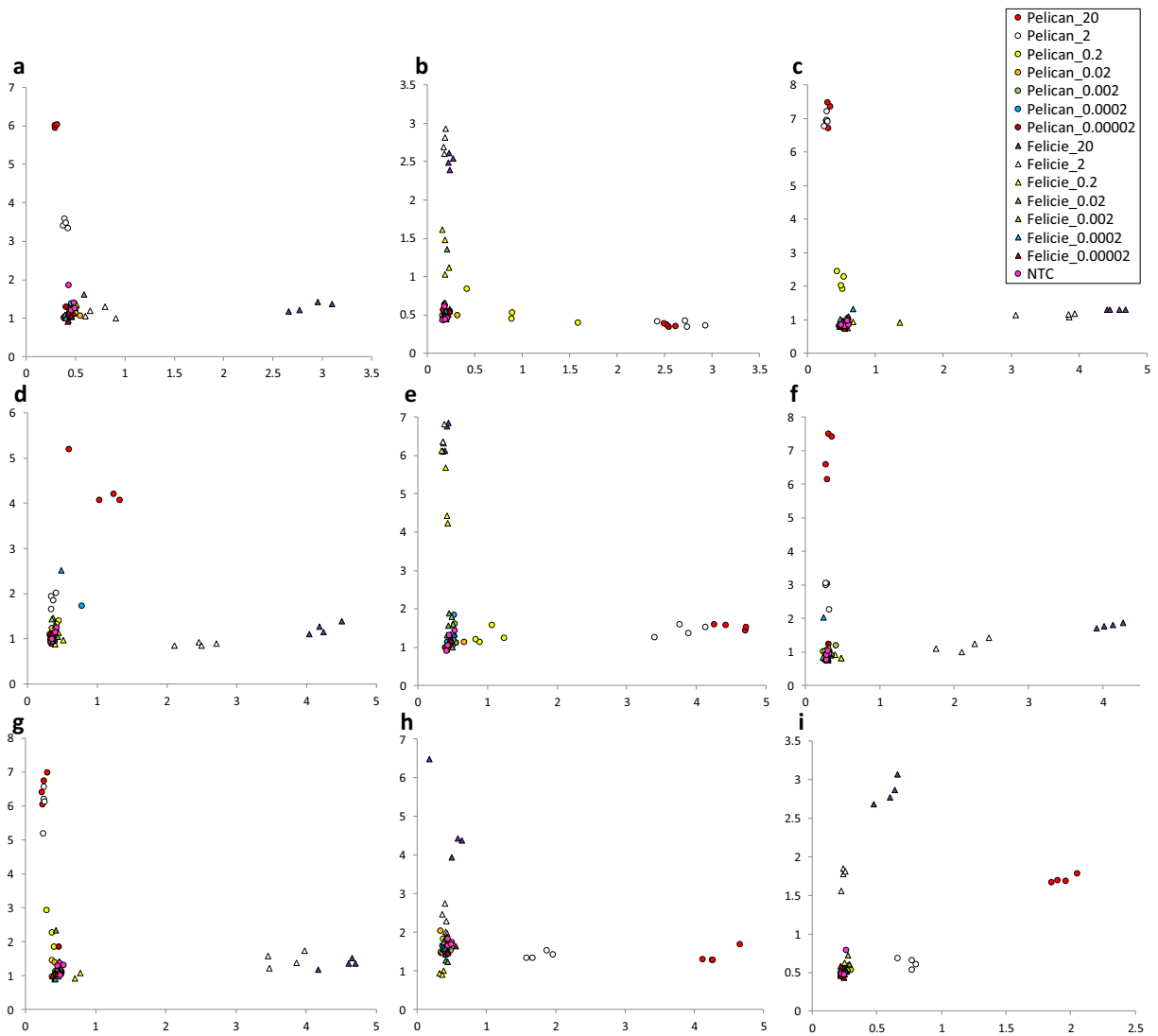
**Fig. 1** Assessment of genetic marker diagnostic for DUS traits for sensitivity across a serial dilution of DNA template concentrations. DNAs extracted from barley cultivars 'Felicie' and 'Pelican', which contrast for all of the eight DUS phenotypes assessed via diagnostic genetic markers here. DNA template concentrations: 20, 2, 0.2, 0.02, 0.002, 0.0002, 0.00002. **a** HvOs03g14380_G125A. **b** Hv11_11299. **c** HvANT2_C4289T. **d** HvPPDH1_A2721G. **e** Hv11_10933_GC. **f** Hv11_10818_CA. **g** HvOs02g01490_G607A. **h** HvCly1_A2604G. **i** HvVRNH1_SNP2

effectiveness of allele calling when two contrasting alleles were present in the DNA template was investigated. DNAs from 'Pelican' and 'Felicie', which contrast for each of the eight DUS traits under investigation, were mixed in nine different proportions as shown in Table 2. These DNA mixtures were used as template for genotyping using the diagnostic KASP genetic markers so that each assay contained a total of 20 ng

DNA template. The genetic markers were evaluated in four technical replicates for each of the 15 DNA templates investigated, and the results shown in Fig. 2. All nine diagnostic KASP markers were found to be co-dominant, i.e. able to detect both alleles when they are present in equal concentrations in the template (DNA templates containing 10 ng 'Felicie' and 10 ng 'Pelican' DNA). Similarly, all nine KASP markers were able to

| DNA template number | ng DNA per KASP reaction, Felicie versus Pelican | Percentage DNA present in samples |
| --- | --- | --- |
| 1 | 20:0 | 100% Felicie |
| 2 | 19.98/0.02 | 99.9% Felicie, 0.1% Pelican |
| 3 | 19.8/0.2 | 99% Felicie, 1% Pelican |
| 4 | 16/2 | 90% Felicie, 10% Pelican |
| 5 | 10/10 | 50% Felicie, 50% Pelican |
| 6 | 2/18 | 10% Felicie, 90% Pelican |
| 7 | 0.2/19.8 | 1% Felicie, 99% Pelican |
| 8 | 0.02/19.98 | 0.1% Felicie, 99.9% Pelican |
| 9 | 0:20 | 100% Pelican |

detect contamination levels of 10% (18 ng versus 2 ng),
irrespective of which variety represented the allele at
low concentration.

### Detection of DUS off-types in bulk DNA extractions from multiple individuals

Having shown the sensitivity of diagnostic genetic
markers selected for study to detect DUS off-types using
in vitro admixture, we next investigated the ability of the
markers to detect DUS off-types in bulk DNA extrac-
tions from multiple individuals. Given the in vitro sen-
sitivity limit was 10%, tissues sampled from individual
'Felicie' and 'Pelican' seedlings were sampled and
bulks of 10 samples used for DNA extraction. Bulks
contained varying proportions of 'Felicie' and 'Pelican'
tissue, the first with 10 'Felicie' samples, and each
subsequent bulk consisting of one less 'Felicie' sample
and one more 'Pelican' sample, with the final bulk
representing 10 'Pelican' samples (Supplementary
Table 2). Genotyping with these templates found seven
markers to robustly detect the presence of a single off-
type in a bulk, irrespective of which allele was present at
the lower frequency: HvOs03g14380_G125A,
Hv11_11299_GC, HvANT2_C4289T,
Hv11_10933_GC, Hv11_10818_CA,
HvOs02g01490_G60, HvCly1_A2604G and
HvVRNH1_SNP2 (Supplementary Fig. 1). However,
while the remaining marker (HvPPDH1_A2721G) was

able to consistently detect a single 'Pelican' individual
within a bulk of 10, it was not able to detect a single
'Felicie' individual within a bulk for all three replicates
undertaken, as the allele call for one rep for this template
clustered with allele calls from 'Pelican' DNA template.

## Discussion

### The use of diagnostic genetic markers for DUS evaluation

Development of new plant varieties represents a long-
term investment for breeders, typically 10–15 years.
This level of investment is protected via the awarding
of plant breeders' rights, which is based on DUS testing
in countries and regions that are signatories to the
UPOV system. If a newly developed submission to
variety testing fails the DUS process, the investment in
its development is lost. For this reason, demonstration
that molecular markers might be relevant to processes
relating to DUS testing is of ongoing interest to plant
breeders and UPOV testing stations around the world.
For the intellectual property vested in a new variety to be
protected, it must first be clearly defined (Jones et al.
2013a). According to UPOV guidelines, a variety is
'defined by its characteristics and that those characteris-
tics are therefore the basis on which a variety can be
examined for DUS' (UPOV 2002). Currently, DUS
testing is carried out using a suite of phenotypic charac-
ters to compare new candidate varieties with relevant
existing varieties. A positive DUS test will result in a
formal and official description of the candidate variety
using its relevant characteristics (Jones et al. 2013a).
Advances in genotyping platforms and reductions in
their cost of use provide opportunities for their potential
application in variety registration (Jones et al. 2013b;
Jamali et al. 2019). Recognising this potential, the Bio-
chemical and Molecular Techniques (BMT) Working
Group of UPOV outlines three possible models for the
use of molecular markers for DUS testing (UPOV
2011): Model 1 involves the use of molecular markers
to directly predict phenotypic DUS characters; Model 2
involves calibration of threshold levels of molecular
distinctness with those attained using traditional DUS
traits to discriminate between varieties, while Model 3
involves the development of a new molecular-based
system independent of phenotypic traits. Here, we in-
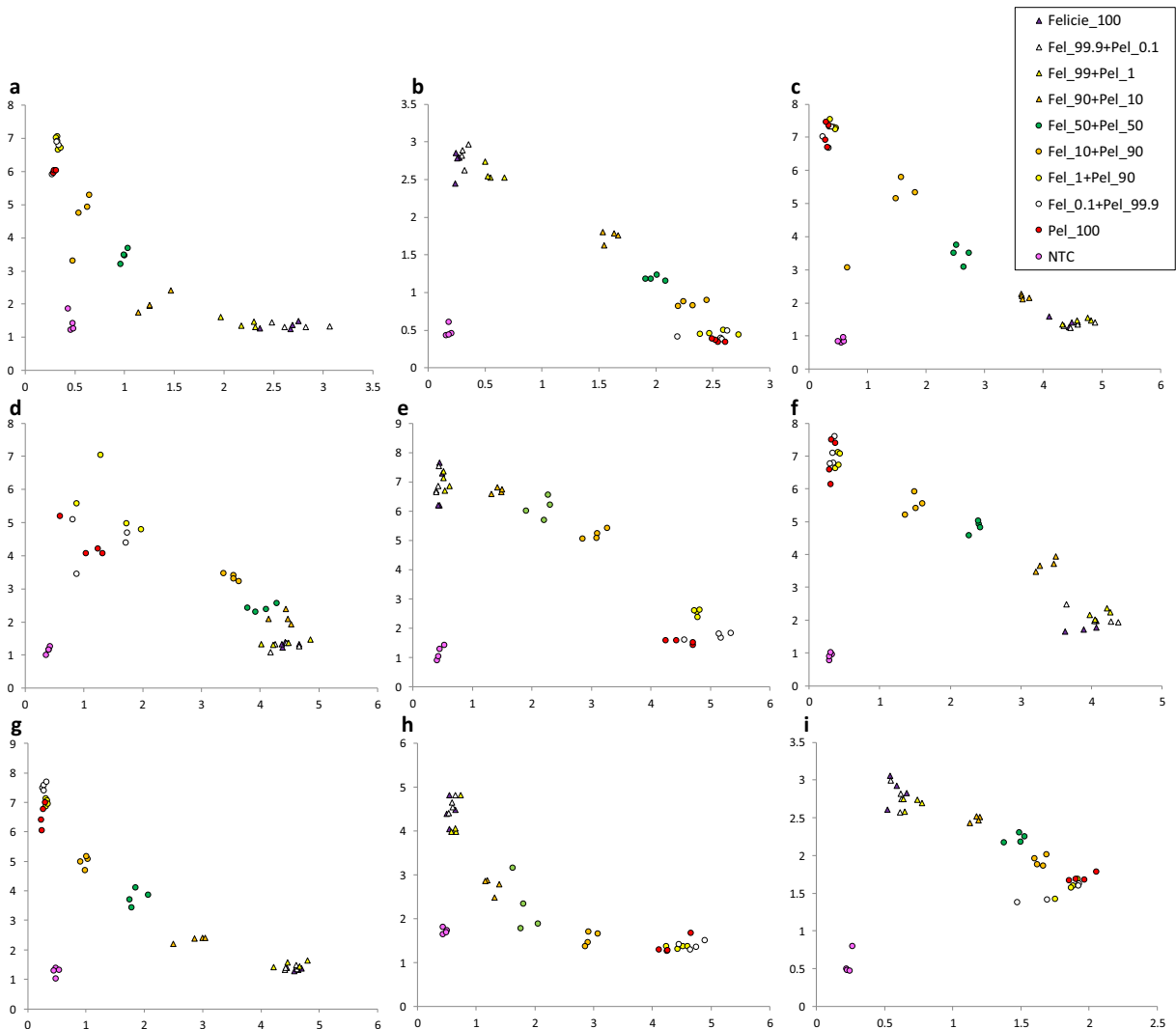vestigate Model 1 specifically the potential for using

**Fig. 2** Use of diagnostic KASP markers to detect in vitro DUS off-types using DNAs spiked at different concentrations with DNA of a contrasting variety (see Table 2). Pel = cv 'Pelican'. Fel = cv 'Felicie'. NTC = no DNA template control. *X*-axis: FAM fluorescence. *Y*-axis: VIC fluorescence. KASP markers assessed: **a** HvOs03g14380_G125A. **b** Hv11_11299_GC. **c** HvANT2_C4289T. **d** HvPPDH1_A2721G. **e** Hv11_10933_GC. **f** Hv11_10818_CA. **g** HvOs02g01490_G607A. **h** HvCly1_A2604G. **i** HvVRNH1_SNP2

molecular markers diagnostic for specific DUS traits for the assessment of uniformity within the DUS context. In barley, we have previously shown that it is possible to identify diagnostic or perfect genetic markers for many barley DUS traits (Cockram et al. 2009, 2010, 2012, 2015; www.naib.com/mas/). However, while these have been shown to be relevant to assessing distinctness (D) and stability (S), to date, the use of barley markers in assessing uniformity (U) had not been shown. In barley, the ability to detect DUS off-types from DNA extracted

from a given sample of grains would rely on the diagnostic genetic markers used being sensitive enough to be able to detect DNA variants, such as SNPs, when they are present in DNAs (a) at low concentration, and (b) as a mixture of alleles. Here, we find the tested molecular assays diagnostic for an array of barley DUS traits are able to reliably detect DUS off-types at frequency of 1 in 10 using DNA extracted from tissue collected from pools of 10 individuals. Thus, using the KASP genotyping system, we demonstrate the potential use of genetic

markers for the characterisation of uniformity within the context of DUS assessment. The methodology should also be of use in other applications where detection of off-types is required. We note that the vast majority of barley varieties released represent inbred lines. In the context of inbreds, if a candidate variety was submitted in which one or more of the 10 seed tested were heterozygous at the candidate locus, based on the results presented here we may not expect the appropriate DNA bulk to be detected, as an off-type DNA proportion of 5% has not been tested.

### Practical use of molecular markers for the assessment of uniformity within a DUS context

The acceptable thresholds for barley uniformity depend on which character is being assessed (UPOV TG 19/10 and CPVO-TP/019/4). For 'B' characteristics (commonly those recorded in field plots) in a sample of 2000 plants, a maximum of 5 off-types are permitted. 'A' characteristics (mostly recorded on samples harvested from the plot) in a sample size of 100 the threshold is 3 off-types. As DNA extractions from bulks of seed are not normally of sufficient quality to use for DUS off-type detection, the most practical and reliable method to implement the markers investigated here would be to germinate batches of seed, and to extract bulk DNAs from leaf tissue sampled from 10 seedlings. For example, in stage 1, 100 seeds could be germinated (or

samples taken from the growing trial) and leaf samples from 10 batches of 10 seed used for DNA extraction, taking ~5 days in total. Each DNA sample would then be used as a template for KASP genotyping, with 3 technical replicates per DNA sample (equating to 30 KASP reactions per KASP marker), followed by data-analysis (2 days). Allowing for the inclusion of the 15 serial dilution control DNAs in four replicates (60 reactions) plus 12 negative controls, this would allow 4 diagnostic KASP markers to be assayed per 384-well plate (Fig. 3). The whole process could be undertaken in 7 days using approximately 1.5 days of technician time, at which point seed batches with 3% or more DUS off-types would be determined. This would be a particularly effective technique for the assessment of the DUS traits 'aleurone layer colouration' or 'hairiness of basal leaf sheaths', both of which are labour intensive. A saving could also be made for the assessment of 'seasonal growth type', which requires an entire trial to determine (although numbers of plants assessed would need to be adjusted to meet protocol requirements). If further investigation to precisely determine the number of DUS off-types was required, a stage 2 experiment could subsequently be carried out: DNAs would be extracted individually from each of the original 100 seedlings sampled in the previous experiment, and the relevant KASP marker used to genotype all 100 individuals. As the complexity of the DNA template is low at this point, only 1 rep would be needed per individual. Thus, stage 2
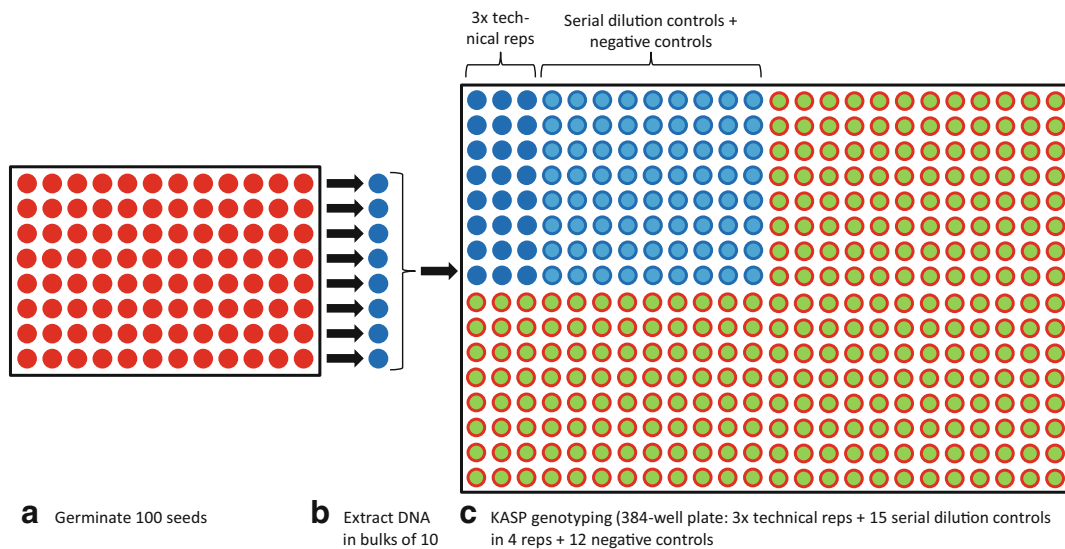


**Fig. 3** Proposed pipeline for molecular testing for DUS off-types in batches of 100 barley seed. In step 3, positions within a single 384-well plate required to assay one KASP marker are indicated in blue. Therefore, at step C, four KASP markers could be assayed in a single 384-well plate, and results would be visualised allowing DNAs containing DUS off-types to be identified

would require 112 reactions per diagnostic KASP marker investigated (including 2 controls in 4 technical replicates each, plus 4 water controls). It should be noted that this approach requires that the underlying genetic variants controlling the target DUS trait of interest are known. In barley, while some of these are already known (summarised by Cockram et al. 2010, 2012; Jones et al. 2013a), implementation under the UPOV Model 1 approach would require the relevant genetic variants for all DUS traits to have been determined. However, it is possible that for traits that are difficult or time consuming to score, such as 'hairiness of leaf sheaths' (UPOV Trait 3), it could be beneficial to employ molecular DUS techniques on a trait by trait basis.

Future prospects

Taken together, these results indicate that the major limiting factor for wider application of this proof-of-principle for the assessment of uniformity in barley within the DUS context is the availability of diagnostic genetic markers for additional DUS traits. The ongoing development of genotyping methodologies in barley that assay increasingly larger number of genetic variants, such as the barley whole-exome capture array (Mascher et al. 2013) and genotyping by sequencing (Poland et al. 2012), means that it should soon be possible to identify tightly linked, or causative, genetic polymorphisms that underlie many more barley DUS traits. Assuming this is possible, implementation would ultimately rely on cost-benefit analysis and consensus around the modification of guidelines that govern variety accreditation.

**Compliance with ethical standards**

**Conflict of interest**   The authors declare that they have no conflict of interest.

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