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A DNA marker assay based on high-resolution melting curve analysis for distinguishing species of the *Festuca–Lolium* complex

Martina Birrer · Roland Kölliker · Chloe Manzanares · Torben Asp · Bruno Studer

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Abstract The grass breeding industry is interested in a fast and cheap method of identifying contamination in seeds of Italian and perennial ryegrass (*Lolium perenne* L. and *L. multiflorum* Lam., respectively). This study shows that high-resolution melting curve analysis in combination with an unlabelled probe assay is an effective method of detecting single nucleotide polymorphisms (SNPs) in diverse Italian and perennial ryegrass backgrounds. This method proved efficient in differentiating ryegrass species and reducing the effect of additional DNA sequence polymorphisms close to the target SNP on the melting curve profiles. For the identification of contamination in Italian and perennial ryegrass seed production, high-resolution melting curve analysis shows great potential, as it is a single closed-tube PCR reaction with an easy workflow, providing results in <2 h after DNA extraction.

Keywords High-resolution melting curve analysis · Italian ryegrass (*Lolium multiflorum* Lam.) · Perennial ryegrass (*Lolium perenne* L.) · Single nucleotide polymorphism · Unlabelled probe assay

Introduction

Ryegrasses (*Lolium* spp.) have a large impact in the grass industry. Italian ryegrass (*L. multiflorum* Lam.) is predominantly used as an annual forage crop while perennial ryegrass (*L. perenne* L.) is used for both forage and turf production. Due to its perennial growth habit and its ability to persist through asexual reproduction by tillers, perennial ryegrass does not need to be sown every year. Therefore, it is highly suitable for lawns and sport turfs where frequent cutting makes it impossible to reproduce through seeds.

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M. Birrer · C. Manzanares · B. Studer (✉)
Forage Crop Genetics, Institute of Agricultural Sciences, ETH Zurich, Universitaetstrasse 2, 8092 Zurich, Switzerland
e-mail: bruno.studer@usys.ethz.ch

M. Birrer
e-mail: martina.birrer@alumni.ethz.ch

C. Manzanares
e-mail: chloe.manzanares@usys.ethz.ch

R. Kölliker
Institute for Sustainability Sciences, ISS, Agroscope, Reckenholzstrasse 196, 8046 Zurich, Switzerland
e-mail: roland.koelliker@agroscope.admin.ch

T. Asp
Department of Molecular Biology and Genetics, Faculty of Science and Technology, Aarhus University, Forsøgsvej 1, 4200 Slagelse, Denmark
e-mail: torben.asp@agrsci.dk

As Italian ryegrass with its annual growth habit does not persist in turf, contaminating annual seed in perennial ryegrass is highly undesirable. The grass breeding and seed production industry is therefore interested in a fast and cheap method of identifying contamination in seed lots. Such methods are already available, the most common being the seedling root fluorescence (SRF) method. The SRF method is based on annuloline, an alkaloid secreted by the seedling roots of Italian but not perennial ryegrass, which is then measured under ultraviolet light (Gentner 1929). However, this method has many disadvantages. For example, it is based on loose linkage between the SRF locus and the annual growth trait (Warnke et al. 2004). Moreover, the SRF test uses living plant material (roots) and is therefore time-consuming. Finally, its accuracy can be questioned as the fluorescence can be impacted by the environment (Floyd and Barker 2002; Rampton 1938). A more recently developed method is based on a quantitative PCR (qPCR) test targeting an insertion/deletion site in the vernalisation gene *LpVRN2* (Chandra-Shekara et al. 2011). Such a DNA-based diagnostic test is advantageous because it is independent from the environment and can be applied at very early growth stages. However, this approach depends on qPCR instrumentation and a sensitive workflow requiring fluorescently labelled TaqMan probes. Moreover, the TaqMan assay needs to be run with both the annual and the perennial ryegrass-specific probe in order to clearly identify the intermediate ryegrass types, i.e. crosses between annual and perennial ryegrasses (*L. hybridum*). There is therefore a need for a fast and simple single-step method with a high throughput for identifying seed contamination from different ryegrass species.

High-resolution melting curve analysis (HRM) has emerged as a technology particularly suitable for such applications due to its simple, fast and flexible workflow (Montgomery et al. 2010). HRM measures the dissociation of double-stranded DNA in the presence of a saturating fluorescent dye and distinguishes different genotypes based on the DNA melting curve profile (Montgomery et al. 2007). Generally, two forms of HRM genotyping can be distinguished: amplicon melting, measuring the melting characteristics of all amplicons that are present in a PCR product, and unlabelled probe melting, where a short unlabelled probe is added to an asymmetric PCR (Zhou et al. 2004). During the melting process of the unlabelled probe assay, two melting peaks can be observed: first, the dissociation of the short unlabelled probe from the full-length PCR product, and second, the dissociation of the full-length product itself (similar to amplicon melting). Because of the difference in the complementarity sequence length (shorter for unlabelled probe melting than amplicon melting), the melting temperature is different. Thus, the genotype calling can either focus on the unlabelled probe melting or the amplicon melting domain.

Single nucleotide polymorphisms (SNPs) are the most common form of DNA sequence variation in genomes, making them excellent targets for the development of species-specific markers. In allogamous forage and turf grass species such as ryegrasses with a generally high degree of heterozygosity, the frequency of SNP occurrence is very high (Ponting et al. 2007). As a consequence, the success rate of SNP genotyping assays can be lower, because polymorphisms close to the target SNP affect genotype calling or the annealing of primers that are used for a locus-specific PCR amplification. This is of particular importance when SNPs are being genotyped in very diverse genetic backgrounds. A publicly available set of gene-derived SNP markers recently became available for perennial ryegrass (Studer et al. 2012). A subset of these SNPs was considered to be interesting candidates for revealing species-specific polymorphisms, clearly separating the Italian from the perennial ryegrass genotypes that have been genotyped using a custom-designed Illumina GoldenGate assay (Studer et al. 2012).

The main aim of this study was to establish a simple, fast and inexpensive method of identifying seed contamination in different ryegrass species. Specifically, we set out (1) to test potential SNPs for their species specificity in a diverse set of genotypes from the *Festuca–Lolium* species complex, (2) to investigate options of using HRM as an efficient method for genotyping species-specific SNP markers and (3) to implement an unlabelled probe assay to specifically address a single target SNP.

Materials and methods

Plant materials

Seeds from a total of 36 cultivars of the species perennial ryegrass, Westerwolds ryegrass (*L. multiflorum* var.

Table 1 SNP locus name, EST sequence and DNA sequence polymorphism (given in *square brackets*) of the potential species-specific SNP markers

SNP locus name[a]	Sequence (5′–3′)
LmsSNP01 (PTA.103.C1)	AAGCCACCGGGACTTACCTTTGAAGTGTAACCAGTGGTGCAATGTTGTTAGATGGGAGTT[T/C]AGCAATCCAACTCCTTTCATAAGGAGCCGTGAATTTCTCTGGCAAGAAGGGCATACAGTT
LmsSNP02 (PTA.1032.C1)	CTTCTCGTGGGGAAGTGAGGCTGTGACCCGCAAGACCCGTCTCCTGGATGTGGTGTACAA[T/C]GCGTCAAACAACGAGCTGGTTCGCACCCAGACTCTTGTGAAGAACGCCATTGTCCAAGTT
LmsSNP03 (PTA.1044.C1)	AATAGATGTGCCAGACCTTCTTAGACGGAGAGCACAGTGCATCATGTAGACAGTAGTAGG[A/G]GAAATAGCTGTGCACACAAATATCCGATCTACACCAGCAAGGTTCAGATTCTCTACCACC
LmsSNP04 (PTA.1535.C1)	CTACAACCAGCTTGCTACAAACACATTGGAGCGGGTGGCCCCTCTGACGCATGCTGTCGG[T/C]AATGTGTTGAAAAGGGTGTTCGTCATTGGTTTCTCGATCATCATCTTTGGCAACAAAATT
LmsSNP05 (PTA.1613.C1)	CTGAACACAAGGAGAAGGCTACTGCTGAAGCCATTGCGCATAACACCCTTACAATGCTGA[A/G]GAGGAGAGTACCACCTGCTGTCCCTGGAATCATGTTCCTTTCTGGCGGACAGTCCGAACT
LmsSNP06 (PTA.2333.C1)	GCAGGAGTATTGTCGGGCGATGTGAGCGATATTGTGCTTCTCGATGTCACGCCACTGTCT[A/C]TAGGTTTGGAGACACTGGGTGGGGTGATGACCAAGATTATCCCAAGGAACACAACCCTGC
LmsSNP07 (PTA.2371.C1)	AGAGCCACCTGGAAGAAGAACCAGACCGTCTCCCTCCGCCGCTACCGTTAAGCCTATGTG[T/C]TTTGCTGTTCTGTCTCTGTGGAATCTGTCCTATCCAGACCTTAGTATGTGTTTGAGCCAG
LmsSNP08 (PTA.240.C2)	AATGCAAGGTGCAGAACCAAATTTGGGAGCAAAAGAAACATTTCAGCGTCAATGCATACA[A/G]AGTAAAGTCTGATCCACATGACATCATGGCAGAGGTCTACAAGAATGGCCCTGTAGAAGT
LmsSNP10 (PTA.32.CB1)	GTGTGGCCGATTGAGGGCATCAAGAAATTCGAGACCCTATCTTACCTGCCACCGCTCTCG[C/G]CCGAGGCCCTCCTGAAGCAGATCGACTTTTTGATCCGCTCCAAATGGGTTCCCTGCTTGG
LmsSNP11 (PTA.43.C1)	GTCTGCATGCCCACTGAACACCCCAAATGGGAGAATGGAAGGTGATTTTGAGATGAAGCA[T/C]ATCGACAAGGTTGGATCATCGACATTCAATATCGCTATTGCACCGTTCTCTCTGTCGATC
LmsSNP12 (r_005b_a08)	GATGGGGCTCTACATGTTCTACATGAACGCGACGCCGGTGGTGGCCGAGGGGAAAGAGGG[A/C]AAGCAGGAGGGGAAACTGCCGGCGGAGGAGCACGTGGTGGTCAACATCGCCAAGCTCAGC

[a] The SNP nomenclature used in Studer et al. (2012) is given in *parentheses*

Westerwoldicum), Italian ryegrass, meadow fescue (*Festuca pratensis* Huds.), tall fescue (*F. arundinacea* Schreb.) and creeping red fescue (*F. rubra*) (Supplementary Table 1) were germinated on sterile filter paper. Three seedlings per cultivar were transplanted into soil-filled pots and cultivated in the greenhouse for 12 weeks. Cultivars were mainly selected from the Swiss list of recommended cultivars (Hirschi et al. 2010). Leaf material from each of the plants was freeze-dried and DNA was extracted using the NucleoSpin® 96 Plant II extraction kit (Machery Nagel, Düren, Germany). Quality and quantity of the DNA was assessed by photospectrometry using NanoDrop and ND-1000 software (Thermo Fisher Scientific, Wilmington, DE, USA).

SNP markers and primer design for HRM genotyping

Expressed sequence tag (EST)-derived SNP markers were tested and preselected for their species specificity in a GoldenGate SNP genotyping assay (Studer et al. 2012). Eleven SNPs being considered as promising candidates for revealing species-specific polymorphisms were selected for this study. EST sequences and the target SNPs (square brackets) are given in Table 1. Primers for HRM genotyping were designed using Primer3 software (Rozen and Skaletsky 2000), targeting an annealing temperature of 60 °C and aiming to keep the amplified PCR product as short as possible (Table 2).

PCR amplification and HRM analysis (amplicon melting)

PCR amplification was conducted using a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) in a total volume of 7 µl, containing 1× LightScanner high-sensitivity master mix (BioFire Diagnostics, Inc, Salt Lake City, UT, USA) and 15 ng DNA as well as 0.3 µM of each forward and reverse primer. The PCR mix was covered with 14 µl mineral oil in order to avoid evaporation during PCR amplification and HRM analysis. Samples were initially denatured for 2 min at 98 °C. This was followed by 44 cycles of 30 s at 95 °C, 30 s at the optimal annealing temperature (T_a) that was determined in a temperature gradient for each primer pair and 30 s at 72 °C for the elongation. The

Table 2 Primer sequences, expected product lengths and annealing temperatures of the primers pairs used for PCR amplification of the target SNPs. The results are described by giving successful PCR amplification and HRM grouping information

Marker/locus name	Sequence (5′–3′) of forward primer	Sequence (5′–3′) reverse primer	Product length (bp)	Annealing temperature (°C)	Successful amplification	Reliable grouping of HRM profiles	Species-specific HRM profiles
LmsSNP01	CACCGGGACTTACCTTTGAA	CAGAGAAATTCACGGCTCCT	100 + intron	60	Yes	No	No
LmsSNP02	GTCTCCTGGATGTGGTGTAC	CAGCTCGTTGTTTGACGC	46	63	No	No	No
LmsSNP03	CATCATGTAGACAGTAGTAGG	TATTTGTGTGCACAGCTATTTC	62	63	No	No	No
LmsSNP04	TCTGACGCATGCTGTCGG	CGAACACCCTTTTCAACACA	76	63	No	No	No
LmsSNP05	CATAACACCCTTACAATGCTGA	CAGCAGGTGGTACTCTCCTC	48	63	No	No	No
LmsSNP06	GCGATGTGAGCGATATTGTG	GGGTTGTGTTCCTTGGGATA	104	63	Yes	Yes	Yes
LmsSNP07	CGCTACCGTTAAGCCTATGT	CCACAGAGACAGAACAGCAAA	45	63	Yes	No	No
LmsSNP08	CATTTCAGCGTCAATGCATAC	GATGTCATGTGGATCAGACTTTACT	50	63	Yes	Yes	No
LmsSNP10	CTTACCTGCCACCGCTCTC	TTGGAGCGGATCAAAAAGTC	66	63	Yes	Yes	No
LmsSNP11	AACACCCCAAATGGGAGAAT	GACAGAGAGAACGGTGCAAT	103 + intron	63	Yes	No	No
LmsSNP12	GGCCGAGGGGAAAGAGGG	GCAGTTTCCCCTCCTGCT	80	63	Yes	Yes	No

PCR reaction was finished by a final extension for 5 min at 72 °C. The LightScanner Instrument (96-well plate format, BioFire Diagnostics) was used for HRM. Measurements were taken from 55 to 98 °C every 0.05 °C, each step with a 1-s hold. The expert scanning module of the LightScanner software Call-IT (version 2.0) was used for the analysis of the HRM data.

Unlabelled probe design and melting

Unlabelled probes were designed using the LightScanner Primer Software version 1.0 (BioFire Diagnostics). The probes were designed to cover the SNPs and were kept as short as possible (21–26 bp). For all probes, a C3-blocker was added at their 3′ end to prevent extension during PCR.

Asymmetric PCR for unlabelled probe assay

The asymmetric PCR for the unlabelled probe assay was conducted using a C1000 Touch Thermal Cycler (Bio-Rad) in a total volume of 7 µl, containing 1× LightScanner high sensitivity master mix, 15 ng DNA and 0.12 µM of the forward primer as well as 0.3 µM of the reverse primer and 0.3 µM of the unlabelled probe. The PCR mix was covered with 14 µl mineral oil in order to avoid evaporation. The HRM analysis was conducted as described above.

Results

The primers designed for species-specific SNPs (given in Table 2) were tested for amplification in a diverse set of 71 genotypes consisting of 24 Westerwolds ryegrass, 24 Italian ryegrass and 23 perennial ryegrass plants. Of the eleven SNPs tested, four primer pairs did not amplify a PCR product suitable for HRM analysis (LmsSNP02, LmsSNP03, LmsSNP04 and LmsSNP05). From the seven primer pairs showing good amplification of the target SNP region, two (LmsSNP01 and LmsSNP11) revealed amplicon melting profiles that could not reliably be assigned to different genotyping groups. This was possibly due to additional polymorphisms being present in the amplified PCR fragment. Sequence homology analysis of the corresponding marker sequences using BLASTn analysis against the nucleotide database of GenBank indicated the presence of an intron between one priming site and the target

Table 3 Unlabelled probe sequences, annealing temperatures (T_a) and product lengths of the unlabelled probes used to specify the PCR amplification of species-specific SNP loci

LunaP/locus name	Sequence (5′–3′)	T_a	Product length (bp)
LunaP01	ATGGGAGTT[C]AGCAATCCAACT	64.6	22
LunaP06	CGCCACTGTCT[C]TAGGTTTGG	63.5	21
LunaP11	AGATGAAGCA[C]ATCGACAAGGT	64.6	22

The target SNP is given in *square brackets*

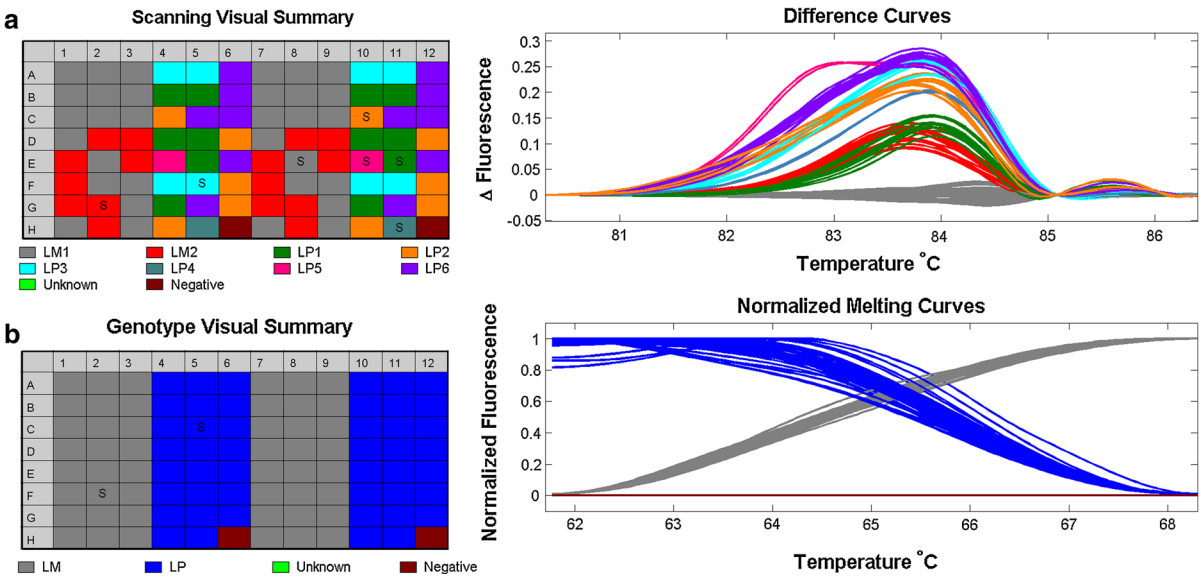

Fig. 1 Visual summary of the genotyping analysis of LMSSNP06 on Italian and perennial ryegrass using high-resolution melting curve analysis (HRM). The visual summary (*left*) and the difference curves (*right*) of the HRM analysis of LmsSNP06 run on DNA isolated from 24 Italian (*LM, columns 1–3*) and 23 perennial ryegrass (*LP, columns 4–6*) genotypes with purified water as a negative control. The analysis was conducted in duplicate. **a** Amplicon melting focussing on the melting peak around 84 °C clearly separated Italian (*red* and *grey*) from perennial ryegrass. The same PCR products were used for unlabelled probe melting (**b**) where genotype calling was based on the melting peak at around 65 °C. Similar to amplicon melting, the unlabelled probe assay successfully discriminated Italian (*grey*) and perennial ryegrass (*blue*)

SNP. This was confirmed by gel electrophoresis of the amplification product (Supplementary Fig. 1). Another four SNPs (LmsSNP07, LmsSNP08, LmsSNP10 and LmsSNP12) showed genotyping groups that did not clearly distinguish the different ryegrass species. The remaining marker LmsSNP06 revealed species-specific HRM profiles, but still showed more than one genotyping group within the same species. This again indicated the presence of additional polymorphisms in the 104-bp fragment that was amplified for this marker.

In order to overcome the effects of additional polymorphisms on the melting profiles, unlabelled probes (Table 3) were designed to shorten the sequence region that is targeted for genotype calling. Using the unlabelled probe assay, the number of genotyping groups decreased for SNP markers LmsSNP06/LunaP06 and LmsSNP11/LunaP11, and reflected the different ryegrass species (Figs. 1, 2). In contrast, LmsSNP01/LunaP01 did not show a similar decrease in genotyping groups.

In order to further evaluate the specificity of LmsSNP06/LunaP06 for distinguishing species within the *Festuca–Lolium* complex, the HRM profiles of the Italian and perennial ryegrass genotypes were compared with 24 genotypes representing meadow fescue, tall fescue and creeping red fescue. While these

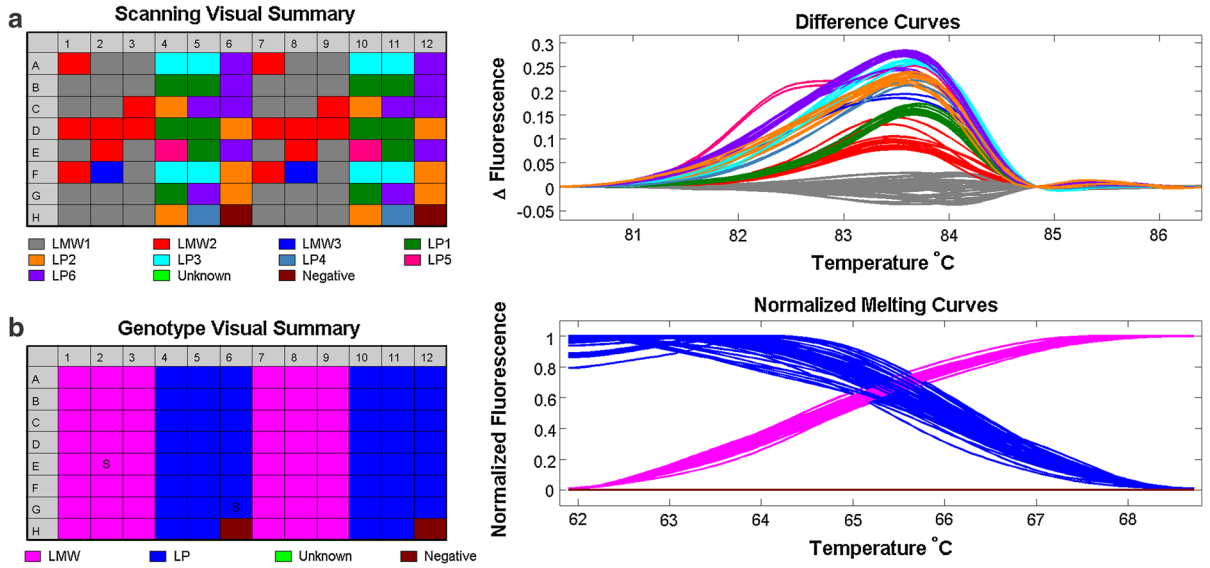

Fig. 2 Visual summary of the genotyping analysis of LMSSNP06 on Westerwolds and perennial ryegrass using high-resolution melting curve analysis (HRM). The visual summary (*left*) and the difference curves (*right*) of the HRM analysis of LmsSNP06 run on DNA isolated from 24 Westerwolds (*LMW, columns 1–3*) and 23 perennial ryegrass (*LP, columns 4–6*) genotypes with purified water as a negative control. The analysis was conducted in duplicate. **a** Amplicon melting focussing on the melting peak around 83.5 °C clearly separated Westerwolds ryegrass (*red*, *dark blue* and *grey*) from perennial ryegrass. The same PCR products were used for unlabelled probe melting (**b**) where genotype calling was based on the melting peak at around 65.5 °C. Similar to amplicon melting, the unlabelled probe assay successfully discriminated Westerwolds ryegrass (*pink*) and perennial ryegrass (*blue*)

fescues could not reliably be separated from perennial ryegrass, they showed clearly distinct HRM profiles when compared to Italian ryegrass (Fig. 3), indicating a common SNP allele constitution of fescues and perennial ryegrass. Moreover, an amplicon melting approach of LmsSNP06 was capable of differentiating meadow fescue, tall fescue and creeping red fescue (Fig. 3).

Homology analysis of the EST sequence underlying the SNP marker LmsSNP06 based on BLASTx analysis against the non-redundant protein database of GenBank revealed a more than 98 % sequence similarity (E value = 0.0) to a 70-kDa heat-shock-related protein localised in the chloroplast but encoded in the nuclear genome.

Discussion

A simple and fast DNA test was successfully used to distinguish different annual and perennial ryegrass species. This test was based on species-specific SNP markers that were genotyped using HRM and proved reliable even when applied in a very diverse set of genotypes belonging to different grass species of the *Festuca–Lolium* complex. This is of major impact for the grass breeding and seed production industry, as the accuracy, time and cost savings of this test to identify seed contamination constitute important competitive advantages. Moreover, this DNA-based method of identifying ryegrass species enables the classification of plants at early growth stages or even directly from seed probes, as successfully applied in maize (Gao et al. 2008). Given the high sensitivity of the HRM approach, an increase in throughput can be achieved by bulking seeds together in a single DNA extraction (Gady et al. 2009; Bush and Krysan 2010; Chen and Wilde 2011).

The approach of testing preselected species-specific SNPs arose from the fact that dominant markers, i.e. PCR-based marker fragments that are present in one but absent in the other species, are of limited impact. For example, it would have been much easier to focus on genes that are involved in the control of the perennial growth habit, such as vernalisation, and are therefore missing in the annual species (Chandra-

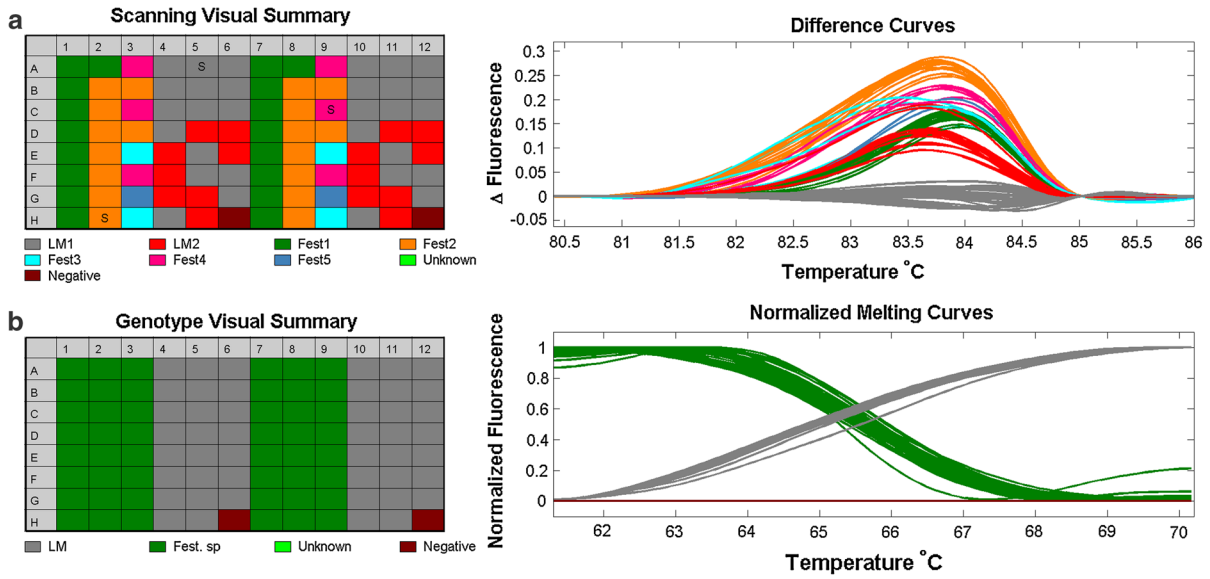

Fig. 3 Visual summary of the genotyping analysis of LMSSNP06 on fescue and Italian ryegrass using high-resolution melting curve analysis (HRM). The visual summary (*left*) and the difference curves (*right*) of the HRM analysis of LmsSNP06 run on DNA isolated from nine meadow fescue, nine tall fescue, six creeping red fescue (*Fest. sp, columns 1–3*) and 23 Italian ryegrass (*LM, columns 4–6*) genotypes with purified water as a negative control. The analysis was conducted in duplicate. **a** Amplicon melting focussing on the melting peak around 84 °C clearly separated Italian ryegrass (*red* and *grey*) from fescues. The same PCR products were used for unlabelled probe melting (**b**), where genotype calling was based on the melting peak at around 65.5 °C. Similar to amplicon melting, the unlabelled probe assay successfully discriminated fescue (*green*) and Italian ryegrass (*grey*)

Shekara et al. 2011). However, with such an approach it would not be possible to distinguish PCR failure from the annual growth habit.

The unlabelled probe assay implemented made it possible to selectively target the species-specific SNPs, thereby reducing the chance of additional DNA polymorphisms interfering with genotype calling. For example, LmsSNP06 showed two different genotype classes in Italian ryegrass when analysed with amplicon melting (*red* and *grey* in Fig. 1a), while the melting curves formed one group when genotyped with the unlabelled probe assay (Fig. 1b). A similar approach can also be used to target other genome locations, where genic SNPs control agriculturally important traits. Considering the high SNP frequency in genomes of outbreeding species (Cogan et al. 2006), an unlabelled probe of 20–26 bp length that specifically targets a single DNA sequence polymorphism constitutes a better option for routine applications when compared with SNP genotyping technologies requiring a 30-bp conserved region flanking the target SNP. Polymorphisms in these regions affect genotyping performance (Grattapaglia et al. 2011; Shen et al. 2005). The unlabelled probe assay could also be applied to SNP markers that have been developed from transcriptome sequencing data. In species such as Italian and perennial ryegrass, where genome sequences have yet to be established, it often remains difficult to predict the exact position of intron/exon junctions based on sequence homology to closely related species such as Brachypodium (*Brachypodium distachyon*) or rice (*Oryza sativa*) (Studer et al. 2012). An unlabelled probe assay therefore provides the option of targeting only a short region of interest with HRM rather than genotyping the entire length of the amplicon containing additional DNA sequence polymorphisms in introns.

In the present study, one out of eleven SNPs (9 %) proved useful for the distinction of Italian and perennial ryegrass. This success rate could be increased by testing additional primer pairs and optimising PCR amplification in combination with unlabelled probe assays. It is worth mentioning that these eleven SNPs have already been tested and preselected for their species specificity in a Golden-Gate SNP genotyping assay, and a much lower success

rate has to be expected for SNPs without any prior information. The use of the first drafts of whole-genome sequence assemblies from Italian and perennial ryegrass would probably result in several thousand additional candidates. However, to reliably predict species, these markers would need to be tested in a large collection of various ryegrass genotypes. A possible solution would be a genotyping-by-sequencing approach in a large set of different Italian and perennial ryegrass genotypes, thereby very quickly providing species-specific SNPs that can be implemented in a HRM approach.

SNP marker LmsSNP06 very reliably distinguished annual from perennial ryegrass species. However, it is worth mentioning that even though 24 genotypes selected from at least six different cultivars is a powerful subset to represent ryegrass species and hence strongly indicates the species specificity of LmsSNP06, it might not be big enough to completely exclude exceptions that might be observed in specific genotypes of a particular cultivar. Moreover, the ability of LmsSNP06 to differentiate fescues from annual ryegrasses opens further opportunities in the development of interspecific hybrids or introgression lines since it allows rapid testing of the origin of the genome region harbouring that particular SNP. For future studies, it might be interesting to localise LmSNP06 in the genome and to compare the genome position of this marker with quantitative trait loci identified for traits such as perenniality.

In conclusion, the results presented here provide the technology and the basic knowledge for its optimisation as well as the marker information to implement a fast and simple method of distinguishing closely related grass species of the *Festuca–Lolium* complex. This will be helpful to the seed production industry and seed testing agencies for efficiently identifying seed contaminants in the grass seed production process. Moreover, grass breeders and the scientific community will be provided with a tool for tracing paternal inheritance and for distinguishing closely related species.

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