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Development of a rapid one-step PCR protocol to distinguish between alfalfa (*Medicago sativa*) and sweet clover (*Melilotus* spp.) seeds

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Summary

Distinguishing seeds of alfalfa (*Medicago sativa*) and sweet cover (*Melilotus* spp.) from one another is difficult because of similarities in the colour, shape, size and weight of the seeds. Existing methods to distinguish these seeds are usually time-consuming and inaccurate. In our study, a polymerase chain reaction (PCR) and polyacrylamide gel electrophoresis (PAGE) method was developed to distinguish alfalfa and sweet clover seeds from one another. Based on differences in the DNA sequences of plastid *matK* spacer regions, a pair of primers was designed and used with simple PCR and PAGE techniques to identify 45 alfalfa and 34 sweet clover (8 white and 26 yellow) accessions that were collected from all over the world. Our results with this pair of primers were clearly different between the two species, supporting the reliability, sensitivity and simplicity of this method.

Introduction

Alfalfa (*Medicago sativa* L.) is a primary forage legume cultivated on more than 40 million hectares worldwide (Bouton, 2007; Zhang *et al*., 2010). Because of its high nutritional quality, alfalfa is referred to as "the queen of forages" and plays important roles in the food processing industry (Liu *et al*., 2007, 2013). Sweet clover (*Melilotus* spp.), mainly consisting of white sweet clover (*M. albus* Desr.) and yellow sweet clover [*M. officinalis* (L.) Lam.], is another widely available annual or biennial legume that is commonly used for ruminant animals all over the world (Tao *et al*., 2009; Tang, 2012). Compared with alfalfa, sweet clover has lower nutritive value and contains coumarin, a chemical substance that is characterised by a bitter flavour and poor palatability (Tang, 2012). These properties have limited the use of sweet clover for animal husbandry. Over the past decade, an increase in the production of alfalfa has been necessary to support growing livestock demands, and alfalfa prices have therefore been steadily increasing in Chinese markets. Because of the increasing price gap between alfalfa and sweet clover, incentives to adulterate alfalfa with sweet clover may increase. The seeds of alfalfa and

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sweet clover share a similar morphology (figure 1) (Sun, 1996). Indeed, when these two types of seeds are mixed, it is very difficult to distinguish them from one another, which greatly reduces the forage value of alfalfa and causes inestimable losses to alfalfa seed production.

Morphological comparisons have traditionally formed the basis for the evaluation of seed purity (Qu *et al*., 2011). However, replication of seed observations is time-consuming and unreliable (Smith and Register, 1998). Many morphological indices of alfalfa and sweet clover such as seed weight and size are similar or overlapping (table 1). This makes it difficult to distinguish alfalfa and sweet clover seeds using morphological approaches, even for well-trained plant taxonomists. Fluorescence identification (FI) is another method to distinguish and test the purity of alfalfa and sweet clover seeds (Sun, 1996). When germinated in an incubator at a constant temperature of 25°C and observed after eight hours in a darkroom with an ultraviolet analyser, only alfalfa seeds exhibit white-yellowish fluorescence. However, the fluorescence of dead and hard alfalfa seeds is too weak to be observed with the naked eye, which prevents the unequivocal identification of these two species with FI.

The rapid development of molecular taxonomic technologies has led to the development of methods to distinguish species for example, on the basis of mitochondrial 16S ribosomal RNA genes in *Anguilla* spp. (Sezaki *et al*., 2005), nrDNA and cpDNA in *Alnus* spp. (Ren *et al*., 2010), ribosomal sequences in *Pyrenophora* spp. (Mavragani *et al*., 2011) and *Elymus* spp. (Baum *et al*., 2012), a partial region of the EF-1 alpha gene in *Fusarium* spp. (Yergeau *et al*., 2005) and a part of the mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene in animal species (Haider *et al*., 2012). However, molecular methods for distinguishing alfalfa and sweet clover have not been reported. In this study, we provide for the first time a reliable one-step PCR protocol to rapidly distinguish between alfalfa and sweet clover seeds by examining different amplicon sizes that result from a pair of primers. The one-step PCR method described in this study provides an accurate, convenient, economical and rapid method to distinguish alfalfa seeds from sweet clover seeds.

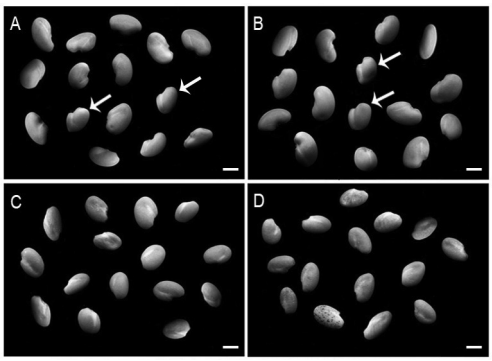

Figure 1. Alfalfa (*Medicago sativa*) and white and yellow sweet clover (*Melilotus albus*, *M. officinalis*) seeds as viewed with a dissecting microscope (Leica, M205). (A) Alfalfa seeds (No. 7, table 2). (B) Alfalfa seeds (No. 10, table 2). (C) White sweet clover seeds (No. 6, table 3). (D) Yellow sweet clover seeds (No. 15, table 3). The white arrows indicate the alfalfa seeds that are morphologically similar to sweet clover seeds. Scale bars =10 mm.

Table 1. Statistical analyses of six morphological indices between the seeds of 45 *Medicago sativa*, 8 *Melilotus albus* and 26 *M. officinalis* accessions. Values are expressed as the mean ± standard deviation (n = 30). Data were obtained using Digimizer software and a high-precision electronic balance.

Species	Seed length (mm)		Seed width (mm)		Radicle length (mm)	
	Range	Mean ± s.d.	Range	Mean ± s.d.	Range	Mean ± s.d.
Medicago sativa	1.8 - 2.9	2.3 ± 0.09	1.1 - 1.8	1.4 ± 0.63	0.6 - 1.2	0.9 ± 0.07
Melilotus albus	1.6 - 2.4	1.9 ± 0.15	1.1 - 1.8	1.3 ± 0.13	0.6 - 1.5	0.9 ± 0.11
Melilotus officinalis	1.6 - 2.5	2.0 ± 0.19	1.0 - 1.9	1.4 ± 0.10	0.6 -1 .6	0.9 ± 0.12

	Cotyledon length (mm)		Radicle length/ Cotyledon length		Thousand seed weight (g)	
	Range	Mean ± s.d.	Range	Mean ± s.d.	Range	Mean ± s.d.
Medicago sativa	1.3 - 2.4	1.9 ± 0.11	0.3 - 0.7	0.5 ± 0.03	1.5 - 2.3	2.0 ± 0.09
Melilotus albus	1.3 - 1.8	1.5 ± 0.14	0.4 - 0.7	0.6 ± 0.14	1.6 - 2.3	1.9 ± 0.11
Melilotus officinalis	1.4 - 1.8	1.5 ± 0.12	0.4 - 0.8	0.6 ± 0.05	1.7 - 2.3	2.0 ± 0.08

Materials and methods

Plant material

A total of 79 accessions of alfalfa and sweet clover were used in this study, which included 45 alfalfa and 34 sweet clover (8 white and 26 yellow) accessions that were obtained from the National Plant Germplasm System (NPGS), the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS) in Beijing and seed markets of Gansu Province, China (tables 2, 3). The alfalfa accessions originated from the following countries: the United States of America (15), China (13), Sweden (8), Australia (3), Canada (3), Austria (1), France (1) and Mexico (1) (table 2). The sweet clover accessions included three landraces (Mo_LX03, Mo_LX05 and Mo_LX06) from China and the other accessions originated from: China (7), the United States of America (7), Estonia (2), Kyrgyzstan (2), Portugal (2), Russia (2), Turkey (2), Belgium (1), Canada (1), Denmark (1), India (1), Poland (1), Sweden (1) and the United Kingdom (1) (table 3).

Seed morphological traits

Bright-field photographs of 30 seeds randomly selected from each accession were obtained using a dissecting microscope (Leica, M205). Six morphological indices, length, width, radicle length, cotyledon length, radicle length/cotyledon length and thousand seed weight were determined using a high-precision electronic balance and Digimizer software that analysed the quantitative characters according to photographs (Singh *et al.*, 1991).

Table 2. Germplasm accessions of 45 *Medicago sativa* accessions that were used in the study.

#	Accession ID	Species	Country of origin	#	Accession ID	Species	Country of origin
1	CF000715	*M. sativa*	China	24	PI 235820	*M. sativa*	Sweden
2	CF000972	*M. sativa*	China	25	PI 235821	*M. sativa*	Sweden
3	CF001084	*M. sativa*	China	26	PI 235823	*M. sativa*	Sweden
4	CF001174	*M. sativa*	China	27	PI 235827	*M. sativa*	Sweden
5	CF001711	*M. sativa*	China	28	PI 235829	*M. sativa*	Sweden
6	CF002722	*M. sativa*	China	29	PI 235830	*M. sativa*	Sweden
7	CF002724	*M. sativa*	China	30	PI 415685	*M. sativa*	Sweden
8	CF005567	*M. sativa*	China	31	PI 235826	*M. sativa*	United States
9	CF005569	*M. sativa*	China	32	PI 467909	*M. sativa*	United States
10	CF020901	*M. sativa*	China	33	PI 467916	*M. sativa*	United States
11	CF020963	*M. sativa*	China	34	PI 467917	*M. sativa*	United States
12	CF021077	*M. sativa*	China	35	PI 467922	*M. sativa*	United States
13	CF021098	*M. sativa*	China	36	PI 467931	*M. sativa*	United States
14	PI 399550	*M. sativa*	Austria	37	PI 467939	*M. sativa*	United States
15	PI 517249	*M. sativa*	Australia	38	PI 467957	*M. sativa*	United States
16	PI 517251	*M. sativa*	Australia	39	PI 536533	*M. sativa*	United States
17	PI 517254	*M. sativa*	Australia	40	W6 22304	*M. sativa*	United States
18	PI 468008	*M. sativa*	Canada	41	W6 22329	*M. sativa*	United States
19	PI 452482	*M. sativa*	Canada	42	W6 22330	*M. sativa*	United States
20	PI 468013	*M. sativa*	Canada	43	W6 22336	*M. sativa*	United States
21	PI 399546	*M. sativa*	France	44	W6 22337	*M. sativa*	United States
22	PI 451728	*M. sativa*	Mexico	45	W6 2507	*M. sativa*	United States
23	PI 235819	*M. sativa*	Sweden				

Table 3. Germplasm accessions of 34 *Melilotus* spp. accessions that were used in the study.

#	Accession ID	Species	Country of origin	#	Accession ID	Species	Country of origin
1	Mo_LX05	*M. albus*	China	18	PI 342891	*M. officinalis*	Estonia
2	Mo_LX06	*M. albus*	China	19	PI 31647	*M. officinalis*	India
3	PI 595324	*M. albus*	China	20	Ames 29037	*M. officinalis*	Kyrgyzstan
4	Ames 19257	*M. albus*	Estonia	21	Ames 29040	*M. officinalis*	Kyrgyzstan
5	Ames 21248	*M. albus*	Portugal	22	PI 342893	*M. officinalis*	Poland
6	PI 593233	*M. albus*	United States	23	PI 342744	*M. officinalis*	Portugal
7	PI 593234	*M. albus*	United States	24	Ames 22891	*M. officinalis*	Russian Federation
8	PI 595392	*M. albus*	United States	25	PI 539021	*M. officinalis*	Russian Federation
9	PI 442522	*M. officinalis*	Belgium	26	PI 90035	*M. officinalis*	Spain
10	PI 595394	*M. officinalis*	Canada	27	PI 342887	*M. officinalis*	Sweden
11	Ames 25658	*M. officinalis*	China	28	PI 172434	*M. officinalis*	Turkey
12	Mo_LX03	*M. officinalis*	China	29	PI 304530	*M. officinalis*	Turkey
13	PI 595335	*M. officinalis*	China	30	PI 342873	*M. officinalis*	United Kingdom
14	PI 595397	*M. officinalis*	China	31	Ames 21623	*M. officinalis*	United States
15	PI 88990	*M. officinalis*	China	32	PI 552552	*M. officinalis*	United State
16	PI 90755	*M. officinalis*	China	33	PI 552553	*M. officinalis*	United State
17	PI 342849	*M. officinalis*	Denmark	34	PI 552554	*M. officinalis*	United State

DNA extraction

Seeds from each accession were placed onto sheets of filter paper that were moistened with 10 ml distilled water in 120 mm-diameter Petri dishes and then incubated under a 16-hours light/8-hours dark photoperiod at 25°C. After five days, the 30 seedlings were combined into one sample and used for the extraction of total genomic DNA, as described by Doyle and Doyle (1987). In the study of the individual plants, 15 individuals of CF002724, 8 individuals of PI 593233 and 7 individuals of PI 88990 were used separately for DNA extraction. The DNA quantity and quality were monitored with a NanoDrop ND1000 instrument (Thermo Scientific) and by electrophoresis through 1% agarose gels, respectively. The working concentration of DNA was adjusted to 25 ng μl^{-1}. All of the DNA samples were stored at -20°C before use.

Primer design

The PCR primers that were used in this study were designed based on differences in the sequences of the alfalfa (GenBank accession HM159582.1) and sweet clover (GenBank accession AF522110.2) plastid *matK* spacer regions. These differences, which included single-nucleotide polymorphisms (SNPs) and insertions and deletions (InDels), allowed for the development of sets of primers by locating unique sequences (figure 2). The sequences of these primers are as follows: MedMel-F, 5'-GGGCAGATAGTCCATTAATGGG-3'; MedMel-R, 5'-GATACATAGTGCGATACAGTC-3'.

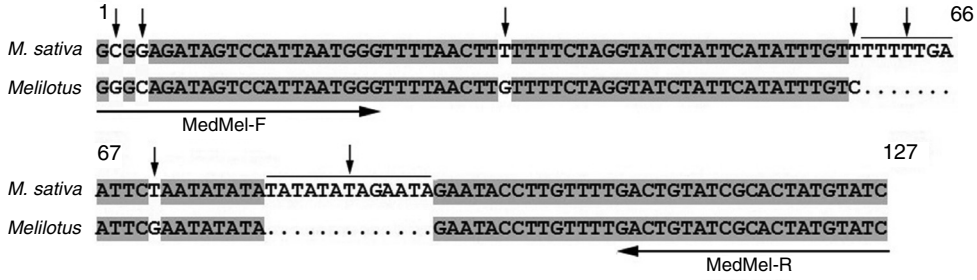

Figure 2. Sequence alignments of a portion of the *matK* regions of alfalfa (*Medicago sativa*) and sweet clover (*Melilotus albus*, *M. officinalis*) cpDNA. The vertical black arrows indicate sequences differences that result from SNPs and InDels. The horizontal black arrows indicate the locations of the primers that were used for PCR.

PCR amplification

PCR amplification was carried out in a 10 µl reaction volume that contained 25 ng template DNA, 1× PCR buffer, 2.0 mM $MgCl_2$, 2.5 mM dNTPs, 4 µM of each primer and 0.8 U Taq polymerase (TaKaRa). The PCR protocol consisted of an initial three minutes denaturation at 94°C, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 20 seconds and a final extension step of 72°C for seven minutes.

PAGE

PCR products (5 µl per lane) were resolved on 8% non-denaturing polyacrylamide gels that were prepared using an acrylamide:bis-acrylamide ratio of 29:1. The gels were run at 200 V (constant) for 90-120 minutes at 4°C. The band sizes were determined by comparison with the DL500 DNA maker (TaKaRa). The gel was stained with ethidium bromide and photographed under UV light using Flourchem FC2 software (Alpha, USA).

Results

There were clear differences between individual alfalfa and sweet clover plants based on the electrophoretic patterns of the PCR products (figure 3). When the DNA samples that were derived from alfalfa were used, there was only one expected band, of approximately 127 bp, that was detected with the MedMel-F and MedMel-R primers. Similarly, when the

DNA samples that were derived from white or yellow sweet clover were used, only one specific, smaller band of approximately 107 bp was generated. This pair of primers was further examined for the ability to distinguish different accessions. The results showed that 45 alfalfa and 34 sweet clover accessions could also be clearly distinguished with the MedMel-F and MedMel-R primers (figure 4).

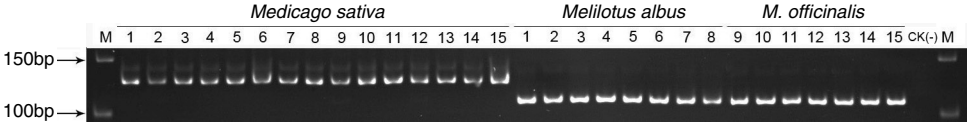

Figure 3. PAGE results of individual alfalfa (*Medicago sativa*) and sweet clover (*Melilotus albus*, *M. officinalis*) seeds. Lanes 1-15 of *Medicago sativa* (No. 7, table 2), 15 alfalfa individuals. Lanes 1-8 of *Melilotus albus* (No. 6, table 3), 8 white sweet clover individuals. Lanes 9-15 of *M. officinalis* (No. 15, table 3), 7 yellow sweet clover individuals. CK(-), negative control. M, molecular markers (150 bp and 100 bp, top to bottom).

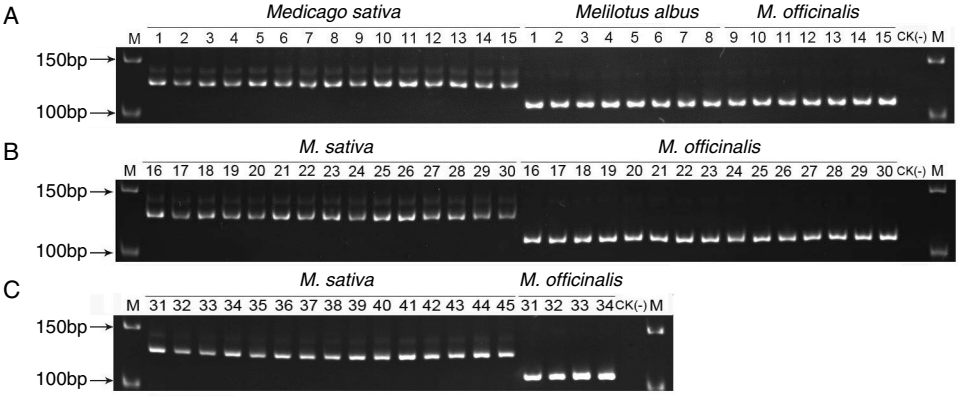

Figure 4. PAGE results of alfalfa (*Medicago sativa*) and sweet clover (*Melilotus albus*, *M. officinalis*) accessions. Lanes 1-45 of *Medicago sativa* (No. 1 to 45, table 2), 45 alfalfa accessions. Lanes 1-8 of *Melilotus albus* (No. 1 to 8, table 3), 8 white sweet clover accessions. Lanes 9-34 of *M. officinalis* (No. 9 to 34, table 3), 26 yellow sweet clover accessions. CK(-), negative control. M, molecular marker (150 bp and 100 bp, top to bottom).

Discussion

Recent progress in DNA sequencing techniques has allowed for short DNA fragments, including chloroplast DNA (cpDNA) with maternal inheritance, to be widely used in the study of pedigree analysis and population differentiation (Birky, 1994; Mogensen, 1996; Penjor *et al*., 2013). Ribosomal RNA maturase (*matK*) is located within the non-coding region of the lysine-tRNA coding region in cpDNA. Because the rate of evolution of this open reading frame region is comparatively fast (Dkhar *et al.*, 2011; Penjor *et al*., 2013), the region is considered to be one of the most informative loci for determining phylogenetic relationships (Hilu *et al*., 2003). The analysis of *matK* may therefore be

useful for the detection of genera within the Leguminosae (Hu *et al*., 2000). In our study, a partial region of *matK* in alfalfa (127 bp) and sweet clover (107 bp) cpDNA were compared and five SNPs and two InDels were found (figure 2). The position and corresponding nucleotide of the SNPs were 2 (C), 4 (G), 32 (T), 59 (T) and 71 (T) in alfalfa. In sweet clover, these nucleotides were 2 (G), 4 (C), 32 (G), 59 (C) and 71 (G). Nucleotides 60-66 (TTTTTGA) and 80-92 (TATATATAGAATA) were identified in alfalfa but were lacking in sweet clover. Through the use of PCR and PAGE, the smaller partial *matK* region that lacked 20 bp in sweet clover could be easily differentiated from that of alfalfa. These findings may therefore be applied to genetically distinguish alfalfa from sweet clover.

A pair of primers (MedMel-F and MedMel-R) were next designed and used to determine whether the PCR amplicons can distinguish individual alfalfa and sweet clover plants. And then this pair of primers was further examined for the ability to distinguish different accessions. Our results with this pair of primers were clearly different between the two species.

By the nucleotide blast analysis, the primers of MedMel-F and MedMel-R not only hit alfalfa and sweet clover, but also hit some related species in the same genera or family. Using the same primers, similarly sized bands might be amplified in the related species. However, we only tested the seeds of alfalfa and white and yellow sweet clover. Therefore, whether the PCR protocol can be successfully applied to other related species such as *Medicago* spp. and *Melilotus* spp. needs further study.

Previously available approaches to distinguish alfalfa and sweet clover required not only extensive experience and professional knowledge but were also time-consuming and unreliable (Sun, 1996; Qu *et al*., 2011). Molecular techniques have recently become the methods of choice to allow for germplasm characterisation and seed purity assays that are highly sensitive, precise and reproducible (Pallavi *et al.*, 2011). Cseke and Talley (2012) provided a reliable method to distinguish wild-type and ornamental varieties of *Imperata cylindrica* (L.) Beauv. that was based on differences in the chloroplast *trnL-F* region. By using a sequence tagged microsatellite site, *Vicia sativa* L. and *Lens culinaris* Medik. could be sensitively distinguished (Pandian *et al.*, 2002). In addition, Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), DNA barcode and DNA sequencing have also been used to distinguish between species (Nagoshi *et al*., 2011; Yazbeck *et al*., 2011; Haider *et al*., 2012; Sarin *et al*., 2013; Cho *et al*., 2014).

However, even with these molecular-based methods, distinguishing species can be complicated and time-consuming or require expensive laboratory facilities. In the present study, the method that we developed to distinguish alfalfa and sweet clover accessions required only one pair of primers, and this approach was highly sensitive, simple and reliable. We believe that this method has the potential to be extensively applied in the production of alfalfa.

Acknowledgements

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