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Commentary

A Sensitive and Specific PCR-Based Discrimination of Split Red Vetch and Lentil Seeds

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Abstract. A PCR-based marker technique was developed to discriminate between morphologically similar split seed of vetch (*Vicia sativa*) and lentil (*Lens culinaris* subsp. *culinaris*). Sequence tagged microsatellite site (STMS) markers were more discriminatory than markers produced from the nontranscribed spacer (NTS) region of the 5S ribosomal RNA gene. A sequence characterized amplified region (SCAR) marker, developed from the 5S rRNA NTS region, was sensitive when resolved on agarose. However, the fluorescent-labeled 5S rRNA SCAR marker was unable to discriminate between vetch and lentil, probably because of the low copy number of the marker, and was not visualized on agarose. An STMS primer-pair (PSMPSAD123), developed from field pea, was able to discriminate split red cotyledon vetch from split red cotyledon lentil because it produced specific markers at 563 bp for lentil and 353 and 474 bp for vetch. The vetch-specific STMS marker was conserved among all species of the *Vicia* genus used in this study and was sensitive enough to discriminate both on agarose gels and on polyacrylamide gel–based fluorescent systems. The fluorescent-tagged STMS analysis revealed peaks for vetch and lentil at the expected sizes in admixtures of milled vetch and lentil seeds, and it was sensitive enough to detect one vetch seed in 1999 lentil seeds. The development of PCR-based tests for detecting the level of vetch seed contamination in lentil export seed may provide a method for quality assurance of export lentil seed.

Key words: fluorescent detection, genotyping, STMS, 5S rRNA

Introduction

In the late 1980s, a new cash-crop market was created in Australia for Blanche fleur, a cultivated red cotyledon vetch cultivar grown for animal fodder. Export of vetch seed increased quickly, from just 50 metric tons during 1988-89 to 9470 metric tons during the 1991-92 season (Tate and Enneking, 1992). Although vetch is used as a high-protein cattle food, it contains neurotoxins (β-cyanoalanine and γ-glutamyl-β-cyanoalanine), which affect pigs, horses, and chickens (Ressler et al., 1969). The spherical cotyledons of the Blanche fleur cultivar are

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morphologically similar to those of the split red lentil, particularly when coated with vegetable oil.

Over the last 10 years, there have been several incidences of split red vetch being exported from Australia, relabeled, and sold at a higher premium as split red lentil (red dhal) for human consumption (Tate et al., 1999). Public concern regarding the neurotoxic effects of vetch has caused a decrease in the Australian vetch export market and has resulted in a temporary ban on imports of Australian lentils into several large consumer countries, including Egypt, India, Pakistan, and Bangladesh (Tate et al., 1999). Ongoing international bans on the import of lentils may be retarding the true export cash potential of this crop. The development of a robust method to discriminate vetch from lentil would help the Australian grain legume industry to regain its former reputation on the international lentil markets through certified quality assurance of export seed.

Intraspecific and interspecific taxonomic discrimination in the Leguminosae are traditionally based on morphology and/or isozyme polymorphisms (Cubero, 1974; Kaser and Steiner, 1983; Ladizinsky, 1975; Mancini et al., 1989). However, for these methods to be accurate, they require all plants to be assessed at the same stage of plant development, under a constant environment. Vetch also has been identified biochemically by measuring the β-cyanoalanine content using chromatographic procedures that are time consuming and laborious and that rely on the availability of a polyclonal antibody (Ressler, 1962). These biochemical methods indicate the presence of vetch neurotoxins but cannot differentiate between vetch cultivars.

Molecular markers are quicker and far more precise in species and genotype identification. The development of a DNA-based test for vetch, particularly Blanche fleur, would remove any uncertainty caused by environmental effects on the expression of the toxins. They provide a more rapid and reliable way of differentiating red cotyledon vetch seed from red cotyledon lentil seed and also may differentiate low- from high-toxin–containing vetches. The advancements of molecular marker technology have broadened the area of genotyping and genetic diversity analysis by potentially revealing a large amount of genetic variation between closely related taxa (Melchinger et al., 1994). A number of molecular marker techniques are available to distinguish between two morphologically similar organisms.

A potential PCR-based method previously examined for the development of species-specific markers was based on nucleotide sequence differences within the nontranscribed spacer (NTS) region of the 5S ribosomal RNA gene (Rogers et al., 1986; Ko and Henry, 1996; Baum and Johnson, 1996). The 5S rRNA gene belongs to the ribosomal gene family, consisting of tandem repeat regions, of which the coding regions are highly conserved (Long and Dawid, 1980). The NTS regions lie in-between the coding regions and are more variable and species-specific (Ko and Henry, 1996; Cespedes et al., 1999). Variability within the NTS regions of the rRNA genes among individuals of *Vicia faba* has been reported previously (Rogers et al., 1986). The numbers and lengths of 5S rRNA spacer regions of lentil were reported to be species-specific (Patil et al., 1995; Ford et al., 1997). Therefore, the NTS regions may be useful for the development of vetch and lentil species-specific probes.

Of the PCR-based methods, markers based on microsatellite sequences and/or their flanking regions have become a popular choice for genotyping. Microsatellites are short DNA sequences of approximately 1-5 bases arranged in a head-to-tail repeat fashion (Lagercrantz et al., 1993). Microsatellite regions exhibit remarkable polymorphism because they are shown to consist of variable numbers of tandem repeats in many species (Levinson and Gutman, 1987; Jeffrey et al., 1988; Zischler et al., 1992). Sequence tagged microsatellite site (STMS) markers are produced using primers designed to the flanking sequences of the microsatellite repeats. The flanking sequences are thought to be more conserved than the internal repeat regions and offer a potential source for species identification markers (Choumane et al., 2000). The transferability of STMS primers among members of the same genera and/or family has been determined previously in accessions of field pea, chickpea, lentil, and vetch (Pandian et al., 2000).

The unambiguous designation of alleles and their precise sizing is crucial in the molecular identification of species and in more in-depth genome analysis. The semiautomated multifluorophore technique involves fluorescent labeling of one primer of a primer-pair with a fluorescein-derived fluorophore, such as 6-FAM, HEX, or NED (Ewen et al., 2000). Once PCR is performed, a precise measure of amplification product size may be made. Furthermore, because the data output are provided as a chromatographic peak, the area under this peak may provide a quantitative measure of the amplification product(s) and, hence, a measure of genomic DNA. Fluorescent analysis has been employed successfully in high-resolution genome analyses of human (Fregeau and Fourney, 1993), soybean (Diwan and Cregan, 1997), and Norway spruce (Pfeiffer et al., 1997) and for genotyping of tomato (Bredemeijer et al., 1998), barley, and wheat (Schwarz et al., 2000).

The aims of this study were to (1) identify vetch and lentil species-specific molecular markers based on 5S rDNA and STMS sequences, (2) verify potential markers for specificity and sensitivity to detect DNA of vetch in admixtures of vetch and lentil seeds, and (3) develop a routine semiautomated fluorescent assay for vetch identification.

Materials and Methods

Plant materials and DNA extraction

Seeds of vetch (*Vicia* spp.) and lentil (*Lens culinaris* subsp. *culinaris*) cultivars were obtained from The Victorian Institute for Dryland Agriculture (VIDA), Horsham, Australia, and the South Australian Research and Development Initiative (SARDI). Admixtures of cultivars of Blanche fleur (vetch) and Cobber (lentil) seeds were milled in the ratios of 1:1 (vetch-lentil), 1:20, 1:50, 1:100, 1:500, 1:1000, 1:1500, 1:2000, 1:2500, 1:3000, 1:4000, and 1:5000 using a grinding machine (MAKLA Mill, Crompton Parkinson, Australia). Three replicates of each admixture of vetch and lentil seeds were used for DNA extraction and further analysis. Genomic DNA was isolated from 100 mg of milled seed by the adapted CTAB method (Taylor et al., 1995).

Table 1. Species-specific markers produced with either the 5S rDNA SCAR or PSMPSAD123 primers

				NTS Marker, bp			STMS Marker, bp			
Grain Legume	Cultivar	Accession	Cotyledon Color	55	105	400	353	474	563	700
Vetch	*Vicia sativa subsp. sativa*	Blanche fleur	Red	-	+	+	+	+	-	-
	Vicia sativa subsp. sativa	Languedoc	Beige/yellow	-	+	+	+	+	-	-
	Vicia sativa subsp. sativa	Velstar	Red	-	+	+	+	+	-	-
	Vicia sativa subsp. sativa	Velero	Red	-	+	+	+	+	-	-
	Vicia sativa subsp. sativa	Vedura	Red	-	+	+	+	+	-	-
	Vicia sativa subsp. sativa	Morava	Beige/white	-	-	-	+	+	-	-
	Vicia sativa subsp. sativa	Cummins	Beige/yellow	-	-	-	-	-	-	+
	Vicia villosa subsp. dasycarpa	Capello	Dark	-	-	-	+	+	-	-
	Vicia villosa subsp. dasycarpa	Namoi	Dark	-	-	-	-	-	-	+
	Vicia benghalensis	Popany	Dark	-	-	-	-	-	-	+
Lentil	*Lens culinaris subsp. culinaris*	Cobber	Red	+	-	-	-	-	+	-
	Lens culinaris subsp. culinaris	Digger	Red	+	-	-	-	-	+	-
	Lens culinaris subsp. culinaris	LairdB	Red	+	-	-	-	-	+	-
	Lens culinaris subsp. culinaris	Indianhead	Red	+	-	-	-	-	+	-
	Lens culinaris subsp. culinaris	ILL2882	Red	+	-	-	-	-	+	-
	Lens culinaris subsp. culinaris	ILL4532	Red	+	-	-	-	-	+	-

PCR amplifications

Several cultivars of vetch and lentil were used to study the specificity of the STMS and 5S rRNA NTS SCAR markers for differentiating red cotyledon vetches from red cotyledon lentil (Table 1). The NTS-based SCAR marker was designed from sequence alignment of the 5S rRNA NTS regions of *Vicia sativa* (cv. Blanche fleur) and *L. culinaris* subsp. *culinaris* (cv. Cobber) using Bestfit analysis within the Australian National Genomic Information Service (ANGIS). The NTS-based SCAR forward primer sequence was 5'-TGTAAACCGATGCAT-TTTTGTTAG-3', and the reverse primer was 5'-TTCACTTCTCAGCGGAAA-AATAC-3'. Twenty-two STMS primer-pairs, each isolated from field pea and chickpea, were used in PCR amplifications as described previously by Pandian et al. (2000). The forward primer sequence of field pea–derived STMS primer-pair

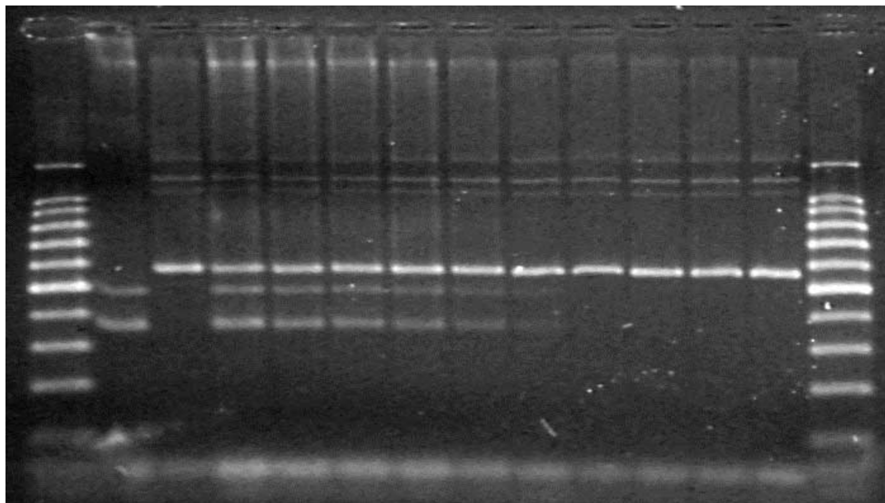

Figure 1. DNA dilution series test using admixtures of milled vetch and lentil seeds amplified with STMS primer PSMPSAD123. Lanes (vetch-lentil): 1 = 1:0; 2 = 0:1; 3 = 1:1; 4 = 1:499; 5 = 1:999, 6 = 1:1499; 7 = 1:1999; 8 = 1:2499; 9 = 1:2999; 10 = 1:3499; 11 = 1: 3999; and 12 = 4999. M = Molecular weight marker.

(PSMPSAD123) was 5'-TCAGGCCCAACGACCATATT-3', and the reverse primer sequence was 5'-TGAGGAAGGAGAAGAATGATGTGA-3'. For 5S rDNA analysis, the PCR conditions were the same except that the annealing temperature was increased to 60°C.

Semiautomated fluorescent analysis

One primer from NTS-based SCAR primer-pair and one from field pea–derived STMS primer-pair (PSMPSAD123) were labeled fluorescently with the fluorophore 6-FAM prior to PCR. Amplification products were electrophoresed through 5% denaturing polyacrylamide gels using a PE Biosystems 377 automated DNA sequencer. Fluorescent products were detected and analyzed with GENESCAN and GENOTYPER software (PE Biosystems, USA).

Results and Discussion

The SCAR primers developed from the 5S rRNA NTS region were able to detect at least 20 pg of pure vetch DNA when mixed with 39.98 ng of pure lentil DNA (0.05% vetch, 99.95% lentil). These SCAR primers produced fragments of 105 bp and 400 bp in vetch and 55 bp in lentil (Table 1). The 400-bp vetch fragment disappeared when PCR amplifications were carried out after vetch DNA was digested with *Mva* I. This probably was caused by the presence of an *Mva* I restriction site at the 5' end of the 5S rDNA coding sequence, as had been shown previously for lentil species (Ford et al., 1997). When tested on milled batch seed admixtures, this marker was able to detect at least one vetch seed in 2999 lentil

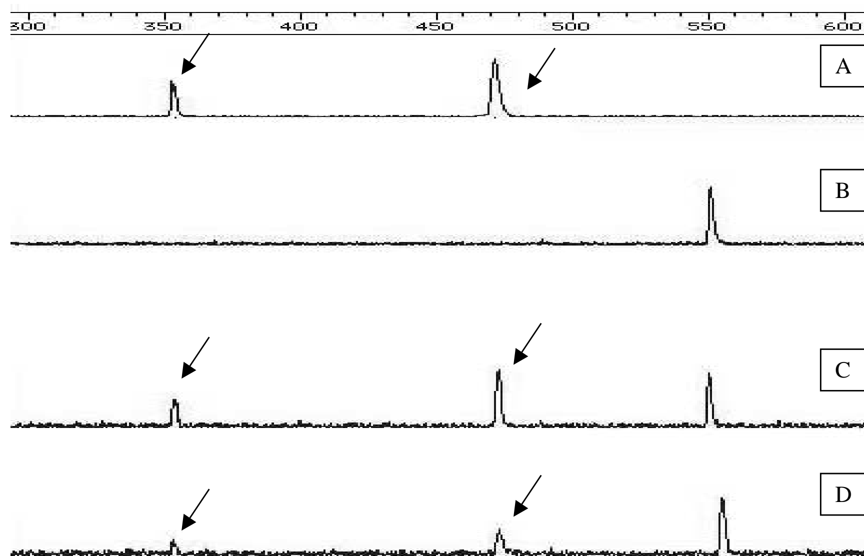

Figure 2. Semiautomated fluorescent analysis of genomic DNA amplified using STMS primers PSMPSAD123. Lanes: A = vetch; B = lentil; C = vetch-lentil (1:1), and D = vetch-lentil (1:100). Arrows indicate vetch-specific peaks.

seeds. The very high sensitivity of the SCAR marker could partly be owing to the abundant occurrence of the 5S rRNA genes in repeat arrays at one or more chromosomal locations (Long and Dawid, 1980; Sastri et al., 1992).

After fluorescent-labeling and detection of PCR products on the PE Biosystems 377 automated DNA sequencer, the SCAR marker was unable to discriminate between the morphologically close cultivars of vetch and lentil. Even at extremely stringent PCR conditions (ie, annealing temperature 66°C), a peak of 105 bp was amplified from both vetch and lentil DNAs. The presence of the 105-bp peak in lentil probably was caused by very high sequence homology between the NTS regions of vetch and lentil. A previous study showed that the dissimilarity among NTS sequences of vetch and lentil is approximately 19% (Ford, 1999). Alternatively, the 105-bp peak in lentil may have been produced in a relatively low copy number, making it impossible to visualize on agarose. Visualization is possible with the semiautomated fluorescent method, which is far more sensitive. The addition of bases to the fluorescent-labeled primer and/or its complementary primer may enhance the discriminatory power of the SCAR marker. However, given the limited sequence variation between vetch and lentil, this may not be possible. Therefore, the 5S rRNA–based SCAR marker ultimately was unable to distinguish between red cotyledon vetch and lentil when the PCR product was analyzed on an automated DNA sequencer.

Forty-four STMS primer-pairs were screened, and 9 produced either vetch or lentil species-specific markers. Of the 22 chickpea STMS primers screened, only 3 primers produced just 1 vetch-specific marker and 2 lentil-specific markers. Of the 22 field pea STMS primers tested, 6 were able to produce 8 vetch-specific and 9 lentil-specific markers. Of these, one primer-pair

(PSMPSAD123) was reliable at discriminating all red cotyledon vetches from all red cotyledon lentils with the semiautomated fluorescent detection. The STMS primer-pairs PSMPSAD123 produced unique peaks at 563 bp for lentil (cv. Cobber) and at 353 bp and 474 bp for vetch (cv. Blanche fleur). This primer-pair also generated a single 700-bp fragment for vetch species, Popany and Namoi (Table 1).

In a DNA dilution series, PSMPSAD123 primers were able to detect at least 1 ng of pure vetch DNA mixed with 39 ng of pure lentil DNA (2.5% vetch, 97.5% lentil), which was less than that of the 5S rRNA NTS primers. In admixtures of milled vetch and lentil seeds, however, PSMPSAD123 was able to detect at least one vetch seed mixed with 1999 lentil seeds (Figure 1). The fluorescent-tagged STMS analysis revealed peaks for vetch and lentil at the expected sizes (Figure 2). In admixtures of milled vetch and lentil seeds, the vetch peak height and area where observed to decrease at a vetch-to-lentil ratio of 1:100 (Figure 2); thus, the STMS-based marker, PSMPSAD123 was efficient for sensitive species identification and can be used in quality assurance of export lentil seed. This would help to identify vetch contamination from rotation crops or from deliberate attempts to substitute split red vetch for split red lentil.

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