Discrimination of the Commercial Seeds of Forage Crops using Ribosomal Intergenic Spacer Analysis

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The precise identification of plant species and their sub-types, and the ability to discriminate between them, are just some of the major advances that have resulted from the application of molecular biology to plant science. Of particular note in this regard is the successful application of molecular techniques to the analysis of plant DNA, which has now made the genotyping of plant materials a routine and much simplified practice. The complete genetic identification of organisms necessarily involves the determination of the genomic DNA sequence, while this is both a costly and time consuming procedure, thus yielding a major concern in most fields of biological science. As an alternative to the sequencing, a series of methodologies has been developed for the genetic identification, such as restriction fragment length polymorphism (RFLP) (Southern 1975), random amplified polymorphic DNA (RAPD) (Williams et al. 1990), amplification fragment length polymorphism (AFLP) (Vos et al. 1995) and simple sequence repeats (SSR) (Akkaya et al. 1992). It is significant, however, that each of these techniques is reliant on the stability and specificity of the genomes of the specific organisms under study, and at the same time their efficiency is dependent upon the degree of variability of corresponding genomic regions between species. Polymerase chain reaction (PCR) based methods have been extensively employed for plant genotyping in particular, due to their simplicity, rapidity and reliability (Breton et al. 2004, García-Beneytez et al. 2002). For PCR analyses, precise primer sets are usually designed to amplify selected sequences from specific organism(s).

There are several limitations of the use of these standard molecular methodologies for plant identification. One such constraint is the difficulty in designing DNA markers that are specific for those plant species, such as most cultivated forage crops, that have been grown and maintained as a heterogeneous population due to their breeding habit including self-incompatibility (Baumann et al. 2000). In these cases, the design of the specific PCR primers or the identification of specific DNA sequences in their genotypes is extremely difficult, both theoretically as well as practically. In spite of this difficulty, the reliable management owing to the traceability of seed products has been a critical element in both plant breeding and in the commercial handling of plant seeds (Auer 2003, McIntosh et al. 2005).

In our previous report (Ikeda et al. 2005), we showed that microbial communities among certain agronomic products could be stably detected using ribosomal intergenic spacer analysis (RISA). RISA is a DNA fingerprinting technique that can identify microbial diversity by providing a unique signature for each agronomic product. In our present study, we report the potential usefulness of RISA for the discrimination of commercial seeds for forage crops and present this as an example of the traceability of agronomic products which have high levels of genetic heterogeneity.

Plant material and DNA extraction

Commercial seeds were obtained from local commercial vendors and consisted of 14 cultivars of Italian ryegrass (Lolium multiflorum) and 9 of timothy (Phleum pratense). Two packages of seeds for each cultivar were obtained, and one sample was collected from each package for duplicate analysis. All of these seeds had been imported from the USA. Four tenth (0.4) g of each sample was transferred into a 2 ml screw-capped tube and subjected to DNA extraction from the microbial community on the surface of seeds. This was performed using the FastDNA SPIN Kit for soil (Qbiogene, Carlsbad, CA, USA) according to the manufacturer’s protocol, except that the washing step was repeated three times and each extract was eluted in a final volume of 50 µl.

Ribosomal intergenic spacer analysis

Ribosomal intergenic spacer analysis (RISA) was performed essentially as previously described by Ikeda et al. (2004), with slight modifications. Briefly, the primer set used...
in the present study was same to the previously described ITSF/ITSErub for eubacteria (Cardinale et al., 2004). Each PCR reaction mixture (final volume of 50 µl) contained 5 µl of 10 × buffer, 10 µg of BSA, 0.5 µM of each primer, 200 µM dNTPs, 4U of Ex Taq HS DNA polymerase (Takara, Kyoto, Japan) and 8 ng of DNA extract. The PCR amplification program consisted of 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C, and a final extension step of 7 min at 72°C. PCR products (3 µl) were then mixed with 2.5 µl of loading dye (95% formamide, 10 mM EDTA, pH 8.0, 0.1% bromophenol blue). After incubation at 80°C for 3 min, these samples were chilled on ice for 5 min, and directly loaded onto 5% polyacrylamide gels (19:1 ratio of acrylamide to bisacrylamide, 0.4 mm thick, 40 cm long) containing 7.7 M urea and 0.5 × TBE. Electrophoresis was conducted at a constant voltage of 2000 V in 1 × TBE for 2.5 hours.

Data analysis

Duplicate samples for each seed product were electrophoresed. Following gel electrophoresis, digital fingerprinting images were then obtained using a fluorescent scanner (Molecular Imager FX, BIO-RAD Laboratories, Inc., Hercules, CA, USA). The RISA banding patterns were then analyzed by Bio-Rad Quantity One™ software to assess the similarities between lanes and then to generate similarity dendrograms (Dice coefficient of similarity) via the UPGMA method. Lane background subtraction was carried out with a rolling disk size of 50 and band detection was performed using the default parameters, with the exception of the sensitivity setting of 800. The range of the DNA band size for data collection was between 186–1722 nt. Similarity dendrograms were then constructed using the band position information (both the presence and absence of bands).

The overall profiles obtained by RISA for both Italian ryegrass and timothy seeds showed stable banding patterns across all samples, with several variable bands in evidence (Fig. 1). A summary of these RISA profiles is shown in Table 1. The Italian ryegrass showed smaller average band number and had less variability than the results for timothy. However, for Italian ryegrass, two cultivars (I-3 and I-6 in Fig. 2) could not be discriminated from other samples using the information from the overall RISA data. Similarly, two cultivars of timothy (T-3 and T-9 in Fig. 3) could not be correctly distinguished by the overall profiles of RISA. Since our RISA experiments did not provide defined profiles for every cultivar, some caution should be taken with this method when low similarities are observed.

In the present study, using commercial seeds of forage crops as an example, we examined the potential usefulness

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<th>Table 1. Summary of the RISA profiles generated using bacterial DNA extracted from the commercial seeds of forage crops</th>
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<td><strong>Number of bands</strong></td>
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<td><strong>Italian ryegrass</strong></td>
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<td><strong>Timothy</strong></td>
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1) Mean±S.D. of the band number for all samples.
2) Total number of band types which is based upon the band locations for each of the DNA fragments detected by RISA.
3) Mean±S.D. of the average inter-cultivar similarities.
4) Mean±S.D. of the average intra-cultivar similarities.

* Statistically significant differences between the inter- and intra-cultivar similarities at the 0.01 level, measured using the U-test.
Discrimination of forage crop seeds by RISA

of RISA for not only the simple profiling of microbial DNA from agronomic products, but also for the discrimination of commercial sources of these products. Moreover, since RISA employs universal primer sets, this method can amplify DNA from a wide range of microbes from various agronomic samples. RISA is similar to random amplified polymorphism (RAPD) (Williams et al. 1990), as it does not require the availability of sequence information prior to obtaining the fingerprinting patterns, and has been used for genetic characterization of a wide variety of microbes. Importantly however, RAPD has not been applied to microbial community analysis as a routine technique due to the high complexity of the genetic compositions within natural microbial populations.

Other types of fingerprinting techniques, such as amplified fragment length polymorphism (AFLP), may also be applicable to microbial community analysis. AFLP might be particularly suitable as, in general, this method is capable to provide reproducible profiling patterns, when compared with other fingerprinting techniques. However, microbial communities in a given pool of natural samples are often composed of heterogeneous populations. Such samples are not appropriate for AFLP as this technique will detect genetic variations underlying at a given bacterial genome among individuals within a species, or even among the subspecies level. In contrast to many fingerprinting techniques, the complexity of the profiles generated by RISA is reduced by focusing on only ribosomal operon regions, which contains conserved sequences and modest levels of variability, and would be genetically stable from the genus to subspecies levels. Furthermore, the high annealing temperatures and the high specificity of the primers used in RISA results in a more stable and reproducible profiling pattern than RAPD. These features of RISA allow the presentation of stable DNA fingerprinting profiles including modest variability resulting from environmental microbial DNA.

It is clear from our current data that, unlike conventional DNA markers, the DNA fingerprinting profiles obtained by RISA cannot discern specific plant genotypes. Moreover, the RISA band patterns can also be strongly affected by several environmental factors, such as differences in the production site, pest management practices and storage conditions. The ambiguity of the results obtained for I-6 in Figure 2 may be reflecting the effects of these environmental factors upon the RISA profiles for these samples. Hence, the RISA method does not always provide stable profiles in the same manner as conventional diagnostic PCR based methods. However, this feature of RISA could be considered as an advantage, since it may be capable of differentiating cultivars based on their production sites or possibly the production year. These kinds of information will be indispensable for the establishment of better traceability systems in the future.

A number of methodologies, such as the measurement of stable isotope compositions and the profiles of volatile compounds, have recently been proposed as potential biological markers that could be used in traceability assessments (Cornu et al. 2001, Schwertl et al. 2005). However, these methods are not routinely applicable to all agronomic products and require special analytical facilities. In contrast, RISA is applicable to any type of biological material and can be performed using the standard facilities that are used for molecular biological techniques. The wide variation in the fragment length of the amplified regions from RISA also allow for the effective visualization of the fragment patterns in either agarose (García-Martínez et al. 1999) or polyacrylamide gels, as shown by the present study. Furthermore, the RISA process could easily be automated if necessary (Ranjard et al. 2001).
In conclusion, the present analyses indicate that RISA profiles are sufficiently stable to discriminate between commercially supplied forage crop seeds. Hence, these profiles may be useful not only in the detection of microbes, but also in the identification of unique signatures for different cultivars of forage crops, which may then be applied to traceability analysis. RISA could therefore be adopted as a complementary tool to conventional PCR markers for agronomic product traceability.

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Literature Cited