The *trn*L-F plastid DNA characters of three *Poa pratensis* (Kentucky bluegrass) varieties

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ABSTRACT

The characterization of crop cultivars (varieties) will come to depend increasingly on molecular characters in addition to traditional morphological and agronomic characters. Three cultivars of Kentucky bluegrass (*Poa pratensis* L.), developed by the Plant Breeding Station Hladké Životice (PBHŽ), were characterized using sequences and PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) patterns from the non-coding plastid *trn*L-F region (*trn*L intron, 549 bp, and *trn*L-*trn*F intergenic spacer [IGS], 344 to 364 bp). These characters could be readily and repeatably determined not only for mature plants, but also for seedlings (less than 12 weeks old), which are difficult to distinguish morphologically. The method is quick and sensitive. When restricted with a combination of *Bsa*J I and *Bsm* I, cultivar Slezanka has one major band, Moravanka has two, and Harmonie has three. When restricted with *Alu* I, the heaviest band migrates most slowly for Slezanka. It is expected that many Kentucky bluegrass cultivars will share the same *trn*L-F sequence, so these characters alone are not sufficient for variety identification.

Keywords: cultivar characterization; Harmonie; Moravanka; non-coding plastid DNA sequence; Slezanka; *trn*L intron; *trn*L-*trn*F intergenic spacer; turf grass

Molecular methods are becoming increasingly important in the characterization of crop varieties. The advantages of most molecular methods are that they are independent of plant age, tissue type, and growing season; they eliminate environmental effects (Tyrka 2002); they provide large numbers of characters (Caceres et al. 2000); they require small sample sizes (Dinelli and Lucchese 1999); and they are relatively faster to perform than morphological and agronomic feature analyses (Budak et al. 2004). Examples of successful molecular cultivar characterization studies in grasses include whole protein electrophoresis in ryegrass (Dinelli and Lucchese 1999) and wheat (Skylas et al. 2001), RFLP in tall fescue (Xu et al. 1994) and creeping bentgrass (Caceres et al. 2000), amplified fragment length polymorphism – AFLP in wheat (Tyrka 2002), and simple sequence repeat markers – SSR and sequence-related amplified polymorphism – SRAP in buffalograss (Budak et al. 2004).

Poa pratensis is widely distributed in the temperate northern hemisphere. It is one of the most economically important grasses, excepting those for human consumption (Soreng and Barrie 1999).

It is used extensively in lawns, pastures, and reclamation projects and has more than 220 recognized cultivars (OECD 2003).

The plastid genes *trn*L (UAA) and *trn*F (GAA) code for transfer RNA and are interspersed with about one thousand base pairs of non-coding sequence (Taberlet et al. 1991). Ridgway et al. (2003) used PCR-RFLP of the *trn*L intron to identify grass roots, including *Poa*, to genus or species. Brysting et al. (2000) used *trn*L-F sequences to determine the maternal parent of a *Poa* hybrid.

In this paper, *trn*L-F sequence for three *P. pratensis* cultivars developed by PBHŽ (Moravanka, Slezanka, and Harmonie) is reported. PCR-RFLP markers subsequently designed from these sequences are discussed.

MATERIAL AND METHODS

Slezanka was first registered in 1987 and is registered in the Czech Republic, Slovakia, Switzerland, Germany, Slovenia, and Poland. It is a forage variety with slow spring growth, high biomass, dependable

seed yield, high tillering ability, and good disease resistance. Recently, it has been successful under trial in Canada and the USA.

Moravanka was registered in 1991 in the Czech Republic and Slovakia. It is a typical turf variety suitable for all lawn types, particularly high-impact areas. It is light green and has long rhizomes. It has excellent lawn properties and good perenniality and disease resistance.

Harmonie was registered in 2002 in the Czech Republic and Slovakia. It is dark green and forms dense sod. It has excellent lawn and turf properties and persistency. It is fully apomictic and has good disease resistance.

Mature plant DNA was from sod collected in PBHŽ field cultures and cultivated in flowerpots in the Brno Botanical Gardens. Seedling DNA was from pooled leaves of 6 to 24 seedlings (5 to 11 weeks old) from PBHŽ seed. The DNeasy Plant Mini Kit (Qiagen) was used to extract DNA from up to 100 mg of fresh leaf tissue disrupted in liquid nitrogen.

The *trn*L-F region was amplified using primers c (cgaaatcggtagacgctacg) and f (atttgaactggtgacacgag) (Taberlet et al. 1991). Reactions, optimised according to Cobb and Clarkson (1994), were in a final volume of 25 µl: 1X *Taq* buffer, 5pmol of each primer, 2.5mM $MgCl_2$, 0.2mM of each dNTP, ~25 ng genomic DNA, and 0.5 U of *Taq*-Purple DNA polymerase (TopBio$_{s.r.o.}$, Prague, Czech Republic). Reactions were overlaid with mineral oil. PCR was on a MiniCycler PTC-150-25 with heated lid (MJ Research, Inc., MA, USA): 95°C for 5 min; 30 cycles of 95°C for 1 min, 54(56)°C for 30 s, and 72°C for 1 min; 72°C for 5 min; then 4°C. For seedling DNA, the annealing temperature was increased to 56°C because this improved readability of the last 100 bases of the *trn*L-*trn*F IGS. PCR products were electrophoresed on an agarose gel and purified from the gel using the Sigma GenElute kit (mature plants) or QIAquick Gel Extraction Kit (seedlings). Sequencing was on an ABIPrism Model 310, Version 3.0 automated sequencer at the Laboratory of Functional Genomics and Proteomics, Masaryk University, using primer f and internal primer d (ggggatagagggacttgaac) and sometimes primer c and internal primer e (ggttcaagtccctctatccc) (Taberlet et al. 1991). Sequences generated by primers d and f were converted to their complements using the program Invert (Holt, unpublished work, 2001). Sequences were aligned using the online program BLAST 2 Sequences (Tatusova and Madden 1999) and adjusted by hand, using colored sequencer printouts and the program ABIView (Klatte 1996).

Sequence differences found with BLAST 2 Sequences (Tatusova and Madden 1999) were analyzed for restriction endonuclease recognition using the online program Webcutter 2.0 (Heiman 1997). Selected enzymes were *Alu* I, *Bsm* I, and *Bsa*J I.

Restriction (50 µl total volume) was carried out on 25 µl of crude PCR reaction product, pipetted out from under the oil into a fresh tube. For mature plants, 12.5 U *Bsm* I, 6.25 U *Bsa*J I, and 25 U *Alu* I in NEB2 buffer were used; for seedlings, 5 U *Bsm* I and *Bsa*J I and 10 U *Alu* I. *Bsm* I and *Bsa*J I were used in a double digest, *Alu* I in a separate assay. Restriction enzymes and buffers were from New England BioLabs$_{Inc}$.

RESULTS

NCBI Entrez Nucleotides database GenBank Accession numbers of the obtained sequences (*trn*L intron, *trn*L-*trn*F IGS) are: Moravanka AY061949, AY061953; Slezanka AY061950, AY061955; and Harmonie AY061951, AY061956 (Figure 1).

The PCR-RFLP patterns created when the cultivars were digested with *Bsa*J I/*Bsm* I and *Alu* I are shown in Figure 2. The point mutation in Slezanka at position 344 in the *trn*L intron is recognized by restriction endonuclease *Bsm* I, which cuts when the mutation is not present (band S1 or bands M1 and M2). The point mutation in Slezanka at position 349 in the *trn*L intron is not recognized by a restriction endonuclease. The 20-base deletion in Slezanka, starting at position 7 in the *trn*L-*trn*F IGS, is recognized by restriction endonuclease *Alu* I, which cuts when the repeat is present (band S1 or band M1/H1). The point mutation in Harmonie at position 192 in the *trn*L-*trn*F IGS is recognized by restriction endonuclease *Bsa*J I, which cuts when the mutation is present (bands H1 and H3 or band M1).

One seedling bulk each was made in 2001 and investigated with both PCR-RFLP and sequencing. The Moravanka bulk clearly showed a mixture of Moravanka and Slezanka sequences. Because Moravanka and Slezanka are not related and the Slezanka haplotype is very rare (2 plants in 135 tested ecotypes, SDSH, unpublished data), the result seemed improbable. Because the two cultivars had been sown in adjacent pots, accidental contamination seemed possible. The bulks were repeated in 2002. One bulk each was made from Slezanka and Harmonie and six were made from Moravanka. All bulks were tested with PCR-RFLP, and some were sequenced. None showed any evidence of unexpected sequence.

DISCUSSION

Plastid DNA apparently does not undergo recombination, and plastids are usually maternally

*trn*L 5' exon

```
M cgaaatcggt agacgctacg gactt  25
```

*trn*L intron

BsaJ I BsaJ I

```
M gattgtattg agccttggta tggaaacctg ctaagtggta acttccaaat tcagagaaac cctggaatta  70
S .......... .......... .......... .......... .......... .......... ..........
H .......... .......... .......... .......... .......... .......... ..........


M aaaaagggca atcctgagcc aaatccgtgt tttgagaaaa caaggggtt ctcgaactag aatacaaagg 140
S .......... .......... .......... .......... .......... .......... ..........
H .......... .......... .......... .......... .......... .......... ..........
```

Alu I

```
M aaaaggatag gtgcagagac tcaatggaag ctgttctaac gaatcgagtt aattacgttg tgttgttagt 210
S .......... .......... .......... .......... .......... .......... ..........
H .......... .......... .......... .......... .......... .......... ..........
```

Alu I

```
M ggaattcctt cgaaattcta aaaagaaggg ctttatacag ctaataaaca cgtatagata ctgagatagc 280
S .......... .......... .......... .......... .......... .......... ..........
H .......... .......... .......... .......... .......... .......... ..........
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Bsm I

```
M aaacgattaa tcacagagcc catattataa tataatattg gttctttatt cttttttaga atgcaatttg 350
S .......... .......... .......... .......... .......... .......... ...a....g.
H .......... .......... .......... .......... .......... .......... ..........


M aaatagaaat gattatgaaa taaaaaattc ataatttttt tagaattatt gtgaatccat tccaatcgaa 420
S .......... .......... .......... .......... .......... .......... ..........
H .......... .......... .......... .......... .......... .......... ..........


M tatttagtaa tcaaatcctt caatttaaag ttttgaggtc tttaaaaaag tggattaatc ggacgaggac 490
S .......... .......... .......... .......... .......... .......... ..........
H .......... .......... .......... .......... .......... .......... ..........


M aaagagagag tcccattcta catgtcaata ctgacaacaa tgaaatttct agtaaaagg 549
S .......... .......... .......... .......... .......... .........
H .......... .......... .......... .......... .......... .........
```

*trn*L 3' exon

```
M aaaatccgtc gactttataa gtcgtgaggg ttcaagtccc tctatcccca  50
```

*trn*L-*trn*F IGS

Alu I

```
M aaccctcctt tattccctaa cttatagctt tattccctaa cttatagtat ttatcctctt tttttctttt  70
S .......--- ---------- -------... .......... .......... .......... ..........
H .......... .......... .......... .......... .......... .......... ..........
```

Alu I

```
M tatcaatggg tttaagattc attagctttt tcattctact ctttcacaaa ggagtgcgaa gagaactcaa 140
S .......... .......... .......... .......... .......... .......... ..........
H .......... .......... .......... .......... .......... .......... ..........


M tggatcttat cctagaatat atttcttttt tattagagta tcgggaagga atcccggtta ttcaatctat 210
S .......... .......... .......... .......... .......... .......... ..........
H .......... .......... .......... .......... .......... .c........ ..........
```

BsaJ I

```
M tttttcagta ttattaagta aaccatgtac aatgcatagg actactcccc cgttttcaaa tttagaatt 280
S .......... .......... .......... .......... .......... .......... ..........
H .......... .......... .......... .......... .......... .......... ..........


M tgaaatactt tatttaattg attttttagt ccctttaatt gacatagata caaatactct actaggatta 350
S .......... .......... .......... .......... .......... .......... ..........
H .......... .......... .......... .......... .......... .......... ..........


M tgcacaagaa aag 363
S .......... ...
H .......... ...
```

*trn*F exon

Alu I

```
M gtcaggatag ctcagttggt agagcagagg actgaaaatc ctcgtgtcac cagttcaaat  60
```

Figure 1. *trn*L-F sequences for *P. pratensis* cultivars Moravanka (M), Slezanka (S), Harmonie (H). Primer sequences are underlined. Moravanka is the reference specimen; dots indicate matching nucleotides. A deletion is indicated with hyphens. For coding regions, only the reference sequence is shown. Labels and arrows indicate restriction sites: full arrows variable sites and thin arrows invariable sites.
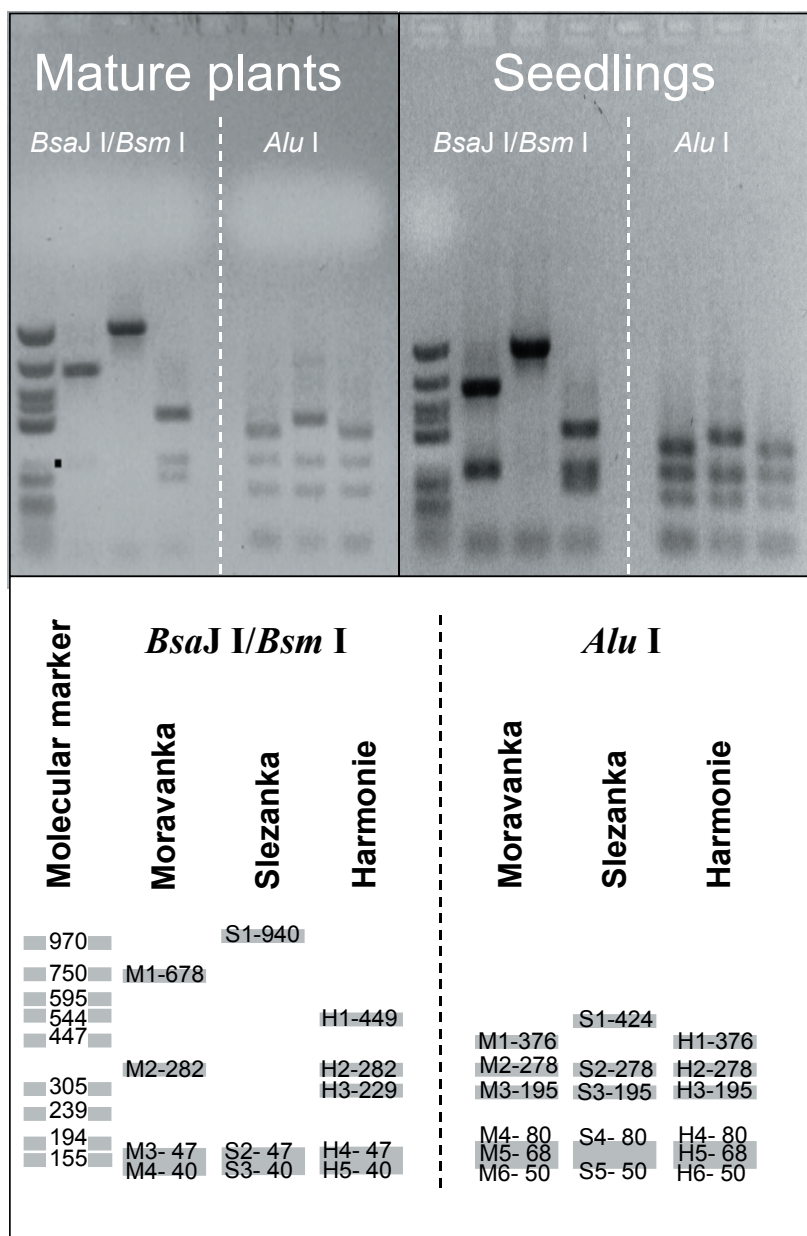
Figure 2. Restriction analyses. The legend below the photographs shows their organization and fragment sizes. Mature plants (left) is DNA from single mature individuals (March 28, 2001); seedlings (right) is from pooled leaves of 9–13 seedlings (39 days old; April 7, 2002). Left of the dotted line is a *Bsa*J I/*Bsm* I double digest. It results in 47 and 40 bp fragments in all three cultivars. Slezanka has only one large fragment, S1. *Bsm* I cuts this band in both Moravanka and Harmonie, resulting in M1 and M2/H2. In Harmonie, *Bsa*J I cuts M1, resulting in H1 and H3. A black dot indicates the very faint M2 from the mature plants *Bsa*J I/*Bsm* I digest. Right of the dotted line is an *Alu* I digest. Moravanka and Harmonie have identical patterns. S1 is slightly heavier than M1/H1. Moravanka and Harmonie have an extra 68 bp band (M5/H5), which is not visible. The predicted fragment sizes, calculated from known sequence, and the molecular marker weights given by the supplier do not perfectly agree.

inherited in angiosperms (Birky 1995, Testolin and Cipriani 1997). Thus, plastid sequence characters can be especially meaningful for cultivars derived from one or a few maternal individuals, particularly those maintained vegetatively or apomictically. *Poa pratensis* spreads by rhizomes (Soreng and Barrie 1999) and produces seed mainly through aposporous apomixis (Mazzucato et al. 1996).

Although reproduction modes can vary, even in cultivars believed to be highly apomictic (Wilton et al. 1972), most individuals from a single culti-var will probably be of the same maternal line, even if they are not genetically identical. Plastid sequence characters are much less likely to be meaningful in strictly outbreeding grasses like ryegrass (Dinelli and Lucchese 1999), creeping

bentgrass (Caceres et al. 2000), and tall fescue (Xu et al. 1994) or dioecious grasses like buffalograss (Budak et al. 2004). In these cases, each cultivar is a heterogenous population of genotypes, and it is highly unlikely that different cultivars each have a single, separate maternal ancestor.

The three *trn*L-F sequences were different, but had low variability. This is expected, because the nucleotide substitution rate in plastid DNA is low (Testolin and Cipriani 1997). This means that these sequences are likely to be uniform across the entire cultivar and remain characteristic of the cultivars, but that unique sequences for each existing *P. pratensis* cultivar should not be expected. Comprehensive characterization would require additional characters.

The PCR-RFLP markers are reproducible and quickly determined and interpreted. It is unlikely a future mutation will affect the ten bases (from a thousand) that result in the *Bsa*J I/*Bsm* I patterns. The 20-base deletion in Slezanka (recognized by *Alu* I) may be prone to parallel occurrence, since it removes a perfect copy, a theoretical precursor for slipped-strand mispairing (Levinson and Gutman 1987). The *Alu* I pattern differences are subtle enough to require a standard of known sequence (Figure 2). However, the one-, two-, or three-major-fragment patterns of *Bsa*J I/*Bsm* I are recognizable even without a known standard.

A variation in the approach described here, in which a known region of sequence was amplified but then screened with restriction endonucleases without foreknowledge of actual sequences present, was undertaken successfully by Lee et al. (2004) in pear cultivars. The PCR-RFLP approach described in this paper was successfully used by Ridgway et al. (2003) to determine grass roots. Although most roots could be ascribed to a particular species using only the *trn*L intron and one to three restriction endonucleases, some species pairs had identical PCR-RFLP patterns. PCR-RFLP markers are limited primarily by the fact that few cultivar-specific sequences are known. Restriction endonucleases are also relatively expensive, only a single region of the genome can be studied at a time, and many mutations cannot be recognized with this method.

The major difference between the two experiments in Figure 2 is that PCR was more successful for seedling bulks. The fresh, tender seedlings probably yielded higher quality DNA. Therefore, all bands are more visible. This is particularly evident for light-weight bands, such as M2. This band is barely visible for mature plants, but very clear for seedlings.

Unique *trn*L-F sequences were found for three *P. pratensis* cultivars. They are likely to be reliable, unvarying characters of these cultivars, though they may not be unique to these cultivars alone. It is possible to quickly recognize these cultivars using restriction endonucleases. This holds true for DNA extracted from juvenile individuals, which do not exhibit cultivar-specific morphological characteristics and would be difficult to properly ascribe to a cultivar using traditional methods. This method requires detailed prior sequence knowledge but can be efficient and useful for solving certain cultivar identification problems.

Acknowledgements

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ABSTRAKT

Charakterizace tří odrůd lipnice luční na základě sekvence plastidové DNA v úseku *trn*L-F

Charakterizace odrůd se bude stále více opírat o molekulární markery, které doplní tradiční a doposud užívané morfologické a agronomické znaky. Tři odrůdy lipnice luční, vyšlechtěné ve šlechtitelské stanici Hladké Životice (PBHŽ), byly charakterizovány pomocí sekvence DNA a PCR-RFLP (polymorfní restrikční fragmenty z produktů PCR reakce) profilů pro nekódující plastidový DNA úsek *trn*L-F (*trn*L intron, 549 pb, a *trn*L-*trn*F intergenový spacer [IGS], 344 až 364 pb). Tyto znaky byly zjištěny opakovaně a spolehlivě nejen u dospělých rostlin, ale i u semenáčků (mladší než 12 týdnů), které jsou morfologicky těžce rozlišitelné. Metoda je rychlá a citlivá. Kombinace restrikční endonukleázy *Bsa*J I a *Bsm* I štěpí Slezanku na jeden hlavní proužek (fragment), Moravanku na dva a Harmonii na tři. Restrikční endonukleáza *Alu* I vyštěpí největší fragment u odrůdy Slezanka. Je však pravděpodobné, že většina odrůd lipnice luční bude mít sekvenci úseků *trn*L-F stejnou, takže tento znak samostatně je pro jistou identifikaci všech odrůd nedostatečný.

Klíčová slova: charakterizace odrůd; Harmonie; Moravanka; nekódující plastidová DNA sekvence; Slezanka; *trn*L intron; *trn*L-*trn*F intergenový spacer; tráva pro trávníky

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