

## Development and testing of novel chloroplast markers for perennial ryegrass from *de novo* sequencing and *in silico* sequences

S. McGrath<sup>1,2</sup>, T.R. Hodkinson<sup>2</sup> and S. Barth<sup>1</sup>

<sup>1</sup>Teagasc Crops Research Centre, Oak Park, Carlow, Ireland; <sup>2</sup> Department of Botany, University of Dublin, Trinity College, Ireland, E-mail: smcgrath@oakpark.teagasc.ie

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**Introduction** Chloroplast DNA is uniparentally inherited and non-recombinant in *Lolium perenne*. These properties make the chloroplast genome a useful tool for studying inter- and intra- specific relationships. Previous genetic studies on *L. perenne* have used chloroplast sequence data. However, the relative lack of variation in the chloroplast genome limits its usefulness for analysis at the single individual level within a species. However, chloroplast SSR markers have recently been shown to have high levels of polymorphism (Provan *et al.*, 2004). This is the first study to design and employ such markers for *L. perenne*. The objectives of this study are (1) to design and (2) optimise novel chloroplast SSR markers and (3) use them to analyse variation and diversity in *L. perenne* and related grass species.

**Materials and methods** DNA from *L. perenne*, *L. multiflorum*, *Festuca pratensis* and *Festuca arundinacea* was extracted using a modified CTAB method. These DNA samples were amplified and sequenced using the primers c and f for the *trn-L* intron and *trn-F* intergenic spacer region (Taberlet *et al.*, 1991), and the primers 1R and 2R for the *atpB-rbcL* intergenic spacer region (Samuel *et al.*, 1997), on an ABI 310 automated DNA sequencer. Further plastid DNA sequences from *L. perenne* and related species were also obtained from GenBank. All *de novo* and *in silico* sequences were analysed for microsatellite motifs using a modified version of the MISA perl script (Thiel, 2003). 27 pairs of primers were designed using Primer 3 software (Rozen & Skaletsky, 1998). PCR conditions were optimised to amplify loci for each primer pair. Of these, 19 were chosen for further analysis. 12 primer pairs were optimised as 5'-fluorescently labelled primers. These primers were used to amplify DNA from various populations of *L. perenne* and other grass species. PCR products were analysed using an ABI 3100 automated DNA sequencer and sized using GeneMapper™ v3.0 software.

**Results** The optimised primers showed little variation within *Lolium* species (Table1). The markers amplified across a broad range of grass species, e.g. from nine species for marker TeaCpSSR1 to 23 for marker TeaCpSSR11. Some of the alleles were shared between *L. perenne* and other species. Certain alleles were species specific, e.g. an allele of marker TeaCpSSR1 was specific to *Arundo donax*.

**Table 1** Example for the allelic range of a set of four chloroplast SSR markers

Primer name	Gene region	<i>L. perenne</i>		Other grass species	
		# alleles	Size range in b.p.	# alleles	Size range in b.p.
TeaCpatpssr2	<i>atpB-rbcL</i>	4	217 - 229	3	227 - 230
TeaCpssr1	<i>23S-5S</i>	3	193 - 196	5	193 - 200
TeaCpstrsr2	<i>trnL-trnF</i>	5	178 - 195	4	176 - 200
TeaCpssr11	<i>trnV</i>	3	193 - 196	5	193 - 201

**Conclusions** As a next step, these optimised primers will be used to investigate the phylogeography of perennial ryegrass including mode and time-point of introduction of *L. perenne* into Ireland. They can also be used to test for maternal inheritance in festulolium hybrids. These primers are also a valuable tool to control cross pollination and selfings in breeding programs. Furthermore, their greatest potential lies in species differentiation, such as in seed testing or at the taxonomic level.

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