*Plant Molecular Biology Reporter* **21:** 249–257, September 2003
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*Commentary*

# Successful Amplification of Rice Chloroplast Microsatellites From Century-Old Grass Samples From the Park Grass Experiment

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**Abstract.** We report the successful amplification of microsatellite markers for the chloroplast genome from century-old samples of 2 grasses growing in the Park Grass Experiment (PGE): *Anthoxanthum ordoratum* and *Festuca rubra*. This opens the possibility of establishing a long-term genetic time series for the PGE, which began in 1856 and is believed to be the oldest ecological experiment in existence. Although the plant samples used were not originally prepared or stored with molecular analysis in mind, the hexadecyltrimethylammonium bromide (CTAB) method of DNA extraction was successfully used. Obtained DNA was degraded but could be amplified by means of PCR. It produced bands around the expected size for chloroplast microsatellite primers derived from rice. When sequenced, bands showed good homology with sequences from rice chloroplast genomes listed in GenBank (accession No X15901).

**Key words:** *Anthoxanthum ordoratum*, archived plant samples, chloroplast microsatellites, *Festuca rubra*, Park Grass Experiment

**Abbreviations:** AFLP, amplified fragment length polymorphism; CTAB, hexadecyltrimethylammonium bromide; ISSR, inter–simple sequence repeat; PGE, Park Grass Experiment.

## Introduction

Making long-term ecological observations is important to understanding climate change. These observations reveal that the warming of the Earth's climate during the twentieth century has had detectable and widespread effects on the distribution, abundance, and phenology of plant and animal species (Parmesan and Yohe, 2003). Evolutionary effects are also to be expected (Thomas et al., 2001); however, as anthropogenic climate change antedates modern genetics, the long-term genetic time-series data that might signal climatically-induced evolutionary change will need to be obtained by means of retrospective analysis of archived samples where these can be found. In this commentary, we report the successful

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amplification of genetic markers for the chloroplast genome from century-old samples of 2 grasses growing in the Park Grass Experiment (PGE). This opens the possibility of establishing a long-term genetic time series for the PGE, which is almost certainly the oldest ecological experiment in existence.

Begun in 1856, the PGE continues to produce unique ecological insights into the long-term dynamics of the meadow plant communities it contains. Findings suggest that climate (Cashen, 1947; Silvertown et al., 1994), mating systems, and genetic diversity may influence species' dynamics (Dodd et al., 1995; Silvertown et al., 2002). Local adaptation to fertilizer and liming treatments applied to PGE plots has been demonstrated in *Anthoxathum odoratum*, one of the species analysed here (Davies and Snaydon, 1976). Establishing a genetic time series in parallel with the ecological time series spanning 1856 to the present should now be possible. Because the chloroplast genome is haploid, maternally inherited in grasses, and unable to recombine, chloroplast microsatellite markers are expected to be particularly sensitive to population bottlenecks, seed dispersal, and other demographic processes (Provan et al., 2001). It should be possible to interpret a genetic time series for this genome, when we have one, in terms of recorded ecological and climatic events in the history of the PGE.

This report is also of methodological interest because success was achieved with samples that were not collected or stored with molecular genetic analysis in mind. A number of authors have reported DNA extraction from dried material (Rogers and Bendich, 1985; Singh et al., 1999; Cheng et al., 1997; Fu et al., 1998; Grunewald et al., 1991; Csaikl et al., 1998) and from archeological and paleontological specimens (see Paabo, 1993, and Wayne et al., 1999, for reviews). However, in most cases, the material was either handled and dried in such a way as to preserve the plant tissues, or samples were large enough so that repeated purifications could be performed to obtain good quality DNA. In contrast, archived PGE leaf samples are, at most, 30-40 mm long and sometimes only 4-5 mm long. They are also brittle and browned with age, which presents formidable problems for DNA extraction. In addition to the usual problems encountered with plant materials (e.g., large quantities of polysaccharides and silicates that are difficult to remove), there are the problems of cellular degradation and the build up of polyphenols with age. The potential amount of DNA in each fragment is small, and separating it from cellular debris is complicated; however, repeated purifications are likely to lose the small amount present.

## Materials and Methods

### *Sampling*

Since 1856, dried plant material has been subsampled from the twice-yearly hay cut taken from each plot of the PGE and stored in airtight tins in an archive at Rothamsted Experimental Station in Harpenden, England, where the experiment is located. We took leaf samples of *Festuca rubra* L. and *Anthoxanthum odoratum* L. from the archive for selected years and plots and, for comparison, analysed fresh and dried samples of these and 7 other grass species (*Agrostis stolonifera* L., *Alopecurus pratensis* L., *Briza media* L., *Bromus hordeaceus* L., *Dactylis glomerata* L., *Holcus lanatus* L., *Agrostis capillaris*) commonly found in

Park Grass. Modern samples were used as references for identification and to screen for molecular markers.

Samples of hay (~ 5.0 g) from plots $4^2$ and 10 with and without lime were taken from the PGE archive from approximately 25, 50, 75, and 100 y ago. Each storage tin was opened and the top layer of hay was removed to a tray, the sample was taken from the centre of the tin, the top layer was replaced, and the tin was resealed.

Fragments of hay were identified to species under a low-power light microscope from gross features (e.g., presence or absence of hairs, veining patterns) using the reference samples for comparison. *Anthoxanthum odoratum* was retrieved from the unlimed subsections of plots where it was abundant: 4 (1900); 4.2 (1925, 1949); 4.2d (1973); 10 (1900, 1925, 1947); and 10d (1973). *Festuca rubra* was sampled from unlimed subplots of plots 4 and 10 (1900) and from limed subplots: 4.2 (1925, 1949); 4.2a (1973); 10 (1925, 1948); and 10a (1974).

In addition to archive samples, grasses of the most common Park Grass species (*Bromus hordeaceus* L., *Alopecurus pratensis* L., *Holcus lanatus* L., *Agrostis stolonifera* L.) were grown in the greenhouse from seeds collected from Park Grass. Leaf samples were collected as follows: (1) snap frozen in liquid nitrogen and stored at -80°C, (2) wilted 1 h (to simulate field conditions), chopped, and dried overnight at 80°C, or (3) wilted 1 h, chopped, left at 4°C overnight, and dried over the weekend (2 cycles of dryer overnight at 80°C, returning to room temperature during the day).

All samples taken by methods 2 and 3 were stored in an airtight tin at room temperature for 6 wk before DNA extraction to simulates the way Park Grass samples have been collected and stored since 1960. Simulating the pre-1960 collection method was not possible because plots were cut and left to dry as hay in the field.

### *DNA extraction*

We compared several methods of DNA extraction recommended for dried or ancient specimens with the hexadecyltrimethylammonium bromide (CTAB) method, which is normally only used for fresh material (Stacey and Isaac, 1994), and found that the latter worked best. Using this method, total DNA was extracted from greenhouse-grown grasses. Dried leaf material was ground with liquid nitrogen in a 1.5-mL Eppendorf tube and combined with 600 µL of CTAB buffer (0.1 M Tris, 0.02 M EDTA, 1.4 M NaCl, 2% CTAB, 50 mM DTT [Dithiothreitol]) and 100 µL of chloroform-octanol (24:1). Samples were incubated for 30 min at 65°C. After cooling to room temperature, 600 µL of chloroform-octanol (24:1) was added, and samples were spun at 15,000 *g* in an Eppendorf bench centrifuge for 7 min. The top layer was removed and 1.6 vol of 95% ethanol was layered over it. Samples were placed on ice for 1 h, mixed, and centrifuged at 8000 *g* for 10 min to pellet the DNA. Pellets were washed in 70% ethanol, dried, and rehydrated in 10 mM Tris–0.1 mM EDTA (pH 8).

In addition to the CTAB method described above, the methods of Singh et al. (1999), Rogers and Bendich (1985), Fu et al. (1998), and Cano et al. (1993) were used to extract archive samples from 1947-1949 and 1973-1974. Methods used were as described in each paper without modifications.

Total DNA was extracted from archived samples of up to 100 y of age using the CTAB method with the following modifications:

- Sterile acid-washed sand was used instead of liquid nitrogen to grind samples from 1900 and 1925.
- Samples were extracted with and without the addition of 1% PVP 40 (polyvinylpyrrolidone MW40000) to the CTAB buffer to assist in polyphenol removal (John, 1992).
- Archived samples were extracted in a lamina flow cabinet to eliminate contamination from the surrounding area.
- Archived samples were washed in 70% ethanol prior to extraction to remove any contamination with microorganisms or hay fragments from other individuals or species and dried in the flow hood.
- A negative control was put through all extraction procedures to check that contamination was not introduced from the equipment or extraction buffers.

DNA from all methods was visualised on 0.8% agarose gels stained with ethidium bromide using a 100-bp ladder for size comparison and 12.5-50 ng of uncut λDNA for quantity comparison.

*DNA analysis - chloroplast microsatellites*

PCR was performed in 50-μL reactions containing 50 ng of template DNA, 10 pmoles of each primer, 200 uM of each dNTP, standard buffer (supplied with the *Taq*) containing 1.5 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase in a Techne Genius thermal cycler, as follows: initial denaturation at 94°C for 4 min; 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 5 min (Ishii and McCouch, 2000).

For archived samples, the PCR mixture was reduced from 50 μL to 20 μL (to increase template concentration), and the number of cycles increased from 35 to 40. Chloroplast microsatellite primers RCt6 forward and reverse and RCt7 forward and reverse developed for rice by Ishii and McCouch (2000) were obtained from MWG Biotech. Bands were visualised on a 6% polyacrylamide sequencing gel by using silver staining.

Bands were recovered from rehydrated silver-stained gels, added to a PCR mix containing the relevant primers, and amplified. Obtained fragments were cloned using the Promega P-Gem-T-easy kit and sequenced on an ABI sequencer using the BigDye sequencing kit (Perkin Elmer). Sequences were aligned using GENEDOC software and matched to the original rice chloroplast sequences in a BLAST search (Figure 1).

*DNA analysis - ISSR and AFLP*

ISSR markers (Zietkiewicz et al., 1994) and AFLP markers (Vos et al., 1995) were used to test both the greenhouse-grown and archived samples.

## Results

DNA of good quantity and quality, as visualised on agarose gels, was obtained from the greenhouse-grown grasses from all 3 sampling methods by using CTAB extraction. The quantity of DNA obtained from dried samples was less than half

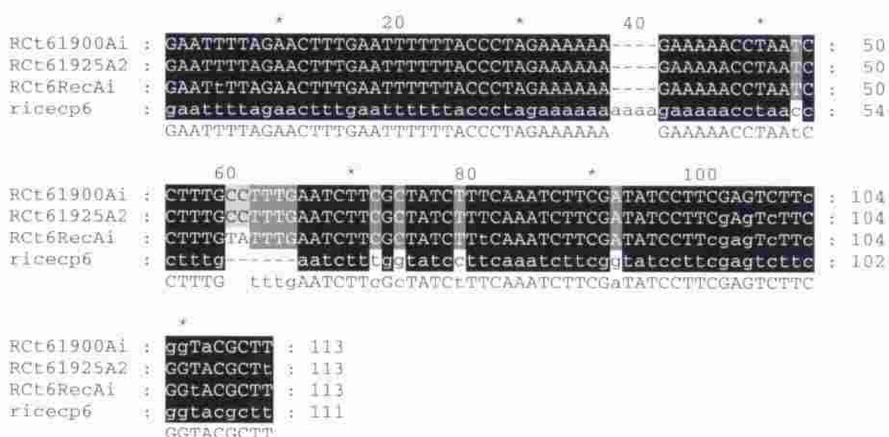



*Figure 1.* Alignment of sequences obtained with RCt6 for *Anthoxanthum odoratum* from archived (denoted by year: 1900, 1925) and recent samples compared with the corresponding rice sequence from Genbank (accession No X15901).

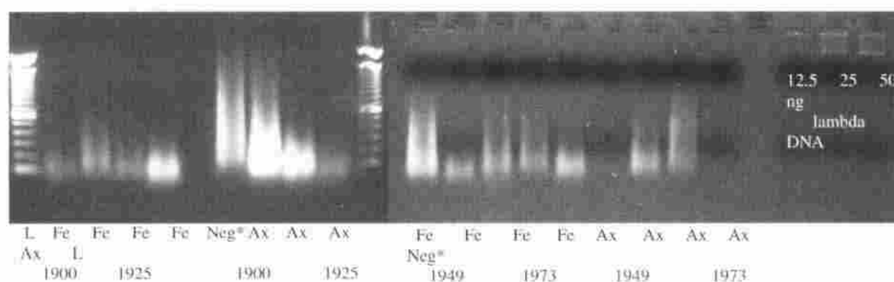
of that obtained from snap frozen samples, presumably because cellular disruption was less efficient. Dried archived samples extracted by using this method produced small quantities of poor quality DNA that was severely degraded; most of the fragments were in the 100- to 600-bp size range (Figure 2).

Four additional methods were tested to try to obtain better quantity and quality DNA from archived samples. Of these, DNA extracted by the methods of Rogers and Bendich (1985), Fu et al. (1998), and Cano et al. (1993) was not visible on a 0.8% agarose gel stained with ethidium bromide, even when half the total sample was loaded. The method of Singh et al. (1999) gave visible DNA but produced between one half and two thirds of the quantity obtained from the CTAB extraction, with a similar amount of degradation.

The 2 modifications of the CTAB method tested were (1) grinding with acid-washed sand instead of liquid nitrogen and (2) adding PVP to the CTAB buffer to assist in polyphenol removal (John, 1992). Each modification increased the yield approximately 2-fold but did not reduce the amount of DNA degradation. However, increased yields made visualising small quantities of high-molecular-weight DNA in the samples possible (Figure 2).

Modifications 3-5 were introduced for quality control of archive samples. Because DNA quantities were so small, applying rigorous control measures were necessary to ensure that samples were not contaminated by other plant materials in the laboratory. Negative controls that were put through all extraction processes without the addition of plant material showed that no contamination from equipment, chemicals, or the environment had occurred.

Initially, ISSR and AFLP markers were tested as methods of DNA amplification. These were only partially successful because amplification of higher-molecular-weight bands in archive samples was not reliable. The use of




*Negative = Treated the same as the samples (ie, all the steps of the protocol and all the reagents used) but no leaf material added

*Figure 2.* DNA extracted from *Festuca rubra* and *Anthoxanthum odoratum* hay samples from the Park Grass Archive using sterile conditions (5 µL of a total of 15 µL).

dominant random markers was clearly unsuitable for archive samples because, with degraded DNA, whether the absence of a band is due to intraspecific variation (polymorphism) is uncertain. It may simply be the loss of the DNA sequence in this region of the genome. Of the available codominant markers, genomic microsatellite markers were not available for these species from the literature; however, chloroplast markers designed for rice and other cereals were (Ishii and McCouch, 2000).

Two sets of primers (one for a conserved region and the other for a variable region of the chloroplast genome) were tested with 6 grass species grown in the greenhouse (*Bromus hordeaceus*, *Anthoxanthum odoratum*, *Alopecurus pratensis*, *Festuca rubra*, *Holcus lanatus*, *Agrostis stolonifera*). Bands of the same length were obtained with all 6 species by using the conserved region (Rct7). Bands were produced in 4 of the 6 species by using the variable region (Rct6). All 4 bands were different sizes. *F. rubra* and *H. lanatus* did not amplify with this primer.

Archived samples of *A. odoratum* of up to 100 y of age produced bands of about the expected size with both primer pairs. *F. rubra* of up to 100 y of age produced bands of the expected size with primer Rct7.

*A. odoratum* bands were sequenced (accession Nos. AY243048 and AY243051) and showed good homology with sequences from rice chloroplast genomes listed in GenBank (accession No X15901) (Figure 1).

## Discussion

Drying greenhouse-grown grasses the same way that Park Grass samples have been dried since 1960 indicated that it is storage, rather than the collection method, that causes degradation. If degradation of DNA occurs while hay samples are being collected and processed, greenhouse grasses that were wilted, chopped, left in the cold room overnight, and dried over the weekend would have shown similar DNA degradation.

Little difference was found in the degradation of DNA recovered from archive samples stored for 25, 50, 75, and 100 y, indicating that initial degradation

occurs rapidly, but further degradation is slow. Even the 100-year-old hay samples yielded some high-molecular-weight DNA when a sufficient quantity of DNA was obtained (Figure 2).

Matsuo et al. (1995) found that just 2 mo storage of dried liver samples caused degradation of the DNA and, although exclusion of oxygen delayed this process, it did not completely stop it. They assumed from this that oxygen stored within the cytoplasm was responsible for continuing lytic enzyme activity. Although little atmospheric oxygen was present in the tins in which the Park Grass archived samples were stored, cytoplasmic oxygen could account for the degradation. Once the lytic enzymes had used up this oxygen, degradation was halted or continued slowly so that little difference existed between the degradation of samples stored for 25 and 100 y.

The use of ISSR and AFLP demonstrated that, although severely degraded, DNA obtained from archive samples was amenable to PCR. However, dominant markers like these are unsuitable for genotyping degraded DNA because the absence of a band could be due to the loss of the DNA sequence in this region of the genome rather than to intraspecific variation (polymorphism). In considering codominant markers, microsatellites from the nuclear genome require sequence information that is usually different for even closely related species. No nuclear microsatellite primer information was available in the literature for the 2 major species used in this study, *A. odoratum* and *F. rubra*.

The chloroplast genome is more conserved, however, and recent work has shown that repeat sequences within this genome can be variable and useful for genotyping (Powell et al., 1995) and that primers designed from one species can be used in related species to amplify the same region (Ishii and McCouch, 2000). Our use of the primers designed for rice by Ishii and McCouch demonstrates that such microsatellite markers can be used in species that are not closely related. A difference in the size of the single base repeat between rice and *A. odoratum* existed; thus, some polymorphism in the microsatellite could possibly be found among individuals of the same species (Figure 1).

By using the techniques outlined above, we have demonstrated the feasibility of extracting DNA from archived samples not specially handled to preserve DNA and have shown this DNA to be amenable to PCR. The chloroplast microsatellite results also demonstrate that markers developed for cereals can be used for grasses, including archived hay samples.

## Acknowledgments

We are grateful to the trustees of the Lawes Trust for permitting access to the archive of plant samples at Rothamsted, to Rothamsted Research for facilities, and to The Open University for funding.

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