# Genetic analysis of forage grasses based on heterologous RFLP markers detected by rice cDNAs

T. YAMADA[1] and T. KISHIDA[2]

[1] National Agricultural Research Center for Hokkaido Region, Hitsujigaoka, Sapporo, Hokkaido, 062-8555 , Japan, E-mail: Toshihiko.Yamada@affrc.go.jp

[2] Yamanashi Prefectural Dairy Experiment Station, Nagasaka, Yamanashi, 408-0021, Japan

*With 1 figure and 2 tables*

*Received March 14, 2002 / Accepted*

*Communicated by*

# Abstract

Genetic polymorphism within and between three species of forage grasses; perennial ryegrass (*Lolium perenne* L.), meadow fescue (*Festuca pratensis* Huds.) and tall fescue (*Festuca arundinacea* Schreb.) was analyzed using RFLP markers detected by rice cDNA probes developed in the Rice Genome Research Program of Japan (RGP). One hundred and ninety seven rice cDNA clones were used for hybridization to genomic DNA of forage grasses. Many of the rice cDNA clones produced no visible band or a smear with no discrete bands. Twenty three clones showed high efficiency cross-hybridization to the genomic DNA of forage grasses. Genetic variation was evaluated for five varieties and one population of forage grasses using twelve polymorphic rice cDNA RFLP probes. Genetic variability within varieties as measured by Rogers' genetic distance was considerably lower for the *F. pratensis* variety 'Tomosakae' than for the *L. perenne* and *F. arundinacea* varieties. To determine genetic diversity between varieties of different species, cluster analysis was performed using data from the twelve RFLP probes. The two accessions of *Lolium perenne* were clustered more closely together than the

three varieties of *F. arundinacea*. Two Japanese varieties of *F. arundinacea* were grouped in the same cluster. The variety-specific RFLP markers were observed among six accessions of *L. perenne*, *F. pratensis* and *F. arundinacea*. Such variety-specific RFLP markers would provide very useful tools for breeding programs such as intergeneric hybridization of *Lolium* and *Festuca* genera.

Key words: – Genetic variability – *Festuca arundinacea* – *Festuca pratensis* – *Lolium perenne* – RFLP – rice genome – variety

Perennial ryegrass (*Lolium perenne* L.), meadow fescue (*Festuca pratensis* Huds.) and tall fescue (*Festuca arundinacea* Schreb.) are very important forage grasses for temperate grassland agriculture, providing the basis for pastoral production systems (Barnes et al. 1995).

These forage grasses show gametophytic self-incompatibility and hence cross-pollinated breeding systems (Lundqvist 1962, Cornish et al. 1979). Consequently, each variety is a heterogeneous population of individual genotypes. Commercial varieties of forage grasses are synthetics developed

by polycrossing of selected parental clones (plants). The foundation clones of varieties are often selected from existing varieties or improved germplasm resources. The levels of genetic diversity within and among varieties is always a concern to researchers and plant breeders working with forage grasses. However, there is currently limited information on degrees of genetic diversity. Selections are often carried out on the basis of limited morphological criteria and agronomic performances.

Intergeneric crosses involving the genera *Lolium* and *Festuca* could potentially lead to the combination of the enhanced palatability and forage quality of *Lolium* species with the higher resistance to disease and tolerance to stress environments such as cold and drought found in *Festuca* species (Thomas and Humphreys 1991). Although crosses between members of the *Lolium* and *Festuca* genera have been performed, the development of commercial hybrid varieties has been limited. A major problem of amphidiploid breeding is the high level of homoeologous pairing between the different genomes, which leads to genetic instability and loss of hybridity in later generations. However, an introgression breeding program involving the transfer of small alien segments of *Festuca* chromatin into the background of a *Lolium* genome would provide a powerful breeding procedure as way of combining desired characters (Humphreys 1989, Humphreys and Pasakinskiene 1996).

Molecular DNA markers based on Southern hybridization such as restriction fragment length polymorphisms (RFLP) as well as PCR-based markers such as randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) have received increased attention from plant geneticists and breeders. Though RFLP analysis is highly labour-intensive compared to the other PCR-based methods, RFLP markers are highly informative in comparative mapping between related species (Kurata et al. 1994b, Devos and Gale, 1998).

The Rice Genome Research Program of Japan (RGP) has been active since 1991 and a high-density rice genetic linkage map has been constructed (Kurata et al. 1994a, Harushima et al. 1998). Detection of heterologous RFLP by rice cDNA clones provides a useful means for detection of genetic variability in forage grasses. In addition, the use of rice cDNA derived markers may be exploited by syntenic analysis between rice and forage grasses leading to gene identification and isolation based on the accumulating information from the RGP and other international rice genomic projects.

The objective of this research was to apply rice cDNA RFLP probes from the RGP to forage grasses in order to investigate genetic variation among and within varieties of grasses and to identify variety-specific RFLP markers for use in breeding programs such as intergeneric hybridization of *Lolium* and *Festuca*.

# Materials and Methods

**Plant materials and DNA extraction:** Genotypes from *Lolium perenne* (two Japanese accessions, variety 'Kiyosato' and population 'Yatsugatake D-10'), *Festuca pratensis* (Japanese variety 'Tomosakae') , *Festuca arundinacea* (Japanese varieties 'Nanryo' and 'Yamanami' and USA variety 'Kentucky 31') were used in the present study (Table 1).

Fresh leaf tissue (8 g) was sampled individually from 25 plants of each variety grown in the greenhouse. DNA of these plants was extracted by the CTAB method (Murray and Thompson 1980).

**RFLP analysis:** Hybridization analysis for RFLP was performed according to the protocol of the RGP (Kurata et al. 1994a). Southern hybridization was performed with DNAs digested with three restriction enzymes; BamHI, Bgl II, Hind III. 2 μ g of each digest was applied per lane on a 0.6 % (w/v) agarose gel and electrophoresed for 12 h. After electrophoresis, DNA was blotted

onto a positively charged Nylon membrane (Boehringer Mannheim) in 0.4 M NaOH for 4 h and the membrane washed in 2 X SSC, air dried and baked at 120 $^{o}$C for 20 min. Rice cDNA probes provided from the RGP were amplified by PCR and labelled with HRP (horseradish peroxidase) according to the protocol of ECL direct nucleic acid labelling and detection system kit (Amersham). Filters were hybridized with HRP-labelled cDNAs and detected by chemiluminescence on a Fuji X-ray film for 1-3 h. C number clones and R number clones of the RGP were selected from a callus cDNA library and a root cDNA library derived from the *japonica* rice variety, 'Nipponbare', respectively.

Data analysis: Each band of the RFLP pattern was considered as a separate variant as the possible allelic nature of the bands was unknown and scored for presence or absence by 1 or 0, respectively. Genetic distance among all possible pairs was estimated from Rogers' distance equation (Rogers 1972) which was adopted by paper of Xu et al. (1994). Average genetic distance was calculated for all pairs among 25 plants each variety. The allelic (band) frequency in variety was obtained by dividing the number of plants containing the allele (band) by the total 25 plants examined. To determine the genetic

diversity between varieties, clustering analysis was performed using Euclidean distance with a single linkage method using averaged data for allele frequency across the 25 genotypes from within each variety (Program: SYSTAT 5 for the Macintosh). A dendrogram was generated based on the data from 12 RFLP clones.

# Results

## Cross-hybridization

One hundred and ninety seven rice cDNA clones were used for hybridization to the genomic DNA of forage grass species. Many of the rice cDNA clones produced either no visible band or a smear with no discrete bands. Twenty three clones showed a high level of efficient cross-hybridization to the genomic DNA of *L. perenne*, *F. pratensis* and *F. arundinacea*. Many of the hybridized probes detected polymorphism among each variety of grass. In many of hybridized clones, RFLP bands that were detected in all individuals of an accession but were absent from all individuals of other accession were observed

among five varieties and one population of *Lolium perenne*, *Festuca pratensis* and *Festuca arundinacea*. These RFLP bands were assumed to be variety-specific RFLP markers.

## Genetic variation within varieties

Twelve RFLP clones showing polymorphism were used for the evaluation of genetic variability within grass varieties (Table 2). The average number of RFLP bands for each clone ranged from 2 (R1012, R2232) to 7 (C385, C1794). The average number of RFLP bands in the *L. perenne* variety, 'Kiyosato' was slightly higher, but other varieties showed almost the same number (4).

The variation within variety is also shown in Table 2. The two accessions of *L. perenne* showed higher within variety variability than the varieties of *F. pratensis* and *F. arundinacea*. For perennial ryegrass, population 'Yatsugatake D-10' showed higher within population variability than that of variety 'Kiyosato'. For *F. arundinacea*, 'Yamanami' showed lower within variety variability within varieties compared to the other two varieties. The *F. pratensis* variety, 'Tomosakae' showed the lowest genetic internal variability among the varieties of grasses that were compared.

## Genetic variation between varieties

The two accessions of *Lolium perenne* were clustered more closely together than the varieties of *F. arundinacea*, while the two Japanese varieties of *F. arundinacea* were grouped in the same cluster (Fig. 1). The *F. pratensis* variety, 'Tomosakae' was placed closer to the cluster that contained the *F. arundinacea* varieties.

# Discussion

The RGP has developed a high-density rice genetic linkage map with 2,275 markers using 186 $F_2$ plants from a single cross between the *japonica* variety Nipponbare and the *indica* variety Kasalath (Harushima et al. 1998) and presently this map has more than 3,000 markers (Rice Genome Research Program, http://rgp.dna.affrc.go.jp/Publicdata.html). The main source of the markers in this map has been RFLPs detected by cDNA clones prepared for expressed sequence tag sequencing from the *japonica* variety Nipponbare callus, root and shoot libraries (Sasaki et al. 1994, Yamamoto and Sasaki 1997).

Some of these rice callus and root cDNA clones of the RGP were used as probes in the present study.

Many of the RGP clones could not be used for analysis because they detected no visible bands, faint bands or only a smeared background. Only 10 % of the clones could be used for the detection of polymorphism in forage grass species. In the case of hybridization to the genomic DNA from the heterozygous parental plant of the *L. perenne* reference mapping family (p150/112) used by the International *Lolium* Genome Initiative (ILGI: Forster et al. 2001), only 17 % of the rice cDNA probes detected clear bands, while in contrast, wheat, oat and barley probes produced higher levels of clear hybridization (Jones et al. 2002). Among the Cornell anchor probe set, the levels of hybridization of rice cDNA (RZ probes) to wheat, barley and oat genomic DNA was lower than for other species such as sorghum, sugarcane and maize (Van Deynze et al. 1998). This trend follows the current taxonomic knowledge of the Poaceae family, as wheat, oat, barley and perennial ryegrass reside in a different subfamily (Pooideae) to rice (Bambusoideae ) (Soreng and Davis 1998).

Cross-hybridization of the rice cDNA probes to the genomic DNA of the selected grasses in many cases produced multiple bands. The forage grasses show DNA contents several times higher ( 4.16 pg/2C nucleus in *L. perenne* (Hutchinson et al. 1979) and 3.96 in *F. pratensis* (Seal and Rees 1982) ) than

that of rice (0.88 pg/2C nucleus) (Arumuganathan and Earle 1991). Although the majority of this variation between Poaceae genome is undoubtedly attributable to differences in the prevalence of dispersed repetitive genomic DNA (Flavell 1980), it is possible that some of the variation may arise from an increased level of gene duplication, such that rice genes may show higher copy numbers in grass genomes.

The genetic variability within an individual variety may be influenced by the number of parental foundation clones involved in the breeding process (Forster et al. 2001). This is a possible explanation for the low variability within variety 'Tomosakae' which is based on only four parental clones. Xu et al. (1994) reported that within-variety variation in *F. arundinacea* based on analysis of RFLP markers increased with the number of parental clones up to a total of 10, then remained relatively stable. The present data on higher genetic variability in variety 'Kentucky 31' coincides with the data of Xu et al. (1994). A lower genetic variability in variety 'Yamanami' was observed although 'Yamanami' is based on a large number(27) of parental clones. The parental clones of 'Yamanami' originated from only North America varieties thus may account for a lower genetic diversity in the basic breeding population. 'Yatsugatake D-10' shows higher variability than 'Kiyosato' although they are both based on 10 parental clones. Some of parental clones of 'Yatsugatake D-10' are from ecotypic germplasm of 'Kangaroo Valley' which has been

shown to be highly variable using AFLPs (Guthridge et al. 2001). Thus higher genetic diversity of 'Kangaroo Valley' may have contribute to an increased variability in 'Yatsugatake D-10'.

The dendrogram based on clustering analysis using RFLP markers reflected the known pedigree of the varieties and the differences between species. As some of the parental clones of 'Yatsugatake D-10' were derived from 'Kiyosato' plants, it is not surprising that the genetic distance between these accessions is relatively low. The two Japanese varieties of *F. arundinacea* were more closely positioned in the cluster analysis than the USA variety 'Kentucky 31'. This observation may reflect divergent selection in different countries of origin due to different environment conditions for ecotypic adaptation and different breeding criteria. The single *F. pratensis* accession is located adjacent to the *F. arundinacea* varieties. Cytogenetic analysis suggests that one of the three genomes of the allohexaploid, *F. arundinacea* is derived from a taxon closely related to contemporary *F. pratensis* (Sleper 1985), supporting a close taxonomic relationship between these species.

A number of variety-specific RFLP markers were found among six accessions of *L. perenne*, *F. pratensis* and *F. arundinacea* in the pesent study. Such variety-specific markers may prove useful for breeding programs such as intergeneric hybridization of *Lolium* and *Festuca* genera. We have crossed

genotypes from the *L. perenne* variety ‘Kiyosato’ and population ‘Yatsugatake D-10’ with plants of the *F. arundinacea* variety ‘Nanryo’ and backcrossed these intergeneric hybrids with genotypes from a tetraploid *L. perenne* variety (unpublished). In this case *F. arundinacea* variety ‘Nanryo’ specific markers may be useful to monitor gene introgression for intergeneric hybridization between *L. perenne* and *F. arundinacea*.

## Acknowledgements

The authors thank Drs. Y. Nagamura, M. Yano and T. Sasaki, the Rice Genome Research Program of Japan, National Institute of Agrobiological Sciences, for advice and encouragement. The authors also thank Dr. John W. Forster, Plant Biotechnology Centre, Agriculture Victoria, La Trobe University, Australia, for critical reading and suggestions for improving the manuscript. This work was supported by the funds from the Ministry of Agriculture, Forestry and Fisheries of Japan.

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# Legend of figure

Fig. 1: Phylogenetic tree showing the relationships between of six accessions of three species of *Lolium* and *Festuca* based on the analysis of 12 polymorphic RFLP loci detected by rice cDNA probes of the RGP.

Table 1: Breeding history of six accessions of *Lolium perenne* , *Festuca pratensis* and *Festuca arundinacea* used in the present study

| Accessions | Number of parental clones | Breeding materials |
|---|---|---|
| *L. perenne* | | |
| Kiyosato | 10 | UK, Dutch, New Zealand and Australian varieties |
| Yatsugatake D- | 10 | 'Kiyosato' and Australian ecotypic variety 'Kangaroo Valley' |
| *F. pratensis* | | |
| Tomosakae | 4 | European varieties and Japanese ecotypes |
| *F. arundinacea* | | |
| Yamanami | 27 | USA and Canadian varieties |
| Nanryo | 8 | European, USA and Japanese varieties and populations |
| Kentucky 31 [1] | - | Ecotypic population from the state of Kentucky, USA |

[1] Not synthetic variety.

Table 2: Within variety variation based on Rogers' genetic distance (RD) according to RFLP bands

| Species | | *L. perenne* | | | | *F. pratensis* | | *F. arundinacea* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Varieties | | 'Kiyosato' | | 'Yatsugatake D-' | | 'Tomosakae' | | 'Yamanami' | | 'Nanryo' | | 'Kentucky 31' | |
| RGP clones | Enzymes | average no. of bands | RD | average no. of bands | RD | average no. of bands | RD | average no. of bands | RD | average no. of bands | RD | average no. of bands | RD |
| C74 | HindⅢ | 6.6 | 0.515 | 6.7 | 0.509 | 4.0 | 0.455 | 2.9 | 0.225 | 6.0 | 0.486 | 4.5 | 0.506 |
| C122 | BamHⅠ | 4.8 | 0.280 | 5.4 | 0.352 | 4.0 | 0.120 | 5.8 | 0.078 | 5.8 | 0.176 | 5.7 | 0.207 |
| C250 | BamHⅠ | 3.2 | 0.215 | 1.2 | 0.261 | 5.0 | 0.000 | 2.5 | 0.240 | 2.6 | 0.381 | 2.1 | 0.433 |
| C256 | HindⅢ | 4.9 | 0.256 | 5 | 0.232 | 3.5 | 0.317 | 4.8 | 0.150 | 4.0 | 0.000 | 4.0 | 0.000 |
| C370 | BamHⅠ | 4.8 | 0.368 | 3.6 | 0.311 | 5.0 | 0.000 | 1.0 | 0.000 | 1.3 | 0.324 | 1.0 | 0.309 |
| C385 | HindⅢ | 6.4 | 0.285 | 4.7 | 0.412 | 6.3 | 0.292 | 7.0 | 0.040 | 7.6 | 0.171 | 7.6 | 0.188 |
| C1794 | HindⅢ | 6.9 | 0.282 | 6.4 | 0.381 | 9.1 | 0.235 | 5.9 | 0.090 | 6.0 | 0.000 | 6.0 | 0.000 |
| R518 | BamHⅠ | 7.4 | 0.544 | 6.8 | 0.379 | 6.3 | 0.488 | 3.0 | 0.463 | 6.1 | 0.521 | 5.5 | 0.553 |
| R1012 | HindⅢ | 2.1 | 0.629 | 1.5 | 0.680 | 1.3 | 0.144 | 1.9 | 0.235 | 2.7 | 0.320 | 2.9 | 0.177 |
| R1607 | BamHⅠ | 1.8 | 0.444 | 2.4 | 0.527 | 3.0 | 0.000 | 2.7 | 0.573 | 3.1 | 0.508 | 3.2 | 0.648 |
| R1928 | BamHⅠ | 4.1 | 0.270 | 4.9 | 0.301 | 3.8 | 0.147 | 5.0 | 0.205 | 4.7 | 0.261 | 4.6 | 0.218 |
| R2232 | HindⅢ | 3.1 | 0.511 | 3.5 | 0.605 | 1.3 | 0.608 | 1.2 | 0.308 | 1.9 | 0.362 | 1.7 | 0.342 |
| mean | - | 4.5 | 0.371 | 4.3 | 0.404 | 4.4 | 0.214 | 3.7 | 0.217 | 4.2 | 0.275 | 4.0 | 0.280 |

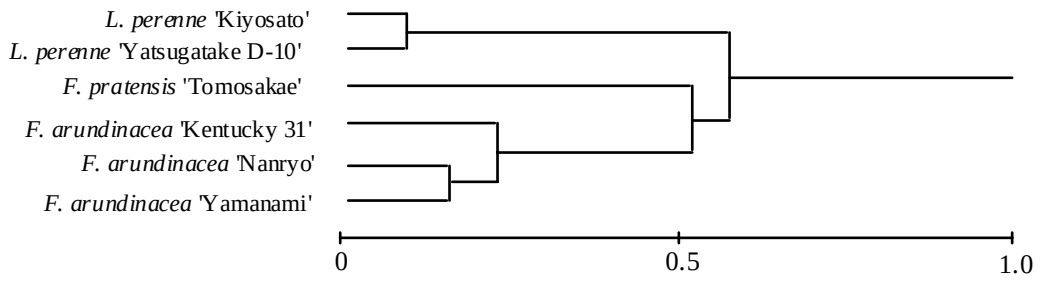


Fig. 1

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