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Author(s)	YAMADA, T.; 山田, 敏彦; KISHIDA, T.
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1 Genetic analysis of forage grasses based on
2 heterologous RFLP markers detected by rice cDNAs

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4 T. YAMADA¹ and T. KISHIDA²

5

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

8

9 ² Yamanashi Prefectural Dairy Experiment Station, Nagasaka, Yamanashi,
10 408-0021, Japan

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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

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

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

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

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

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

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

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

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

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

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3 Materials and Methods

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

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

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

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

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

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

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9 Results

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11 Cross-hybridization

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

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

4 5 Genetic variation within varieties

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

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

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1 Genetic variation between varieties

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

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11 Discussion

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13 The RGP has developed a high-density rice genetic linkage map with 2,275
14 markers using 186 F₂ plants from a single cross between the *japonica* variety
15 Nipponbare and the *indica* variety Kasalath (Harushima et al. 1998) and
16 presently this map has more than 3,000 markers (Rice Genome Research
17 Program, <http://rgp.dna.affrc.go.jp/Publicdata.html>). The main source of the
18 markers in this map has been RFLPs detected by cDNA clones prepared for
19 expressed sequence tag sequencing from the *japonica* variety Nipponbare
20 callus, root and shoot libraries (Sasaki et al. 1994, Yamamoto and Sasaki 1997).

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

3 Many of the RGP clones could not be used for analysis because they
4 detected no visible bands, faint bands or only a smeared background. Only
5 10 % of the clones could be used for the detection of polymorphism in forage
6 grass species. In the case of hybridization to the genomic DNA from the
7 heterozygous parental plant of the *L. perenne* reference mapping family
8 (p150/112) used by the International *Lolium* Genome Initiative (ILGI: Forster
9 et al. 2001), only 17 % of the rice cDNA probes detected clear bands, while in
10 contrast, wheat, oat and barley probes produced higher levels of clear
11 hybridization (Jones et al. 2002). Among the Cornell anchor probe set, the
12 levels of hybridization of rice cDNA (RZ probes) to wheat, barley and oat
13 genomic DNA was lower than for other species such as sorghum, sugarcane
14 and maize (Van Deynze et al. 1998). This trend follows the current
15 taxonomic knowledge of the Poaceae family, as wheat, oat, barley and
16 perennial ryegrass reside in a different subfamily (Pooideae) to rice
17 (Bambusoideae) (Soreng and Davis 1998).

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

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

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

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

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

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

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

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1 **Legend of figure**

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3 Fig. 1: Phylogenetic tree showing the relationships between of six accessions
4 of three species of *Lolium* and *Festuca* based on the analysis of 12 polymorphic
5 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
<i>L. perenne</i>		
Kiyosato	10	UK, Dutch, New Zealand and Australian varieties
Yatsugatake D-	10	'Kiyosato' and Australian ecotypic variety 'Kangaroo Valley'
<i>F. pratensis</i>		
Tomosakae	4	European varieties and Japanese ecotypes
<i>F. arundinacea</i>		
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		<i>L. perenne</i>				<i>F. pratensis</i>		<i>F. arundinacea</i>					
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	BamH I	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	BamH I	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	BamH I	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	BamH I	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	BamH I	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	BamH I	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

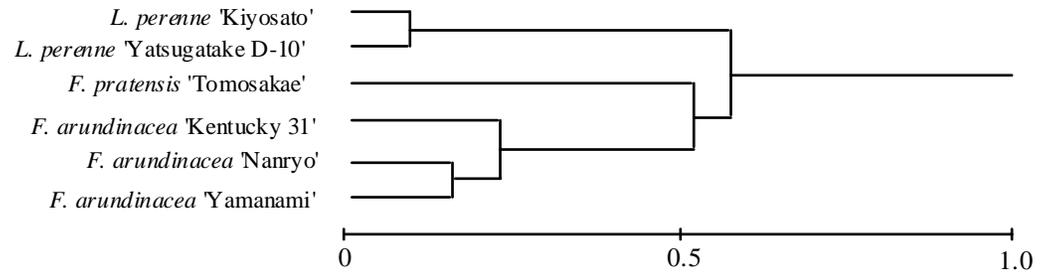


Fig. 1

Yamada and Kishida