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H. Akagi · Y. Yokozeki · A. Inagaki
T. Fujimura

Highly polymorphic microsatellites of rice consist of AT repeats, and a classification of closely related cultivars with these microsatellite loci

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Abstract Microsatellites consisting of AT repeats are highly polymorphic in rice genomes and can be used to distinguish between even closely related japonica cultivars in Japan. Polymorphisms of 20 microsatellite loci were determined using 59 japonica cultivars, including both domestic and modern Japanese cultivars. Although the polymorphisms of these 20 microsatellite loci indicated that the Japanese cultivars were genetically quite similar, microsatellites consisting of AT repeats showed high gene diversity even among such closely related cultivars. Combinations of these hypervariable microsatellites can be employed to classify individual cultivars, since the microsatellites were stable within each cultivar. An identification system based on these highly polymorphic microsatellites could be used to maintain the purity of rice seeds by eliminating contamination. A parentage diagnosis using 17 polymorphic microsatellite loci clearly demonstrated that plants which carried desired chromosome regions had been selected in breeding programs. Thus, these hypervariable microsatellites consisting of AT repeats should promote the selection of plants which carry desired chromosomes from genetically similar parents. Backcrossing could also help to eliminate unnecessary chromosome regions with microsatellite polymorphisms at an early stage in breeding programs.

Key words Parentage diagnosis · Microsatellites · AT repeats · Japonica rice · Breeding

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H. Akagi (✉) · Y. Yokozeki · A. Inagaki · T. Fujimura
Plant Biotechnology Laboratory, Life Science Institute, Mitsui Toatsu Chemicals Inc., Togo 1144, Mobara 297, Japan

Introduction

Rice is one of the most important crops, and most cultivated rice is *Oryza sativa* L. This has been classified into two groups, indica and japonica, based on characteristic morphological and physiological traits. The japonica varieties have been divided into tropical and temperate subgroups, formerly called javanica and japonica, respectively (Oka 1991). These subspecies are easily distinguished by their morphology and isozyme patterns. However, local varieties of japonica in both Japan and Korea are quite similar and have been classified into the same group by isozyme analysis (Glaszmann and Arraudeau 1986). Modern cultivars in Japan have been bred by crossing among these domestic cultivars. Therefore, these modern Japanese cultivars show extremely high genetic similarity.

RFLP is preferable to examining isozyme patterns, since RFLP can distinguish between several japonica cultivars belonging to the same isozyme group (McCouch and Tanksley 1991). However, in cultivated rice, there are few polymorphisms of RFLP markers (Wang and Tanksley 1989). Since there are few polymorphisms of RFLP markers within closely related cultivars in Japan, these Japanese cultivars cannot be distinguished by RFLP analysis. The infrequency of polymorphisms of RFLP markers limits the application of RFLP analysis in practical breeding, especially using closely related cultivars. Thus, highly polymorphic and easily assayed DNA markers are needed to promote the breeding of new varieties. Classification methods which use such polymorphic DNA markers should also simplify the management of rice seeds.

Microsatellites consist of tandemly arrayed di-, tri- and tetra-nucleotide repeats, and are hypervariable and ubiquitously distributed throughout eukaryotic genomes. Microsatellite DNA markers, which can be directly amplified by PCR, have been developed using unique sequences on both sides of the flanking region of each microsatellite (Litt and Luty 1989; Tautz 1989;

Weber and May 1989). In several crop plants, including soybean (Akkaya et al. 1992), rice (Wu and Tanksley 1993), barley (Becker and Heun 1995), wheat (Röder et al. 1995), maize (Senior and Heum 1993) and brassica (Lagercrantz et al. 1993), specific amplification of microsatellite loci has indicated that microsatellite DNA markers are more variable than RFLP markers. In rice, microsatellite DNA markers showed polymorphism not only between but also within subspecies. The microsatellite markers of rice were several times more polymorphic than RFLP markers (Wu and Tanksley 1993; Yang et al. 1994). Closely related bread-wheat cultivars have also been distinguished by microsatellite polymorphisms (Plaschke et al. 1995). These results suggest that highly polymorphic microsatellites could be used to identify closely related rice cultivars, such as Japanese cultivars, using PCR.

We have developed several DNA markers based on PCR to promote rice breeding (Akagi et al. 1995). We recently developed microsatellite DNA markers and assigned them to rice chromosomes (Akagi et al., in press). In the present study, we show that microsatellites consisting of AT repeats were highly polymorphic and could be used to detect allelic variations even within closely related Japanese cultivars. Using these hypervariable microsatellites, we successfully distinguished 59 closely related japonica cultivars in Japan.

Materials and methods

Plant materials

Fifty nine cultivars, including local Japanese varieties and modern varieties which had been bred from crossing within these local varieties, were studied (see Figs. 4, 5). Indica cultivars IR8 and IR24 were also used.

DNA extraction

Crude DNA was extracted from the leaves of young seedlings or from mature seeds. After being dried at 70°C for 2 h, leaves were homogenized with small glass beads in Eppendorf tubes using a vortex mixer. Crude DNA was then extracted according to the method of Edwards et al. (1991). Rice seeds were crushed using a hammer. Extraction buffer (400 µl; Edwards et al. 1991) was added to the crushed seeds and the mixture was incubated at 100°C for 10 min. DNA was precipitated from the supernatant by an equal volume of 2-propanol.

Microsatellites and PCR amplification

Twenty microsatellites were analyzed. For the amplification of these microsatellites, primers were synthesized according to the original reports (Wu and Tanksley 1993; McCouch et al. 1995; Akagi et al. in press).

PCR amplification was performed in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 or 1 unit of TAKARA *Taq* (TAKARA), 4 nmol dNTP, 10 pmol primer, and 10 ng or genomic DNA per 20 µl using a Thermal Cycler 9600 (Perkin-Elmer). Thirty five PCR cycles, each consisting of 30 s denaturation at 94°C, 30 s annealing at 55°C, and 1 min polymerization at 72°C, were performed.

Nucleotide sequence of amplified DNA fragments

Amplified DNA fragments were subcloned into pCR™II using a TA cloning kit (Invitrogen). Nucleotide sequences were determined using a DNA sequencing system (ABI 373S, Applied Biosystems Inc.). DNA sequences were analyzed using GENETYX-MAC software (Software Development, Tokyo).

Data analysis

Lengths of alleles were determined by ethidium bromide staining after electrophoresis on 3% MetaPhor Agarose gels (FMC). Gene diversity (GD) was calculated as follows:

$$GD = 1 - \sum_{i=1}^{m} x_i^2$$

where x_i indicates the population frequency for a marker and the summation extends over n patterns (Nei 1973).

Dendrograms were constructed by multivariate cluster analysis using the Euclidean maximum-length algorithm. Before this analysis, the data regarding allele length for each microsatellite were standardized. MUVA software (1993, developed by Dr. T. Tanaka of Osaka Medical College) was used for this analysis.

Results and discussion

Highly polymorphic microsatellites among Japanese cultivars

The degrees of polymorphism of 20 microsatellites were examined in 59 japonica cultivars in Japan. The microsatellites OSR1, OSR2, OSR3, OSR6 and OSR20 showed 5–10 alleles, while OSR26, RM3 and RM6 showed no polymorphism within the 59 cultivars. The remaining 12 microsatellite loci showed only 2–3 alleles (Table 1, Fig. 1). The allele lengths detected in OSR1 are shown in Fig. 2. Seven alleles could be clearly distinguished on 3% MetaPhor agarose. OSR2 was the most

Table 1 Allelic variation and gene diversity for microsatellite loci

Microsatellite	Repeat units	Chromosome position	Number of alleles	Gene diversity
OSR1	(at)(ct)	11	7	0.801
OSR2	(at)	1	10	0.777
OSR3	(at)	1	5	0.702
OSR6	(at)	11	7	0.821
OSR8	(ag)	2	3	0.159
OSR9	(ag)	2	3	0.244
OSR11	(ag)	2	2	0.097
OSR15	(ag)	4	2	0.066
OSR18	(ac)	6	3	0.159
OSR20	(ct)	12	5	0.685
OSR22	(ct)	7	3	0.434
OSR26	(tg)	2	1	0.000
OSR28	(aga)	9	2	0.483
OSR29	(aag)	9	2	0.483
RM1		1	3	0.637
RM3		6	1	0.000
RM6		2	1	0.000
RM7		3	2	0.033
RM164		5	3	0.445
RM167		11	2	0.344

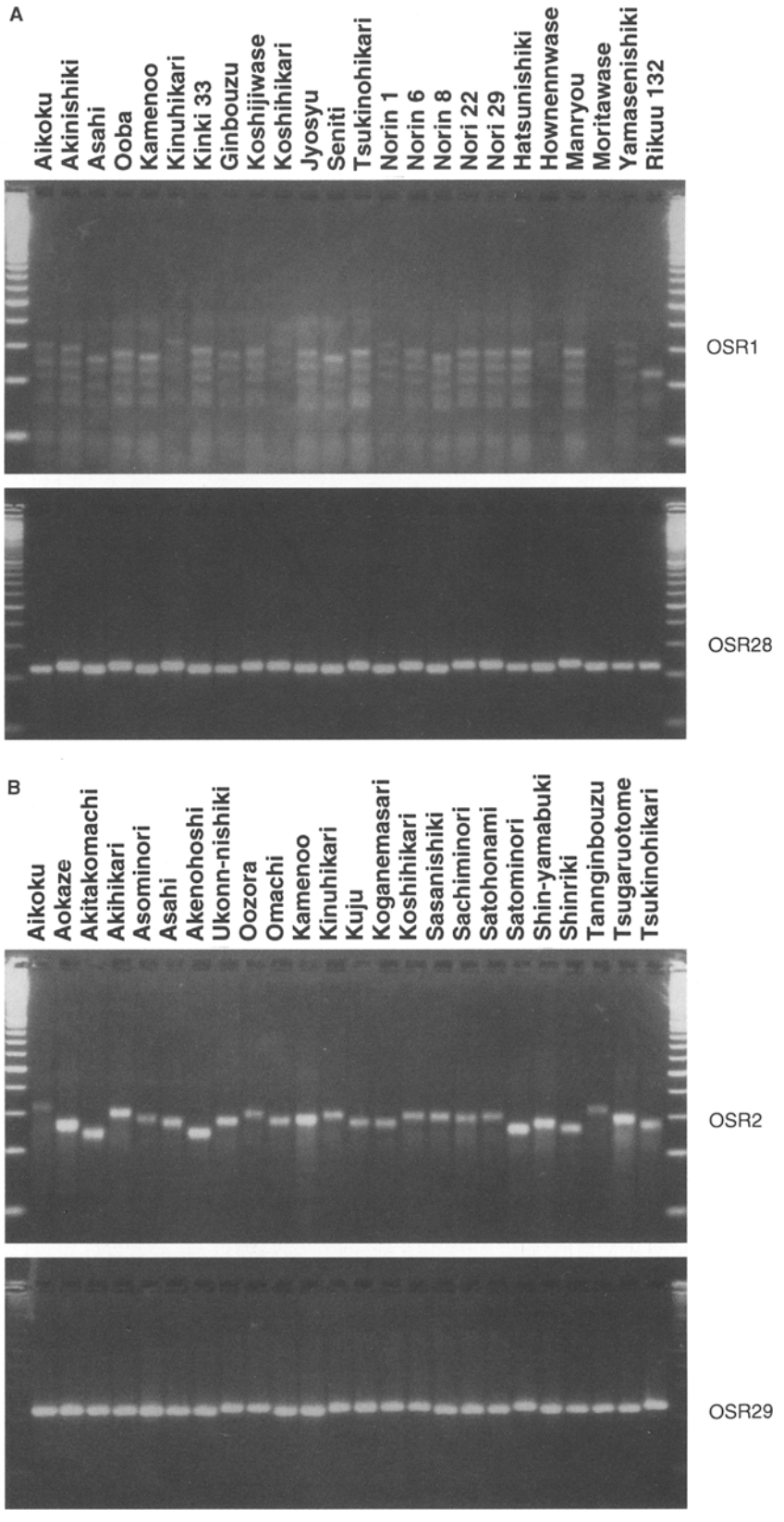

Fig. 1A, B Polymorphisms of PCR-amplified microsatellite DNA markers within closely related japonica cultivars in Japan. Panel **A** shows polymorphisms for OSR1 and OSR28 for 24 of the 59 cultivars. Panel **B** shows polymorphisms for OSR2 and OSR29 for another 24 cultivars. 100-bp/500-bp DNA ladders were used as DNA size markers

polymorphic of these microsatellites; ten alleles were detected at this locus (Table 1, Fig. 1B).

High genetic diversity within japonica subspecies has been observed for RM164 and RM167, 0.74 and 0.60, respectively (Wu and Tanksley 1992). Similar gene diversity for these microsatellites has also been observed in japonica landraces and cultivars (Yang et al. 1994). However, we found relatively low gene diversities

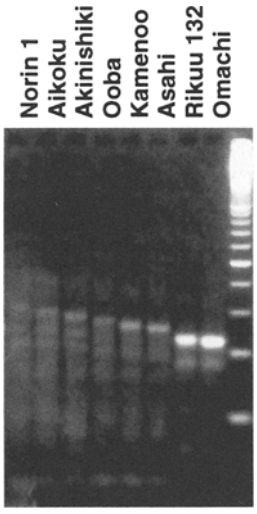

Fig. 2 Discrimination of seven alleles detected in OSR1 among 59 Japanese cultivars. The PCR products of OSR1 were electrophoresed on 3% MetaPhor agarose gels (FMC) and then stained with ethidium bromide. DNA size-marker lanes contain 100-bp/500-bp DNA ladders

(= heterozygosity) for RM164 and RM167 within Japanese cultivars, 0.445 and 0.344, respectively (Table 1). The gene diversities for these microsatellites indicated that the japonica cultivars bred in Japan have a very low gene diversity.

As shown in Fig. 1 and Table 1, there were several highly polymorphic microsatellites within these Japanese cultivars, although they were genetically quite similar. The most common repeat unit consisted of AT, which was detected in more than five alleles. Gene diversities of microsatellites consisting of AT repeats were also high, ranging from 0.702 to 0.821 (Table 1). OSR1 had two types of repeat unit consisting of AT and CT repeats, respectively (Fig. 3). The high polymorphism of OSR1 was mainly due to variation in the number of AT repeats (Fig. 3). These results suggest that microsatellites consisting of AT repeats are the most variable in rice genomes. Therefore, these highly polymorphic microsatellites might be useful for identifying closely related rice cultivars. Microsatellites with AT repeats are also highly polymorphic genetic markers in soybean (Akkaya et al. 1992). Thus, like the CA repeat in humans (Dib et al. 1996), microsatellites based on A and T may be hypervariable genetic markers in plants. Although AT repeats were the most abundant in plant genomes, only a few such microsatellites have been reported to-date (Lagercrantz et al. 1993; Wang et al. 1994).

Stability of hypervariable microsatellites within a cultivar

The higher polymorphisms of microsatellite loci suggested that they were quite variable. The number of repeats in microsatellites must be uniform within each cultivar if we wish to use them for the practical identification of cultivars. We determined the stability of the number of repeats for the microsatellites within a cultivar using 96 plants of cv Kinuhikari. The highly polymorphic loci OSR6 and OSR20, which contain AT and CT repeats, respectively, showed no polymorphism within this population (data not shown). This result indicates that alteration of the microsatellites within a cultivar must occur at a frequency of less than 1%. Thus, these highly polymorphic microsatellites are stable within individual rice cultivars. We concluded that the allele length of these microsatellites could be used as an index of each cultivar.

Cultivar identification using microsatellites

To identify individual cultivars using these microsatellites, agglomerative cluster analysis was performed using standardized allele lengths. All 59 of the closely related cultivars were classified using 17 polymorphic microsatellites (Fig. 4). Most of these cultivars could also be classified by the five highly polymorphic microsatellites, OSR1, OSR2, OSR3, OSR6 and OSR20, except for the three combinations of Koshihikari and Hatsuboshi,

Fig. 3 Comparison of the nucleotide sequences of the microsatellites in OSR1, amplified from Nipponbare, IR24 and IR8

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Nipponbare  ACCATGGATGGTACCAACTCCTCCTATA--CTCTCTCTCTCTCTCTCTCTCTCTCTAT  58
IR24        ACCATGGATGGTACCAACTCCTCCTATA--CTCTCTCTCTCTCTCTCTCTCTC--TAT  56
IR8         ACCATGGATGGTACCAACTCCTCCTATATACTCTCTCTCTCTCTCTC----------TAT  50
            ***************************  ****************        ***

Nipponbare  ATATATATATATATATATATATATATATATATATATATATATATATATATATATATAT 118
IR24        ATATATATATATATATATATATATATATATATATA------------------------  91
IR8         A-------------------------------------------------------------  51

Nipponbare  ATATATATTTATTTATTTTCACCTACCTACTTCTATTGCACACCTACCAAATTAATGCTC 178
IR36        ----------------TTTCACCTACCTACTTCTATTGCACACCTACCAAATTAATGCTC 135
IR8         ----------------TTTCACCTACCTACTTCTATTGCACACCTACCAAATTAATGCTC  95
                            ****************************************

Nipponbare  TGAAACCAATTAATTAGTTAGAAACTAAAAAAGTTTTGCTTTACCACACGCGCGGCACAC 238
IR24        TGAAACCAATTAATTAGTTAGAAACTAAAAAAGTTTTGCTTTACCACACGCGCGGCACAC 195
IR8         TGAAACCAATTAATTAGTTAGAAACTAAAAAAGTTTTGCTTTACCACACGCGCGGCACAC 155
            ************************************************************

Nipponbare  GGCAAAAGCATAGTAGATGCAAGCGAA 265
IR24        GGCAAAAGCATAGTAGATGCAAGCGAA 222
IR8         GGCAAAAGCATAGTAGATGCAAGCGAA 182
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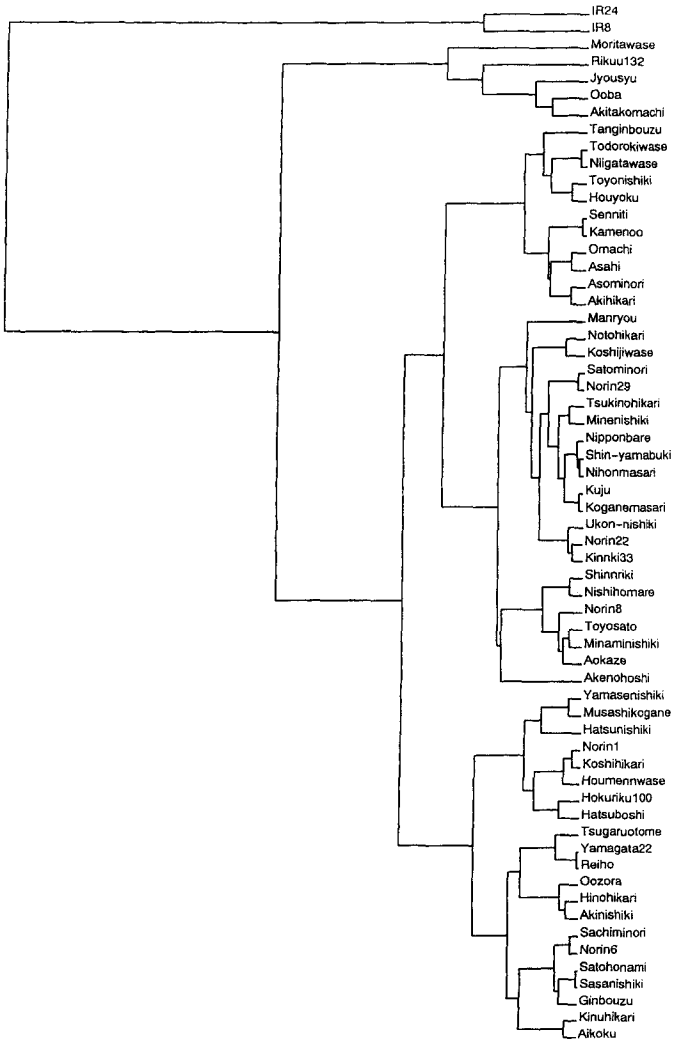

Fig. 4 Dendrogram showing a cluster analysis of 59 japonica cultivars and two indica cultivars, based on polymorphisms of 17 microsatellite loci. Polymorphic data for each microsatellite were standardized before the cluster analysis

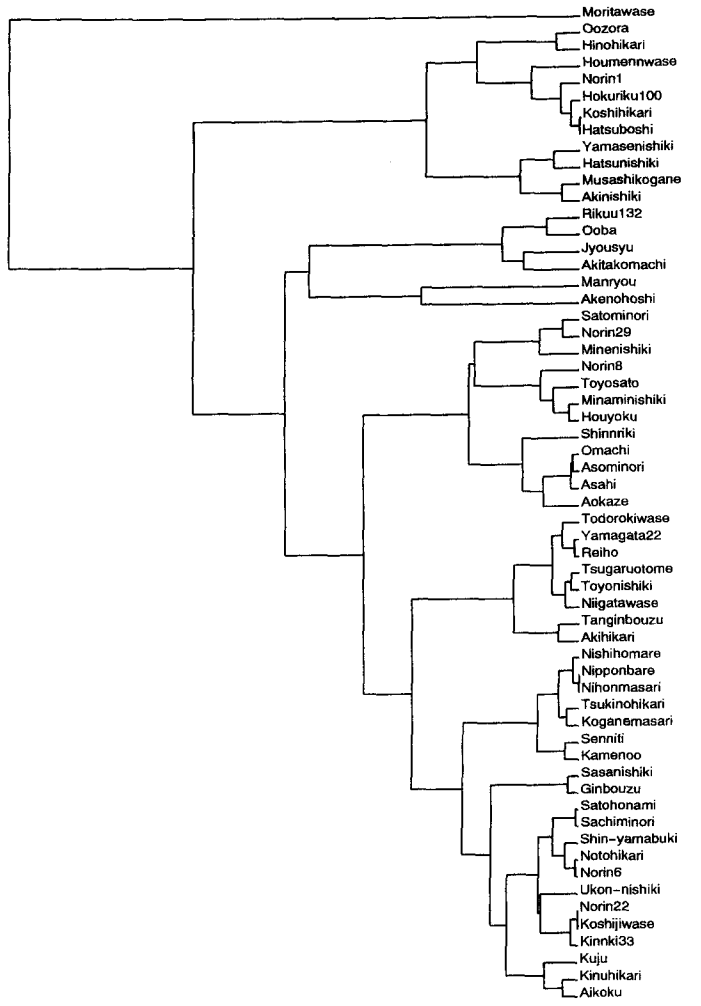

Fig. 5 Dendrogram showing a cluster analysis of 59 japonica cultivars with five highly polymorphic microsatellites: OSR1, OSR2, OSR3, OSR6 and OSR20. Polymorphic data for each microsatellite were standardized before the cluster analysis

Nipponbare and Nihonmasari, and Norin 22 and Koshijiwase (Fig. 5). These three combinations reflect a parent-offspring relationship, indicating that these five microsatellite loci have been conserved in the breeding of Hatsuboshi, Nihonmasari and Koshijiwase, as discussed below. Only a few closely related cultivars were classified using the microsatellites developed by Wu and Tanksley in 1993 (Yang et al. 1994). Thus, microsatellites consisting of AT repeats were especially useful for identifying cultivars.

The present results indicate that combinations of these highly polymorphic microsatellites enable us to easily identify individual cultivars from among a population of closely related Japanese cultivars using PCR. This identification system, based on highly polymorphic microsatellites, could also be used to maintain the purity of rice seeds by eliminating contamination.

Application of microsatellite polymorphisms to breeding

One Japanese pedigree includes several elite cultivars, such as Koshihikari. The polymorphisms of 17 microsatellite loci in the closely related cultivars in this pedigree were analyzed. The origins of these microsatellite loci could be traced back three generations, based on gaps between modern cultivars and progenitor domestic varieties (Fig. 4, arrows). Domestic varieties generally consisted of a heterogeneous population. Therefore, the plants analyzed here might not be identical to those used for crossing as a breeding parent. In the case of Rikuu 132, the allele lengths of 10 of the 17 microsatellite loci are inconsistent with those of the parents (data not shown). This suggests the possibility of a mutation or else that a hybrid plant with contaminated pollen was

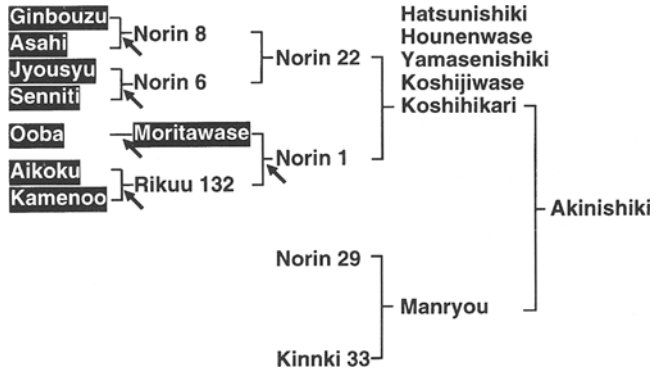

Fig. 6 Pedigree of some elite varieties in Japan. The varieties by *white letters* are domestic varieties in Japan. Gaps in allele length detected by microsatellite loci are indicated by *arrows*

selected during the breeding process. Thus, a parentage diagnosis using microsatellite loci can identify chromosome regions which contribute to the individuality of a cultivar.

Within this family, crossing Norin 1 with Norin 22 gave five elite cultivars: Koshihikari, Koshijiwase, Hatsunishiki, Yamasenishiki and Hounennwase. The constitution of chromosomes from each parent was determined for nine microsatellite loci which showed different alleles in the two parental varieties (Table 2). The percentages of chromosome regions in Koshijiwase, Koshihikari, Yamasenishiki, Hatsunishiki and Hounennwase from Norin 1 were estimated to be 22%, 67%, 78%, 78% and 89%, respectively. None of these showed an identical chromosome constitution (Table 2). These results indicate that molecular markers based on microsatellites clearly demonstrate that plants carrying the desired chromosome regions had been selected in breeding programs, even though only a limited number of microsatellite loci were analyzed here (Table 1).

Microsatellites consisting of AT repeats are hypervariable, which enables us to distinguish between even closely related cultivars using PCR. We have developed microsatellite DNA markers and have assigned them to rice chromosomes (Akagi et al., in press). Thus, highly polymorphic microsatellites which are distributed throughout rice chromosomes should promote the selection of plants carrying desired chromosomes from genetically similar parents. This approach should also promote backcrossing to eliminate unnecessary chromosome regions in the early stage of breeding programs. Consequently, hypervariable microsatellites consisting of AT repeats should promote the practical breeding of rice both by shortening the breeding period and saving space in the field.

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Table 2 Origin of chromosome regions of five cultivars derived from crossing Norin 1 with Norin 22 based on highly polymorphic microsatellites

Cultivars	Markers and chromosome postions								
	OSR1 1	OSR3 1	RM1 1	OSR9A 2	OSR28 9	OSR29 9	OSR1 11	OSR6 11	OSR20 12
Koshijiwase	N22	N22	N22	N1	N22	N22	N22	N22	N1
Koshihikari	N1	N1	N1	N22	N22	N22	N1	N1	N1
Hatsunishiki	N22	N1	N1	N22	N1	N1	N1	N1	N1
Yamasenishiki	N1	N1	N1	N22	N1	N1	N1	N1	N22
Hounennwase	N1	N1	N1	N22	N1	N1	N1	N1	N1

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