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Genetic diversity and phylogeny of Japanese sake-brewing rice as revealed by AFLP and nuclear and chloroplast SSR markers

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Abstract Japanese rice (*Oryza sativa* L.) cultivars that are strictly used for the brewing of sake (Japanese rice wine) represent a unique and traditional group. These cultivars are characterized by common traits such as large grain size with low protein content and a large, central white-core structure. To understand the genetic diversity and phylogenetic characteristics of sake-brewing rice, we performed amplified fragment length polymorphism and simple sequence repeat analyses, using 95 cultivars of local and modern sake-brewing rice together with 76 cultivars of local and modern cooking rice. Our analysis of both nuclear and chloroplast genome polymorphisms showed that the genetic diversity in sake-brewing rice cultivars was much smaller than the diversity found in cooking rice cultivars. Interestingly, the genetic diversity within the modern sake-brewing cultivars was about twofold higher than the diversity within the local sake-brewing cultivars, which was in contrast to the cooking cultivars. This is most likely due to introgression of the modern cooking cultivars into the modern sake-brewing cultivars through breeding practices. Cluster analysis and chloroplast haplotype analysis suggested that the local sake-brewing cultivars originated monophyletically in the western regions of Japan. Analysis of variance tests showed that several markers were significantly associated with sake-brewing traits, particularly with the large white-core structure.

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Introduction

Rice (*Oryza sativa* L.) is one of the most important staple food crops supporting the world population. Rice is cultivated throughout the world, mainly in Asian countries and in the United States, western Africa, and some areas in Europe. Three subspecies, i.e., *indica*, *japonica*, and *javanica*, compose a large reservoir of rice germplasms including a variety of local landraces and cultivars (Chang 1976). In addition, there are a number of wild relatives that provide potentially valuable resources for the improvement of cultivated rice (Tanksley and McCouch 1997). The genetic diversity in the world rice germplasms is quite large, with a higher diversity in *indica* rice than in *japonica* rice (Yang et al. 1994; Aggarwal et al. 1999; Yu et al. 2003). In contrast to the richness of genetic variation in the world rice resources, rice cultivars in Japan generally display a much narrower genetic variation (Akagi et al. 1997; Kubo et al. 1998; Ashikawa et al. 1999). Modern Japanese rice cultivars have been bred through selection among limited resources, and, as a result, their genetic diversity has become even less than that in local cultivars.

In Japan, rice is also used for the production of sake, a traditional and popular Japanese alcoholic beverage. Sake is brewed from steamed rice grains through the simultaneous and carefully controlled processes of saccharification by *koji* (*Aspergillus oryzae*) and fermentation by yeast (*Saccharomyces cerevisiae*). Rice cultivars preferred for sake brewing are characterized by common traits such as large grain size with low protein content and a large white-core. The presence of a large white-core (called *Shinpaku* in Japanese) in the center of rice grains has long been considered as the single most important trait for sake-

brewing, because it can stimulate a uniform spread of *koji* mycelia within rice grains. Sake-brewing cultivars also possess several undesirable traits, such as a high lodging susceptibility because of the tall plant height. Breeding efforts have been devoted to improve agronomic traits of sake-brewing rice. However, a single cultivar named Yamada-nishiki has retained its leading status as the most popular and highest-quality sake-brewing cultivar in Japan for the last seven decades.

To breed better-quality sake-brewing rice, the genetic structure controlling the sake-brewing traits has to be understood. A number of research studies have been conducted to understand the inheritance of traits related to the sake brewing such as grain size (Takita 1985; Kato 1989; Takeda 1990) and protein content (Okamoto 1994). We have recently conducted a quantitative trait locus (QTL) analysis, employing several DNA marker systems to detect significant chromosomal regions possessed by Yamada-nishiki (Yoshida et al. 2002). Despite such efforts, the genetic structure of sake-brewing rice remains largely unknown. Furthermore, almost no information is available on the genetic diversity and phylogeny of sake-brewing cultivars. Such knowledge is important for designing effective breeding programs for sake-brewing rice. Based on the historical knowledge of sake-brewing rice in Japan, we assume that these cultivars have retained certain unique genome structures that are associated with their lineages. We therefore attempted to study these aspects of sake-brewing rice in comparison with cooking rice.

A number of DNA marker systems have been developed and efficiently utilized in genetic and breeding studies. These include restriction fragment length polymorphism [(RFLP) Botstein et al. 1980] and polymerase chain reaction (PCR)-based marker systems such as amplified fragment length polymorphism [(AFLP) Vos et al. 1995] and simple sequence repeat [(SSR) Tautz 1989]. The AFLP marker system has proven to be a useful approach for genetic mapping, genotyping, and marker-assisted breeding in rice (Mackill et al. 1996; Maheswaran et al. 1997; Zhu et al. 1998). The SSR marker system has been developed for both nuclear and chloroplast genomes in rice (Wu and Tanksley 1993; Panaud and McCouch 1996; Chen et al. 1997; Ishii and McCouch 2000; Temnykh et al. 2000) and has been used as a powerful tool in a variety of genetic and breeding studies (Yang et al. 1994; Olufowote et al. 1997).

We herein report our study on the genetic diversity and phylogeny of Japanese sake-brewing rice cultivars in comparison with cooking rice cultivars, using AFLP and nuclear and chloroplast SSR markers. Associations of markers with important sake-brewing traits including the expression of *Shinpaku* were also studied by the analysis of variance (ANOVA) test.

Materials and methods

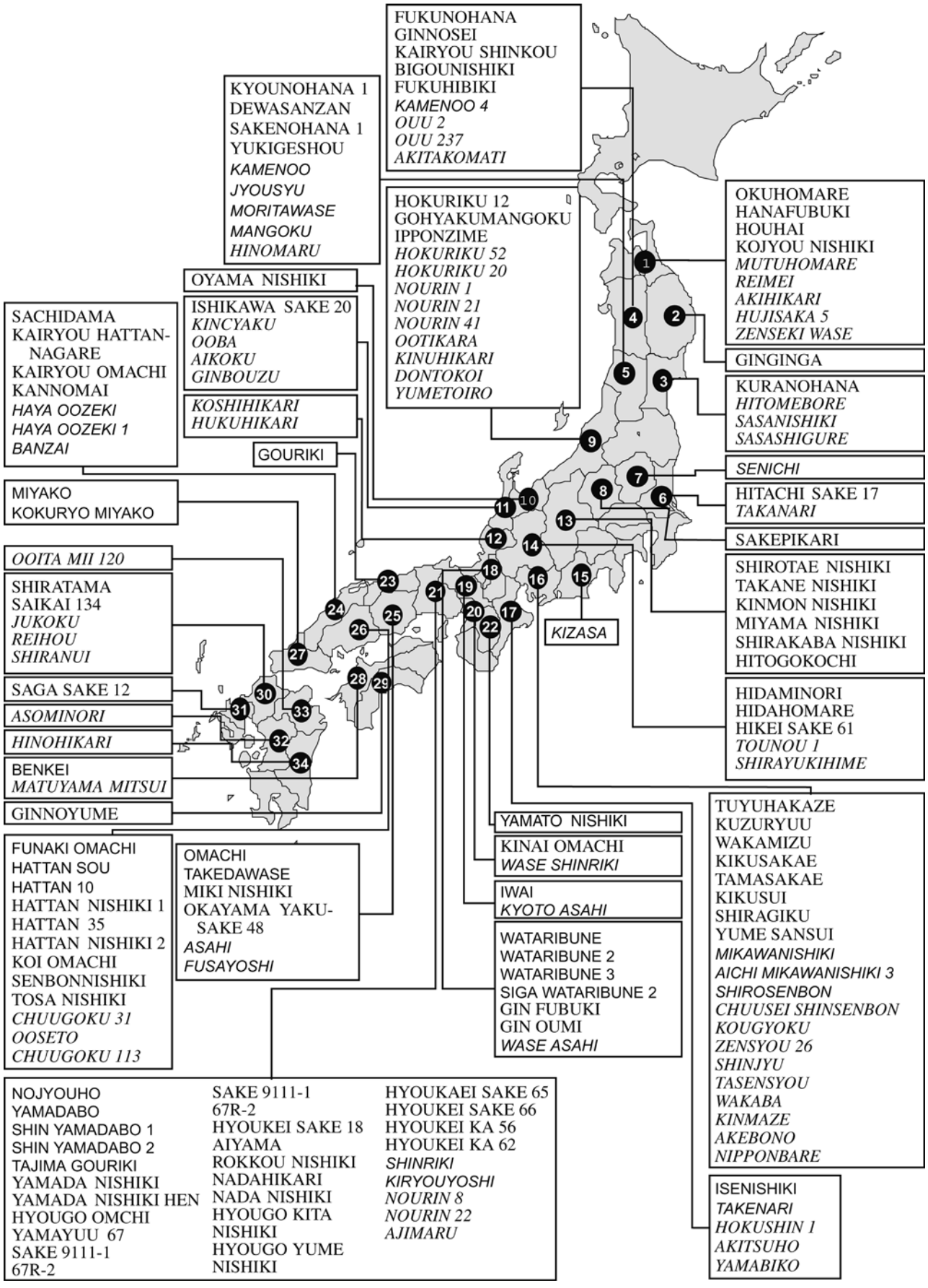
Plant materials

Rice cultivars used in this study covered almost all collections of sake-brewing cultivars and major cooking cultivars in Japan, including 28 local and 67 modern sake-brewing cultivars as well as 29 local and 47 modern cooking cultivars. Local cultivars have not been bred through modern breeding procedures; therefore, their precise pedigrees are often unknown. Modern cultivars have been bred through crossings among local cultivars and/or with other genetic sources, and therefore, their recent pedigrees are mostly known. In addition, 13 non-sake-brewing and non-cooking cultivars including two cultivars derived from an *indica*/*japonica* cross were used. Figure 1 shows geographical distribution of the Japanese sake-brewing rice cultivars used in this study.

AFLP and SSR analyses

Total DNA was extracted from leaves of greenhouse-grown plants by the cetyl trimethyl ammonium bromide method (Murray and Thompson 1980). A high-efficiency AFLP genome-scanning system (Mano et al. 2001), a modified version of the original AFLP method (Vos et al. 1995), was applied for fingerprinting. Total DNA (150 ng) was digested with *Eco*RI and *Mse*I, and the adaptors were ligated to the ends of the restriction fragments. A total of 20 cycles of PCR were performed for pre-amplification (30 s at 94°C, 1 min at 56°C and 1 min at 72°C). Selective amplification was performed using *Eco*RI and *Mse*I primers that additionally contained three selective nucleotides at the 3′ ends (Table 1). Before selective amplification, a 2-min denaturation step was performed at 94°C. The first phase of the selective amplification was for one cycle (30 s at 94°C, 30 s at 68°C, and 1 min at 72°C). The second touchdown phase was for 16 cycles, with the annealing temperature decreasing stepwise from 67.3°C to 56.8°C. The last phase was for 22 cycles (30 s at 94°C, 30 s at 56°C and 1 min at 72°C). Amplified products were fractionated by electrophoresis through 13% non-denaturing polyacrylamide gels and stained using the silver-staining kit Sil-Best Stain for protein/PAGE (Nacalai Tesque, Japan).

For nuclear SSR analysis, 32 markers covering all 12 rice chromosomes were examined (Table 2). For chloroplast SSR analysis, ten markers designated RCt1–RCt10 (Ishii and McCouch 2000) were used. These chloroplast SSR markers consisted of 10 to 17 repeats of either A or T nucleotides (Table 2). PCR was performed in the following program: 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, and a post-extension for 5 min at 72°C. Amplified products were fractionated by electrophoresis through 6% denaturing polyacrylamide gels and stained using a staining kit, Silver Sequence DNA Sequencing System Staining Reagents (Promega, USA).


FUKUNOHANA
GINNOSEI
KAIRYOU SHINKOU
BIGOUNISHIKI
FUKUHIBIKI
KAMENOO 4
OUU 2
OUU 237
AKITAKOMATI
KYOUNOHANA 1
DEWASANZAN
SAKENOHANA 1
YUKIGESHOU
KAMENOO
JYOUSYU
MORITAWASE
MANGOKU
HINOMARU
HOKURIKU 12
GOHYAKUMANGOKU
IPPONZIME
HOKURIKU 52
HOKURIKU 20
NOURIN 1
NOURIN 21
NOURIN 41
OOTIKARA
KINUHIKARI
DONTOKOI
YUMETOIRO
OKUHOMARE
HANAFUBUKI
HOUHAI
KOJYOU NISHIKI
MUTUHOMARE
REIMEI
AKIHIKARI
HUJISAKA 5
ZENSEKI WASE
GINGINGA
KURANOHANA
HITOMEBORE
SASANISHIKI
SASASHIGURE
SENICHI
HITACHI SAKE 17
TAKANARI
SAKEPIKARI
SHIROTAE NISHIKI
TAKANE NISHIKI
KINMON NISHIKI
MIYAMA NISHIKI
SHIRAKABA NISHIKI
HITOGOKOCHI
HIDAMINORI
HIDAHOMARE
HIKEI SAKE 61
TOUNOU 1
SHIRAYUKIHIME
TUYUHAKAZE
KUZURYUU
WAKAMIZU
KIKUSAKAE
TAMASAKAE
KIKUSUI
SHIRAGIKU
YUME SANSUI
MIKAWANISHIKI
AICHI MIKAWANISHIKI 3
SHIROSENBON
CHUUSEI SHINSENBON
KOUGYOKU
ZENSYOU 26
SHINJYU
TASENSYOU
WAKABA
KINMAZE
AKEBONO
NIPPONBARE
ISENISHIKI
TAKENARI
HOKUSHIN 1
AKITSUHO
YAMABIKO
OYAMA NISHIKI
ISHIKAWA SAKE 20
KINCYAKU
OOBA
AIKOKU
GINBOUZU
KOSHIHIKARI
HUKUHIKARI
SACHIDAMA
KAIRYOU HATTAN-NAGARE
KAIRYOU OMACHI
KANNOMAI
HAYA OOZEKI
HAYA OOZEKI 1
BANZAI
GOURIKI
MIYAKO
KOKURYO MIYAKO
OOITA MII 120
SHIRATAMA
SAIKAI 134
JUKOKU
REIHOU
SHIRANUI
SAGA SAKE 12
ASOMINORI
HINOHIKARI
BENKEI
MATUYAMA MITSUI
GINNOYUME
KIZASA
YAMATO NISHIKI
KINAI OMACHI
WASE SHINRIKI
IWAI
KYOTO ASAHI
WATARIBUNE
WATARIBUNE 2
WATARIBUNE 3
SIGA WATARIBUNE 2
GIN FUBUKI
GIN OUMI
WASE ASAHI
FUNAKI OMACHI
HATTAN SOU
HATTAN 10
HATTAN NISHIKI 1
HATTAN 35
HATTAN NISHIKI 2
KOI OMACHI
SENBONNISHIKI
TOSA NISHIKI
CHUUGOKU 31
OOSETO
CHUUGOKU 113
OMACHI
TAKEDAWASE
MIKI NISHIKI
OKAYAMA YAKU-SAKE 48
ASAHI
FUSAYOSHI
NOJYOUHO
YAMADABO
SHIN YAMADABO 1
SHIN YAMADABO 2
TAJIMA GOURIKI
YAMADA NISHIKI
YAMADA NISHIKI HEN
HYOUGO OMCHI
YAMAYUU 67
SAKE 9111-1
67R-2
SAKE 9111-1
67R-2
HYOUKEI SAKE 18
AIYAMA
ROKKOU NISHIKI
NADAHIKARI
NADA NISHIKI
HYOUGO KITA NISHIKI
HYOUGO YUME NISHIKI
HYOUKAEI SAKE 65
HYOUKEI SAKE 66
HYOUKEI KA 56
HYOUKEI KA 62
SHINRIKI
KIRYOUYOSHI
NOURIN 8
NOURIN 22
AJIMARU

◀ **Fig. 1** Geographical distribution of the 181 Japanese rice cultivars used in this study. *Roman letters* indicate sake-brewing cultivars, *italics* cooking cultivars and others, *sans serif* local cultivars, and *Serif letters* modern cultivars. The map specifies the distribution of each cultivar in the name of prefecture. *1* Aomori, *2* Iwate, *3* Miyagi, *4* Akita, *5* Yamagata, *6* Ibaragi, *7* Tochigi, *8* Gunma, *9* Niigata, *10* Toyama, *11* Ishikawa, *12* Fukui, *13* Nagano, *14* Gifu, *15* Shizuoka, *16* Aichi, *17* Mie, *18* Shiga, *19* Kyoto, *20* Osaka, *21* Hyogo, *22* Nara, *23* Tottori, *24* Shimane, *25* Okayama, *26* Hiroshima, *27* Yamaguchi, *28* Ehime, *29* Kochi, *30* Fukuoka, *31* Saga, *32* Kumamoto, *33* Oita, *34* Miyazaki. Three cultivars (M7, SLG1, and Hokushi Tami) are not included; M7 is a modern cooking cultivar bred from Watari-bune in the United States, SLG1 is a non-registered *japonica* line, and Hokushi Tami is a Chinese local cultivar

Table 1 List of amplified fragment length polymorphism (AFLP) markers

Marker no.	Marker name	Selective primer sequence[a]
1	Me0238	M-AAG/E-TGG
2	Me1848	M-GAG/E-TCC
3	Me5801	M-CTG/E-AAA
4	Me5816	M-CTG/E-ACC
5	Me5821	M-CTG/E-GGA
6	Me5907	M-CTT/E-AGT
7	Me6326	M-CCT/E-GTG
8	Me6433	M-CCC/E-TAA
9	Me6455	M-CCC/E-CGT
10	Me6457	M-CCC/E-CTA
11	Me6460	M-CCC/E-CTC

[a] *M Mse*I adaptor, *E Eco*RI adaptor

AFLP and SSR data analysis

In the AFLP analysis, genetic distance (d), defined as the number of pair-wise nucleotide changes per site, and the nucleotide diversity (π), defined as the average number of pair-wise nucleotide changes per site (Nei and Li 1979), were calculated according to Innan et al. (1999). This method is especially designed for an AFLP data set, and according to the authors, it gives a reasonably accurate estimate as long as the π is small (Innan et al. 1999). Based on all pair-wise d values, phylogenetic trees were constructed by the UPGMA method (Sneath and Sokal 1973). In the SSR analysis, the size of the stained band was determined for each marker locus based on its migration distance relative to the Nipponbare allele. The allelic diversity was calculated according to the diversity index, H, described by Nei (1987), in the following formula; $H_i = 1 - \sum_{j=1}^{n} {p_{ij}}^2$, where p_{ij} is the frequency of the jth pattern for the ith marker locus and summation extends over n patterns. Based on the percentages of common fragments, dendrograms were constructed by the UPGMA method (Sneath and Sokal 1973). Chloroplast haplotypes (plastotypes) were identified based on ten SSR loci. Phylogenetic relationships of the detected plastotypes were evaluated based on the number of alleles different among haplotypes, and the summary network of the plastotypes was constructed by the neighbor-joining method (Saitou and Nei 1987).

Table 2 List of nuclear and chloroplast simple sequence repeat (SSR) markers

Marker[a]	Location	Repeat
RM5	Chr. 1	$(GA)_{14}$
RM237	Chr. 1	$(CT)_{18}$
RM84	Chr. 1	$(TCT)_{10}$
RM212	Chr. 1	$(CT)_{24}$
RM259	Chr. 1	$(CT)_{17}$
RM315	Chr. 1	$(AT)_4(GT)_{10}$
RM145	Chr. 2	$(GA)_{31}$
RM262	Chr. 2	$(CT)_{16}$
RM208	Chr. 2	$(CT)_{17}$
RM240	Chr. 2	$(CT)_{21}$
RM7	Chr. 3	$(GA)_{19}$
RM232	Chr. 3	$(CT)_{24}$
RM255	Chr. 4	$(CT)_{16}$
RM241	Chr. 4	$(CT)_{31}$
RM252	Chr. 4	$(CT)_{19}$
RM13	Chr. 5	$(GA)_{16}$
RM31	Chr. 5	$(GA)_{15}$
RM253	Chr. 6	$(CT)_{25}$
RM225	Chr. 6	$(CT)_{18}$
RM2	Chr. 7	$(GA)_{13}$
RM234	Chr. 7	$(CT)_{25}$
RM256	Chr. 8	$(CT)_{21}$
RM44	Chr. 8	$(GA)_{16}$
RM219	Chr. 9	$(CT)_{17}$
RM257	Chr. 9	$(CT)_{24}$
RM216	Chr. 10	$(CT)_{18}$
RM258	Chr. 10	$(CT)_{21}$
RM21	Chr. 11	$(GA)_{18}$
RM229	Chr. 11	$(CT)_{11}$
RM235	Chr. 12	$(CT)_{24}$
RM247	Chr. 12	$(CT)_{16}$
RM101	Chr. 12	$(CT)_{37}$
RCt1	Intron (*trnK*)	$(A)_{10}$
RCt2	*rpoC2*	$(A)_{11}$
RCt3	Intergenic	$(A)_{10}$
RCt4	*psbG*	$(T)_{12}$
RCt5	Intergenic	$(T)_{10}$
RCt6	Intergenic	$(A)_{10}$
RCt7	*infA*	$(T)_{10}$
RCt8	Intron (*rpl16*)	$(T)_{17}$
RCt9	Intergenic	$(T)_{10}$
RCt10	Intergenic	$(T)_{10}$

[a] Nuclear SSR markers are after Panaud et al. (1996), Chen et al. (1997) and Temnykh et al. (2000). Chloroplast SSRs are after Ishii and McCouch (2000). *RM* Nuclear SSR marker, *RCt* chloroplast SSR marker, *Chr.* chromosome number

ANOVA for identification of markers associated with sake-brewing traits

Five agronomic traits (*Shinpaku* size and grain weight, length, width, and thickness) were measured in two consecutive years, using 50 rice cultivars including 38 representative sake-brewing and 12 cooking cultivars grown in pots in a greenhouse. The ANOVA test was employed to identify AFLP and nuclear SSR markers significantly associated with the sake-brewing traits. Two contrasting groups were classified based on the presence or absence of informative markers in case of AFLP markers and the allelic differences in case of SSR markers. Significant associations of marker loci with particular traits were evaluated by comparison of the two groups for each trait.

Results and discussion

Estimation of the genetic diversity among Japanese rice cultivars, based on AFLP and nuclear SSR markers

To study genetic diversity, we fingerprinted the genome, using AFLP and SSR markers. In the AFLP analysis, 16 representative cultivars including both sake-brewing and cooking rice were first studied to select for informative primer combinations that could detect clear polymorphisms. Among 48 selective primer combinations, 11 produced 28 polymorphic bands in a total of 776 major countable bands. The polymorphism frequency was thus 3.6%. We next studied 184 cultivars, using the selected 11 primer combinations (Table 1). Of 138 major countable bands, 25 (18.1%) showed clear polymorphisms. π was calculated within each of the following four subgroups, i.e., 28 local and 67 modern sake-brewing cultivars and 29 local and 47 modern cooking cultivars (Table 3). A total of 13 non-sake-brewing and non-cooking cultivars including two cultivars derived from an *indica*/*japonica* cross were excluded from this analysis. The overall average value of π estimated in this study (π=0.0021) was about one fifth and a half the value of that estimated in *Arabidopsis thaliana* (π=0.0106, Miyashita et al. 1999) and *Dioscorea tokoro* (π=0.0050, Innan et al. 1999), respectively. The π estimated within the sake-brewing cultivars was even lower than that within the cooking cultivars (Table 3). Within the sake-brewing cultivars, the π value was much smaller in the local cultivars (π=0.0011) than in the modern cultivars (π=0.0021). By contrast, within the cooking cultivars, the average genetic diversity in the modern cultivars (π=0.0020) was equivalent to that in the local cultivars (π=0.0021). The local sake-brewing cultivars showed the lowest π: the π value of this subgroup was about half of that in the other three subgroups.

Genetic diversity was also evaluated based on the nuclear SSR data for 32 loci covering all 12 rice chromosomes (Table 3). A total of 27 loci (84%) were found to be polymorphic among 171 Japanese rice cultivars. Overall number of alleles per locus ranged from 1 to 13, with an average of 4.1. An overall average *H* value based on the nuclear SSR data was 0.33 within the Japanese cultivars, which was exactly the same as the *H* value (0.33) obtained previously in five *japonica* cultivars (Ishii et al. 2001). The average *H* value of Japanese cultivars was about half of that estimated for world rice accessions (*H*=0.68), which included 83 landraces, 15 breeding lines, and 95 improved varieties from eight major rice-growing regions of the world (Yu et al. 2003). These

Table 3 Genetic diversity estimated within local sake-brewing rice, modern sake-brewing rice, local cooking rice, and modern cooking rice cultivars using nuclear and chloroplast DNA markers. π Nucleotide diversity, *Nc* nuclear genome, *Ct* chloroplast genome, *H* diversity index

Group	No. of cultivars	AFLP[a] $\pi\times100$	Nc-SSR[b] *H* (No. allele)	Ct-SSR[b] *H* (No. allele)
Sake-brewing rice				
Local	28	0.11	0.16 (2.2)	0.00 (1.0)
Modern	67	0.21	0.31 (2.9)	0.05 (1.4)
Average	95	0.19	0.30 (3.2)	0.03 (1.4)
Arithmetic mean[c]	–	0.16	0.24 (2.6)	0.03 (1.2)
Cooking rice				
Local	29	0.21	0.34 (2.7)	0.07 (1.3)
Modern	47	0.20	0.32 (3.1)	0.09 (2.0)
Average	76	0.21	0.35 (3.5)	0.08 (2.0)
Arithmetic mean[c]	–	0.20	0.33 (2.9)	0.08 (1.7)
Total				
Average	171	0.21	0.33 (4.1)	0.06 (2.0)
Arithmetic mean[c]	–	0.18	0.28 (2.7)	0.05 (1.4)

[a] π was estimated by AFLP analysis of total genome
[b] *H* was calculated for both nuclear and chloroplast SSR markers. Average number of alleles per marker locus (*No. allele*) found in each group is also shown
[c] Arithmetic mean was calculated by taking the arithmetic mean value of local rice group and modern rice group

results support the previous reports that in contrast to the richness of genetic variation in the world rice resources, rice cultivars in Japan generally display a much narrower genetic variation (Akagi et al. 1997; Kubo et al. 1998; Ashikawa et al. 1999).

The average *H* values calculated within the four subgroups agreed relatively well with the π estimated based on the AFLP data (Table 3). Our data thus clearly show that the local sake-brewing cultivars possess the narrowest genetic base. On the other hand, the genetic diversity within the modern sake-brewing cultivars was comparable to that in the cooking cultivars. The modern sake-brewing cultivars have been bred for the last seven decades through crossings of the local sake-brewing cultivars with the cooking rice cultivars. This introgressive breeding process likely has contributed to increase the genetic diversity in the modern sake-brewing cultivars.

A catalog of Japanese rice cultivars was constructed based on the AFLP and nuclear SSR fingerprints. Among 171 cultivars, 117 haplotypes were distinguished based on 25 AFLP bands. Among these haplotypes, 95 consisted of single cultivars and could therefore be identified based on their unique AFLP fingerprints. Similarly, these 171 cultivars were classified into 157 distinguishable haplotypes, based on 27 nuclear SSR loci. Among these haplotypes, 150 consisted of single cultivars. Accordingly, a total of 150 cultivars including 55 that could not be distinguished by AFLP analysis were distinguished based on their unique nuclear SSR fingerprints. The remaining 20 cultivars could not be cataloged using these two marker systems.

Phylogenetic relationships among the Japanese rice cultivars estimated by AFLP and nuclear SSR markers

We first constructed a phylogenetic tree based on 11 AFLP markers, using only the local cultivars (Fig. 2A). Because the modern cultivars were bred through hybridization between local cultivars, we considered it better not to include these to better illustrate phylogenetic relationships. For comparison, we also constructed a dendrogram using all 184 cultivars. The 57 local cultivars, including 28 sake-brewing and 29 cooking cultivars, showed essentially a similar clustering feature in the two dendrograms (data not shown). Most of the local sake-brewing cultivars formed a single cluster designated I-1. A tree constructed based on 32 nuclear SSR loci revealed a very similar clustering (Fig. 2B). According to these two trees, it was strongly suggested that most of the local sake-brewing cultivars were derived monophyletically. All cultivars belonging to cluster I are typical for the western regions of Japan (Fig. 1). On the other hand, most cultivars belonging to clusters II and III are typical for the eastern and northern regions. Apparently, regional differences exist in the overall genomic structure of the Japanese local rice cultivars. The cluster analysis showed that Omachi and Watari-bune, which are the two major local sake-brewing cultivars in the western regions, were genetically very close. This agrees with the presumption that Watari-bune was likely derived from Omachi (Fig. 3). It was noted that three local sake-brewing cultivars, Benkei, Shin-yamada-ho 1, and Yamato-nishiki, were not included in cluster I-1. Because agronomic traits of these local cultivars clearly differed from those reported for the original cultivars (data not shown), we considered that they were probably miss-classified accessions. It is unclear why Hattan-sou, which is another local sake-brewing cultivar, did not belong to cluster I-1 based on the AFLP loci (Fig. 2A).

Phylogenetic relationships among the local cultivars and the modern cultivars were examined next. In this analysis, 13 cultivars of non-sake-brewing and non-cooking cultivars including two cultivars derived from an *indica*/*japonica* cross were used in addition to 171 sake-brewing and cooking cultivars. Since trees constructed independently based on 11 AFLP and 32 SSR loci were essentially equivalent, we show the one constructed using the combined data (Fig. 4). Almost all of the local sake-brewing cultivars were clustered in I-1, which basically corresponds to cluster I-1 in Fig. 2. This cluster also contained about half of the modern sake-brewing cultivars, including Yamada-nishiki and its related cultivars. Because Yamada-nishiki and its closely related cultivars were bred through crossings with local sake-brewing cultivars (Fig. 3), it is reasonable to assume that they are genetically similar to these parental local cultivars. The other modern sake-brewing cultivars that did not belong to this cluster were dispersed in cluster II and many in subcluster II-3. The result shows that these modern sake-brewing cultivars possess genetic architectures introgressed from the cooking cultivars. Cluster II contained one major modern sake-brewing cultivar, Gohyaku-mangoku, and other cultivars that are genetically close to it. Many cultivars in this cluster II are cultivated in the northern regions and possess characteristic traits such as cold resistance and early heading, both of which are required for adaptation to this region and were likely incorporated into these cultivars from the local cooking cultivars. Gohyaku-mangoku is considered to have received sake-brewing traits from Omachi (Fig. 3). However, Gohyaku-mangoku was not clustered with Omachi but instead clustered with cooking rice cultivars including Kamenoo and other northern cultivars. About 100 years ago, Kamenoo was one of a few major cooking cultivars that were well adapted to the northern climate of Japan because of their resistance to the cold. Because Kamenoo has small grains and no *Shinpaku*, it does not have typical sake-brewing traits. Therefore, the adaptive traits of Gohyaku-mangoku have probably been introgressed from Kamenoo, while the sake-brewing traits derived from Omachi have been retained.

Genetic diversity and phylogenetic relationships estimated by chloroplast SSR markers

Among ten chloroplast SSR markers examined (Table 2), two intron (RCt1 and RCt8) and four intergenic (RCt3, RCt5, RCt6, and RCt9) markers showed polymorphisms

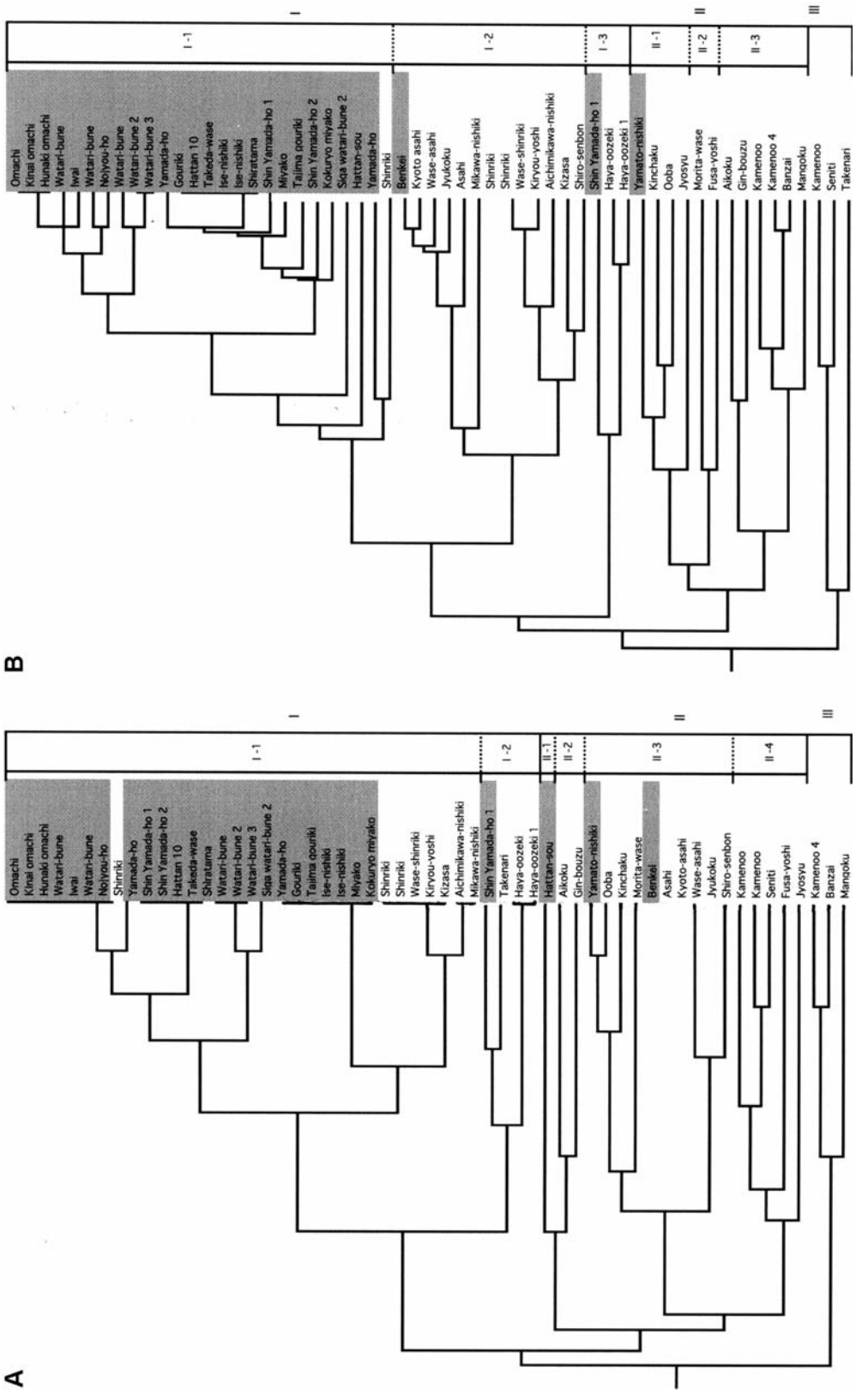


Fig. 2 Phylogenetic trees constructed based on amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) analyses. The trees were constructed based on **A** 11 AFLP markers and **B** 32 SSR markers in 28 local sake-brewing cultivars and 29 local cooking cultivars. *Shaded boxes* indicate sake-brewing cultivars

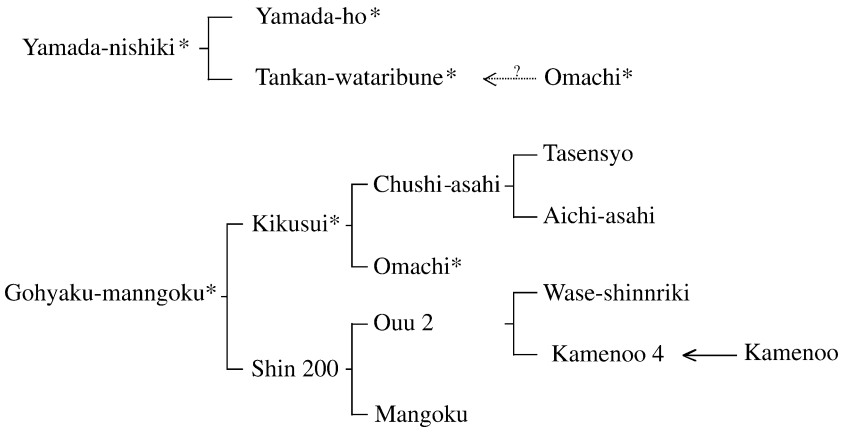


Fig. 3 A pedigree of the two most popular modern sake-brewing rice cultivars, Yamada-nishiki and Gohyaku-mangoku. *Asterisks* indicate sake-brewing cultivars. Cultivar Kamenoo was mainly used for cooking but also used for sake brewing in the past

in 171 Japanese rice cultivars. The average number of alleles was smaller in the sake-brewing rice than in the cooking rice and the smallest in the local sake-brewing rice (Table 3). The average *H* values calculated based on the chloroplast SSR loci within the four subgroups were also much smaller than those calculated based on the nuclear SSR loci. Seven plastotypes were recognized among all the cultivars. A majority of the sake-brewing cultivars possessed either one of the two major plastotypes A (85%) or B (9%). All the local sake-brewing cultivars and 88% of the modern sake-brewing cultivars possessed the major plastotype A (Fig. 5). Plastotype B occurred in 10% and 8% of the modern sake-brewing and the modern cooking cultivars, respectively. This second major plastotype, however, was not found in the local cooking cultivars. These results suggest that plastotype B appeared in the modern cultivars through either mutation or introduction from unknown sources into the modern cultivars. The local cooking cultivars possessed plastotype D in addition to plastotypes A and C, while the modern cooking cultivars possessed two other plastotypes, E and F, in addition to plastotypes A, B, and C . Apparently, the modern cooking cultivars possess the largest genetic variability in their chloroplast genome, which is in contrast to their nuclear genome diversity. The local sake-brewing cultivars possess the smallest variability in both nuclear and chloroplast genomes.

Phylogenetic relationships among these plastotypes were studied based on the allele differences in each Ct-SSR marker. Figure 6 shows the summary network of the plastotypes constructed based on the neighbor-joining method (Saitou and Nei 1987). Since all of the local sake-brewing cultivars belong to the major plastotype A, our analysis of the maternal lineage also strongly supports the monophyletic origin of the sake-brewing rice in Japan. Two *japonica* cooking rice cultivars, Kinuhikari and Dontokoi, possessed plastotype F, which was close to plastotype G found in the two cultivars derived from an *indica*/*japonica* cross, Yumetoiro and Takanari. Kinuhikari was bred using an *indica* cultivar, IR8, as a mother, while Dontokoi was bred using Kinuhikari as a mother. Plastotype F therefore was likely derived from plastotype G through mutation.

A history of Japanese sake-brewing cultivars inferred from the molecular variations

We have provided evidence that the Japanese sake-brewing rice cultivars have a much narrower genetic base than the cooking cultivars. This is most likely the result of the monophyletic origin of the local sake-brewing cultivars that possessed desirable traits for sake brewing. Based on the AFLP and SSR data, a phylogenetic history of sake-brewing cultivars in Japan can be inferred as follows: Cultivars having characteristic large grains with low protein content and large *Shinpaku* were found in a small group of rice cultivated in the western regions. Some of these cultivars became recognized as suitable sake-brewing cultivars and disseminated throughout Japan. In recent years, genetic diversity in these limited local resources has become enlarged as a result of introgression from modern cooking cultivars through breeding practices. The modern sake-brewing cultivars can be classified into two main subgroups, i.e., a group closer to the western local cultivars and a group closer to the eastern and northern cultivars. This suggests that a genetic structure preferred for sake brewing originally found in the western local cultivars has been introgressed into the genetic backgrounds of various modern cultivars through breeding for adaptation to different local environments. Therefore, limited number of local cultivars might have contributed the genes for sake-brewing traits to the modern cultivars.

Identification of DNA markers associated with sake-brewing traits

We performed an ANOVA test that combined the trait data and DNA fingerprinting data obtained from 38 cultivars of sake-brewing rice and 12 cultivars of cooking rice. This ANOVA test is based on pedigree relationships and is thus expected to reflect linkage disequilibrium that has been retained among the sake-brewing cultivars. Significant associations were detected for 12 AFLP markers and nine SSR markers with several sake-brewing traits. Among these, seven AFLP markers showed association at the 1% significance level with *Shinpaku* size, grain weight, width, and/or thickness, while two SSR markers showed signif-

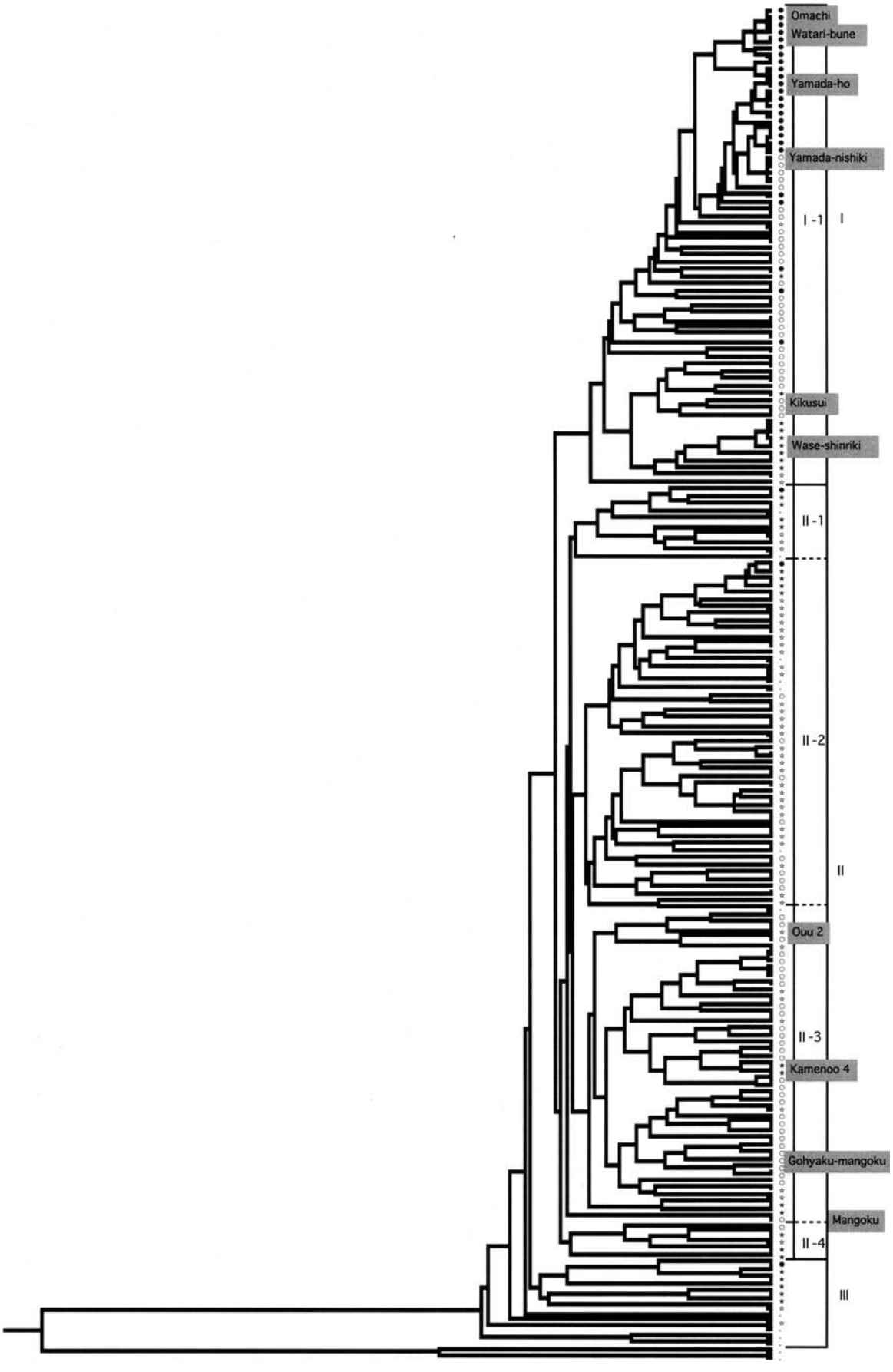


Fig. 4 A phylogenetic tree constructed based on the combined AFLP and SSR data in 184 Japanese rice cultivars. *Filled circles*, *open circles*, *filled stars*, and *open stars* indicate local sake-brewing, modern sake-brewing, local cooking, and modern cooking rice, respectively

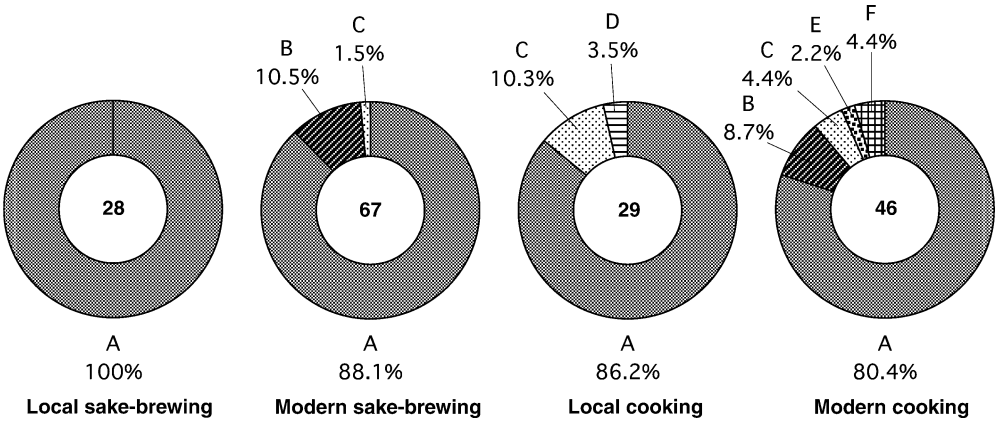


Fig. 5 Frequencies of six chloroplast haplotypes (plastotypes) in 171 Japanese rice cultivars. The other 13 cultivars consisting of non-sake-brewing and non-cooking and cultivars derived from an *indica*/*japonica* cross possessed plastotype A (seven cultivars), plastotype B (four cultivars), and plastotype G (two cultivars)

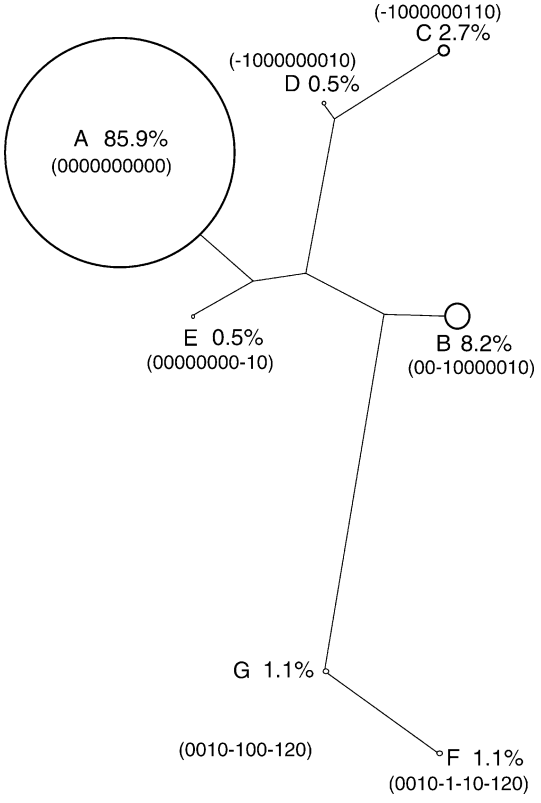


Fig. 6 A summary network of plastotypes detected in this study. The *ten digits in parentheses* indicate the difference of the SSR length between the standard marker allele in cultivar Nipponbare at RCt1 (10 bp), RCt2 (11 bp), RCt3 (10 bp), RCt4 (12 bp), RCt5 (10 bp), RCt6 (10 bp), RCt7 (10 bp), RCt8 (17 bp), RCt9 (10 bp), and RCt10 (10 bp), respectively (+ increase in SSR length, − decrease in SSR length). Diameter of circle is proportional to the numbers of cultivars, and relative distance represents the genetic distance

icant association with *Shinpaku* size (Table 4). An AFLP marker, 5801b, was previously shown to specify a QTL for *Shinpaku* size of Yamada-nishiki (Yoshida et al. 2002). This QTL that was detected by single-point analysis, using doubled haploid lines explained 17.3% of the phenotypic variation in *Shinpaku* size. One SSR marker (RM259) associated with one QTL for *Shinpaku* size, which was detected in a F_2 population derived from a cross between a sake-brewing cultivar, Hyogo-kitanishiki, and a cooking cultivar, Yumetoiro (Kuze et al., unpublished result). Another SSR marker (RM234) that showed significant association with *Shinpaku* size coincided with the QTL marker for grain width and thickness. These results validate the significance of marker associations with the sake-brewing traits that were detected by the ANOVA test. Usefulness of these and other DNA markers in the marker-assisted breeding of sake-brewing rice has to be further examined.

Table 4 Marker association with four sake-brewing traits detected by analysis of variance tests. *Shinpaku* in Japanese stands for the large white-core in a rice grain

Marker	*Shinpaku* size	Grain weight	Grain width	Grain thickness
0238a	**	**	*	
1848a			*	*
1848b			*	*
5801a			**	**
5801b[a]	**	**	**	
5907b	*			
6326a	*		**	
6433a		*		
6433c	**			
6455b	*			
6457d	*		**	
6460c	**		*	
RM5			*	*
RM31	*			
RM101		*		
RM232	*			
RM234	**		*	
RM235	*			
RM257	*			
RM259	**			
RM315			*	

Significance levels: *P<0.05, **P<0.01

[a]The AFLP band 5801b is identical to the AFLP band designated as 5801a in Yoshida et al. (2002). *RM* represents SSR markers and the others AFLP markers

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