Evolutionary relationships among rice species with AA genome based on SINE insertion analysis

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Previous studies based on morphological and molecular markers indicated that there are two cultivated and five wild rice species within the *Oryza* genus with the AA genome. In the cultivated rice species, *Oryza sativa*, a retroposon named *p-SINE1* has been identified. Some of the *p-SINE1* members characterized previously showed interspecific insertion polymorphisms in the species with the AA genome. In this study, we identified new *p-SINE1* members showing interspecific insertion polymorphisms from representative strains of four wild rice species with the AA genome: *O. barthii*, *O. glumaepatula*, *O. longistaminata*, and *O. meridionalis*. Some of these members were present only in strains of one species, whereas the others were present in strains of two or more species. The *p-SINE1* insertion patterns in the strains of the Asian and African cultivated rice species *O. sativa* and *O. glaberrima* were very similar to those of the Asian and African wild rice species *O. rufipogon* and *O. barthii*, respectively. This is consistent with the previous hypothesis that *O. sativa* and *O. glaberrima* are derived from specific wild rice species. Phylogenetic analysis based on the *p-SINE1* insertion patterns showed that the strains of each of the five wild rice species formed a cluster. The strains of *O. longistaminata* appear to be distantly related to those of *O. meridionalis*. The strains of these two species appear to be distantly related to those of three other species, *O. rufipogon*, *O. barthii* and *O. glumaepatula*. The latter three species are closely related to one another with *O. barthii* and *O. glumaepatula* being most closely related. A phylogenetic tree including a hypothetical ancestor with all loci empty for *p-SINE1* insertion showed that the strains of *O. longistaminata* are related most closely to the hypothetical ancestor. This indicates that *O. longistaminata* and *O. meridionalis* diverged early on, whereas the other species diverged relatively recently, and suggests that the *Oryza* genus with AA genome might have originated in Africa, rather than in Asia.

INTRODUCTION

Rice is one of the most widely cultivated cereal crops in the world. The genus, *Oryza*, is comprised of two cultivated rice species and twenty-one wild rice species (Vaughan, 1994; Khush, 1997). The wild rice species represent a reservoir of useful genes and have great potential for aiding in the improvement of the cultivated rice variety (Tanksley and McCouch, 1997; Brar and Khush, 1997). The clarification of the genetic relationships of the wild rice species is thus not only theoretically important for understanding the evolution of rice, but also essential for effectively utilizing these wild

resources. Based on cytogenetic analysis (Li et al., 1964; Morinaga, 1964), genomic DNA hybridization (Aggarwal et al, 1997) and DNA sequence analysis of two nuclear genes (Ge et al., 1999), all species in the *Oryza* genus are classified into six diploid genome types (AA, BB, CC, EE, FF and GG) and four tetraploid genome types (BBCC, CCDD, HHJJ and HHKK) (Vaughan, 1994; Khush, 1997; Ge et al., 1999).

Species with the AA genome have been widely studied because these include the cultivated rice species. The species with the AA genome are classified into two cultivated rice species, *O. sativa*, which is of the Asian origin and cultivated worldwide, and *O. glaberrima*, which is endemic to Africa, as well as five wild rice species, *O. rufipogon*, *O. barthii*, *O. longistaminata*, *O. meridionalis* and *O. glumaepatula*. *O. rufipogon* is distributed throughout

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tropical Asia and Oceania, and has a life cycle ranging from perennial to annual. *O. barthii* (previously called *O. breviligulata*) is typically annual and is distributed throughout west Africa. *O. rufipogon* and *O. barthii* are thought to be progenitors of *O. sativa* and *O. glaberrima*, respectively (Morishima et al., 1992; Khush, 1997). *O. longistaminata* is another African wild rice species which is perennial and rhizomatous, as well as being partly self-incompatible and allogamous (Chu et al., 1969a; Ghesquiere, 1986). *O. meridionalis*, the wild rice species in Australia, is annual, whereas *O. glumaepatula*, the Latin American relative, varies in perenniality. Various levels of hybrid sterility have been observed in interspecific crossings among these species (Chu et al., 1969b; Morishima, 1969; Chu and Oka, 1970; Naredo et al., 1998), which resulted in a reproductive isolation mechanism among species.

The nomenclature and taxonomy of the AA genome species have been in confusion because of rich variation, even within the strains of a species. Isozyme analysis pointed out for the first time that *O. meridionalis* and *O. longistaminata* represent two distinct species, whereas *O. barthii*, *O. rufipogon* and *O. glumaepatula* are closely related (Second, 1985). Results obtained on the basis of nuclear RFLP (Wang et al., 1992; Bautista et al. 2001), RAPD (Ishii et al., 1996; Bautista et al. 2001), AFLP (Aggarwal et al., 1999) and polymorphisms exhibited by other molecular markers (Bautista et al. 2001) are largely consistent with this classification. However, the detailed phylogenetic relationship is still inconsistent between studies.

Short interspersed elements (SINEs) are eukaryotic mobile elements whose transposition uses RNA as an intermediate in a process called retroposition (Maraia, 1995). SINEs range from 70 to 500 bp with each having an internal promoter for RNA polymerase III. Many SINEs appear to be related to tRNAs, whereas a few, such as the primate Alu and rodent B1 family elements, are related to 7SL RNA. As a consequence of retroposition of SINE, descendent members are replicated from the parent member(s) and inserted into the chromosome randomly. The probability that independent insertion occurs at the same locus, or that a SINE is precisely removed, is virtually nil (Nikaido et al., 1999; Shedlock and Okada 2000). The unique and irreversible event of SINE insertion endows them with a perfect character to infer a phylogenetic relationship, which is generally not available for markers based on DNA sequence variation (Shedlock and Okada 2000). Alu insertions have been used to evaluate genetic relationships between human and higher primates (Bailey and Shen, 1993; Bailey and Shen, 1997; Hamdi et al., 1999). Polymorphic insertions of Alu have been used to support the Africa origin of humans (Batzer et al., 1994). SINE insertion analysis has successfully clarified the previously contentious phylogeny of salmonids (Murata et al., 1993), African cichlid

tribes (Takahashi et al., 1998), and whales in relation to even-toed ungulates (Shimamura et al., 1997; Nikaido et al., 1999). An insertion analysis of a SINE element has been used to study the relationship of rice (Mochizuki et al., 1993; Hirano et al., 1994) and *Brassica* species (Tatout et al., 1999).

p-SINE1 is the first plant SINE identified in *O. sativa* and its related rice species (Umeda et al., 1991; Mochizuki et al., 1992). Copy numbers of *p-SINE1* have been estimated to be about 6500 and 3000 per haploid genome of *O. sativa* and *O. glaberrima*, respectively (Motohashi et al., 1997). The enormous volume of SINE amplifications per organism suggests an important evolutionary role for shaping the diversity of *Oryza* genomes. We have previously identified several *p-SINE1* members from the *O. sativa* genome, which showed insertion polymorphisms in rice strains with the AA genome (Mochizuki et al., 1993; Hirano et al., 1994). We have recently identified many *p-SINE1* members from the *O. sativa* genome (Motohashi et al., 1997; Cheng et al. unpublished data). Of these, many are present at the respective loci in all the AA genome strains examined, but some show interspecific insertion polymorphism. The polymorphic *p-SINE1* members include those which form a subfamily (named RA) with three correlated mutations from the *p-SINE1* consensus sequence and are specifically present in the *O. sativa*-*O. rufipogon* population (Cheng et al. unpublished data).

In this study, we isolated new *p-SINE1* members from representative strains with the AA genome of four wild rice species other than *O. rufipogon*, and investigated the presence and absence of these *p-SINE1* members at the respective loci in the strains of all species with the AA genome. Based on these results, we classified the rice strains and inferred their phylogenetic relationships. We will show that the method used in this study is a powerful new tool for studying the classification of the cultivated and wild rice strains and for inferring the relationships between them.

MATERIALS AND METHODS

Rice strains used. Rice strains used are shown in Tables 1 and 2. Most of the strains were obtained from Dr. N. Kurata, the Genetic Stock Research Center of National Genetic Institute, Japan. Total genomic DNAs were isolated from these strains with a Nucleon Phytopure kit (Amersham).

Polymerase chain reaction (PCR). PCR was performed with *Ex Taq* DNA polymerase (Takara) as described previously (Motohashi et al., 1997). PCR products were electrophoresed in a 1.8% agarose gel. In most cases, when determining the presence or absence of each *p-SINE1* member by PCR, one unique fragment with or

Table 1. The presence of *p-SINE1* members at respective loci in the representative strains of AA genome and other diploid rice species^a

Member	AA								BB	CC	EE	FF
	Os		Or	Ogla	Oba	Oglu	Om	Ol	Op	Oo	Oa	Obr
	Nippon. ^b	IR36	W1954	W440	W1581	W1189	W2077	W1624	W1582	W0002	W1538	W1401
r1	+	+	+	+	+	+	+	+	/	/	/	/
r6	+	+	+	—	+	+	/	—	/	/	/	/
r29	+	+	+	+	+	+	+	—	—	—	—	/
r32	+	+	+	+	+	+	—	+	—	—	—	—
r34	+	+	—	+	+	+	—	—	/	—	—	—
r46	+	+	+	+	+	+	—	+	—	/	/	/
r55	—	+	+	—	—	—	—	—	—	/	—	/
r63	+	+	+	+	+	+	—	—	/	/	/	/
r68	+	+	+	+	+	+	+	—	—	—	—	/
r69	—	+	+	+	+	+	+	+	/	—	—	/
r70	+	+	+	+	+	+	—	—	—	—	—	/
r72	+	+	+	+	+	+	—	/	/	/	/	/
r217	+	+	+	+	+	+	+	+	/	/	/	/
r509	+	+	+	+	+	+	—	+	/	/	/	/
r502	+	+	+	—	—	—	—	—	—	—	—	—
<hr/>												
r601	—	—	—	+	+	+	—	—	/	—	—	/
r605	+	+	+	+	+	+	+	+	/	/	—	/
r606	+	+	+	+	+	+	+	—	/	/	/	/
r607	+	+	+	+	+	+	/	/	—	/	/	/
r701	—	—	—	—	—	—	—	±	/	—	—	/
r702	—	—	—	—	—	—	—	+	/	—	/	/
r703	—	—	—	—	—	—	+	+	—	—	—	/
r705	—	—	—	—	—	—	—	+	—	—	/	/
r706	+	+	+	+	+	+	+	+	+	+	+	+
r707	—	—	—	—	—	—	+	+	/	/	/	/
r708	+	+	+	+	+	+	+	+	—	—	—	/
r709	+	+	+	+	+	+	+	+	/	/	/	/
r801	—	—	—	—	—	+	—	—	/	/	/	/
r803	+	+	+	+	+	+	—	—	/	—	—	/
r804	+	+	+	+	+	+	+	+	/	/	/	/
r806	—	—	—	+	+	+	—	—	/	/	/	/
r807	—	—	—	+	+	+	—	—	/	—	/	/
r808	+	+	+	+	+	+	+	—	—	—	/	/
r901	—	—	—	—	—	—	+	—	/	/	/	/
r902	—	—	—	—	—	—	+	—	/	/	/	/
r903	+	+	+	+	+	/	+	+	/	+	/	/
r904	—	—	—	—	—	—	+	—	—	—	—	/
r905	—	—	—	—	—	—	+	—	—	—	—	—
r906	—	—	—	—	—	—	+	—	/	—	/	/
r907	—	—	—	—	—	—	+	—	/	—	/	/
r908	/	/	/	/	/	/	+	/	/	/	/	/

^a *p-SINE1* members identified in the cultivated and wild rice genomes are shown in the first column. Members shown in boldface are those of RA subfamily. The wild or cultivated rice strains of the species, *O. sativa* (Os), *O. rufipogon* (Or), *O. glaberrima* (Ogla), *O. barthii* (Oba), *O. glumaepatula* (Oglu), *O. meridionalis* (Om), *O. longistaminata* (Ol), *O. punctata* (Op), *O. officinalis* (Oo), *O. australiensis* (Oa), and *O. brachyantha* (Obr), are shown at the top together with their genome types, AA, BB, CC, EE, and FF. The presence (+) or absence (—) of *p-SINE1* members at the respective loci was determined by analysis of PCR-amplified fragments (see Materials and Methods). ± indicates the strain that generates fragments both with and without *p-SINE1*. A slash shows that no PCR fragments was amplified by PCR. The presence of *p-SINE1* members identified originally in the respective strains are shown by + in boldface. +* and +[†] indicate *p-SINE1* members with *Tnr8* and solo-LTR of a retrotransposon (*RIRE11*), respectively, whereas +‡ indicates the r217 member with a LINE (RILN20) in its flanking region.

^b Nipponbare.

Table 2. The distribution of *p-SINE1* members at respective loci in the strains of AA genome species^a

[illegible]

^a The name, ecotype and origin of wild and cultivated rice strains with AA genome were shown in the left columns. Ecotypes of the *O. sativa* strains, japonica (JA) and indica (IN) are shown. Ecotypes of the other strains, annual (A), perennial (P) and intermediate (M) are also shown. *O. rufipogon* strains, whose ecotypes are reported as perennial based on one classifier or annual based on another, are indicated by (P, A). Thirty-five *p-SINE1* members or other transposable elements investigated are shown at the top. Two polymorphic *p-SINE1* members (r905 and r907) in Table 1 were not used for the analysis, because they showed the same polymorphic pattern as r904 and r906. Other features are the same as those shown in Table 1.

without a *p-SINE1* member was clearly visible in the agarose gel. When the fragments differed in size or when two or more bands were present, the presence or absence of *p-SINE1* in the fragments was confirmed by Southern hybridization (Mochizuki et al., 1993) or direct sequencing of the PCR products. Primers used for the analysis

will be informed upon request.

ADL-PCR (Adaptor-Ligation Based PCR) (Spertini et al., 1999) was performed with *Ex Taq* DNA polymerase (Takara) as follows: total DNA from W1581 (*O. barthii*), W1189 (*O. glumaeapatula*), W2077 (*O. meridionalis*) or W1624 (*O. longistaminata*) was digested with *Hind*III,

which does not cut the *p-SINE1* sequence. T4 DNA ligase (New England Biolabs) was used to ligate the digested DNA with the oligonucleotide adaptor. PCR first was performed with a ligated sample as the template using primers that hybridize to the adaptor and the *p-SINE1* consensus sequence to obtain fragments with the proximal portion of the *p-SINE1* sequence and its flanking sequence. PCR was then performed with primers that hybridize to the adaptor and a different portion of the *p-SINE1* sequence. To isolate the fragments with the *p-SINE1* sequence of the RA subfamily, the 3' ends of the primers used were arranged to match the mutated nucleotides in the *p-SINE1* sequence of the RA subfamily (see Fig. 1 for primers used). To obtain fragments that included the entire *p-SINE1* sequence, ADL-PCR was

performed with primers that hybridize to the flanking sequence of each identified member and the adaptor.

IPCR (Inverse PCR) was performed as described previously (Tenzen et al., 1994) with total DNA as the template and primers that hybridize to the *p-SINE1* consensus sequence. In an attempt to isolate RA *p-SINE1* members, the 3' ends of two primers for the 1st PCR reaction were arranged to match the mutated nucleotides in the *p-SINE1* sequence of the RA subfamily (see Fig. 1 for primers used).

Cloning and DNA sequencing. PCR products were cloned into the pGEM-T Easy Vector plasmid (Promega), according to the supplier's instructions. DNA sequencing was performed with a BigDye Terminator kit (PE Bio-

Fig. 1. Nucleotide sequences of the *p-SINE1* members. Members identified previously in *O. sativa* (A) and new members identified in *O. barthii* (B), *O. longistaminata* (C), *O. glumaepatula* (D) and *O. meridionalis* (E), are shown. RA-subfamily members are shown in boldface. A consensus sequence (CON) derived from all *p-SINE1* members (Motohashi et al., 1997) is shown at the top; a consensus sequence (RA-CON) of RA-subfamily members is shown at bottom. Sequences corresponding to the A- and B-boxes of the pol III promoter are shown in boldface. In each *p-SINE1* sequence, nucleotides identical to those in the consensus sequence are shown by dashes, and deleted nucleotides by slashes. Underlined nucleotide(s) in r706 and r908 indicates duplication. The three diagnostic mutations of the RA-subfamily members are shown in boldface. Members r32 and r63 respectively have a transposon *Tnr8* and a solo LTR of a retrotransposon (*RIRE11*), whose target site sequences are boxed. Positions of two pairs of primers [IP1-1 and IP1-2 for first PCR and IP2-1 and IP2-2 for second PCR] used to isolate the *p-SINE1* members by IPCR are shown by the horizontal arrows at the top, while positions of two primers [PS1 and PS2] used to isolate the *p-SINE1* members by ADL-PCR are shown at the bottom. Note that in attempts to isolate RA-subfamily members, the 3' nucleotides of primers of IP1-1 and IP1-2 as well as PS1 and PS2 are arranged to match the two diagnostic mutations of RA-subfamily members in A box or B box, respectively.

system) and an ABI PRISM 377 DNA sequencing system. Some PCR product was directly sequenced after electrophoresis in an agarose gel and recovered using SUPREC-01 cartridges (Takara). Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB088070 - AB088081 and AB088122 - AB088135.

Computer analysis. Primary nucleotide sequences were analyzed with the GENETYX-Mac 10.1 software system. Nucleotide sequence searches made in the DDBJ/Genbank/EMBL databases were performed with the programs, BLAST (Altschul et al., 1990) and Smith-Waterman search (Smith and Waterman, 1981). Multiple sequences were aligned using CLUSTAL W (version 1.7) with some manual modifications.

To construct the phylogenetic tree for the various rice strains, the presence or absence of the *p-SINE1* members at particular loci was organized into a data matrix; the presence of a *p-SINE1* member at a given locus was coded 1, and its absence at the same locus coded 0. Some strains generated two PCR-amplified fragments with or without a *p-SINE1* member, indicating both the presence and absence of a *p-SINE1* member, and this case was coded 1, the presence state. The neighbor-joining (NJ) method was performed using PAUP* 4.0b8 software (Swofford, 1998).

RESULTS

Isolation of new *p-SINE1* members present in wild rice strains with AA genome. We have previously identified a large number of *p-SINE1* members present at particular loci in the genomes of three *O. sativa* strains (Nipponbare, IR36 and C5924). Of these, 13 members were found to be polymorphic for insertion in strains of two cultivated and five wild rice species with AA genome (Mochizuki et al., 1993; Motohashi et al., 1997; Cheng et al. unpublished data). The nucleotide sequences of these polymorphic members are shown in Fig. 1, together with two non-polymorphic members, r1 and r217. Of these, four members (r34, r55, r63, and r502) belong to the RA subfamily with three diagnostic mutations. Table 1 shows the presence or absence of all *p-SINE1* members at the respective loci in representative rice strains from all species with the AA genome, as demonstrated by PCR with a pair of primers that hybridize to the flanking regions of each *p-SINE1* member.

In this study, we attempted to isolate new polymorphic *p-SINE1* members from representative strains of the four wild rice species with AA genome, *O. barthii*, *O. glumapatula*, *O. longistaminata* and *O. meridionalis*, by inverse PCR (IPCR) with primers matching the *p-SINE1* consensus sequence. Because a few *p-SINE1* members belonging to the RA subfamily showed insertion polymorphism

in strains of *O. sativa/O. rufipogon* as well as in some species with the AA genome, we also attempted to isolate more RA-subfamily members by IPCR and adaptor-ligation based PCR (ADL-PCR) from the rice strains used above with primers matching to an RA-subfamily *p-SINE1* consensus sequence (see the bottom of Fig. 1). Twenty-six members were obtained, and their nucleotide sequences were aligned together with those of the 15 members identified previously (Fig. 1). There were no subfamily members with a set of correlated mutations on the basis of the *p-SINE1* consensus sequence, except for a few members belonging to the RA subfamily (Fig. 1).

We investigated the presence or absence of all *p-SINE1* members at the respective loci in representative rice strains from all species with the AA genome, by PCR with a pair of primers that hybridize to the flanking regions of each *p-SINE1* member. Some *p-SINE1* members, such as r217 and r804, were present in all the rice strains with the AA genome, similar to member r1 which has been shown to be present in all the strains of *O. sativa* and *O. rufipogon* (see Table 1). Most of the other members, belonging to either the RA or non-RA subfamily, showed interspecific *p-SINE1* insertion polymorphisms (Table 1). Most of the members identified in strains of *O. longistaminata* or *O. meridionalis* were present only in those of the same species, whereas most of the members from the other species were present in the strains of two or more species (Table 1).

We also investigated the presence or absence of all *p-SINE1* members at the respective loci in representative rice strains from four species with non-AA genome (BB, CC, EE or FF), as above. We failed to amplify the fragments with most of the members from the strains with non-AA genome (see Table 1), probably because the binding sites of primers used for PCR were lost due to mutations.

Distribution of *p-SINE1* members among various rice strains with AA genome. To understand relationships among various rice strains with the AA genome, we investigated the distribution of 32 *p-SINE1* members in 72 cultivated and wild rice strains of all species with the AA genome (see Table 2). The three non-polymorphic *p-SINE1* members, including r1, were present at their respective loci in all the rice strains (Table 2), clearly indicating that all these strains are derived from a common source. The 29 polymorphic *p-SINE1* members, however, showed different distribution spectra in the rice strains examined. Some members, such as r801, were present only in the strains of one species, and the others, such as r68, were present in several species (Table 2). Several of these members, such as r34, r803, r6 and r69, showed intraspecific polymorphisms in one or two species (Table 2). The five RA members, such as r601, identified in species other than *O. sativa* did not show any

intraspecific insertion polymorphisms (Table 2).

Some members were specifically present in the strains of each of the wild rice species (*O. longistaminata*, *O. meridionalis*, *O. glumaepatula*, or *O. rufipogon*), delineating the strains of each species from others. Note, however, that a few of these members showed insertion polymorphisms among the strains of the species (Table 2). Interestingly, several *p-SINE1* members were present in the strains of all the species, except for *O. longistaminata* or *O. meridionalis*, which delineates the strains of these two species from the others (Table 2).

In the strains of *O. sativa* and *O. rufipogon*, which showed a very similar pattern, no particular *p-SINE1* members could be used to distinguish the strains of *O. sativa* from those of *O. rufipogon* (Table 2). Similarly, in the strains of *O. glaberrima* and *O. barthii*, which showed a very similar pattern, no *p-SINE1* members could be used to distinguish the strains of *O. glaberrima* from those of *O. barthii* (Table 2).

Notes on transposable elements found within *p-SINE1* or in regions nearby. In the study of the distribution of *p-SINE1* members, three kinds of transposable elements were found to be present within *p-SINE1* or in

regions nearby. One is a DNA type transposable element, called *Tnr8*, which was inserted in a *p-SINE1* member r32 (see Fig. 1 and Table 1; for details, see Cheng et al. 2000). This element was found to be specifically present in r32 in the strains of *O. barthii* and *O. glaberrima* (Table 2). The second element is a solo-LTR of a retrotransposon, here called *RIRE11*, which was inserted in another *p-SINE1* member r63 (see Fig. 1 and Table 1). This element was present in all the strains with r63 (Table 2). The third element is a LINE-like retrotransposon, called *RILN20*, which was inserted in the flanking region upstream of another *p-SINE1* member r217 (see Table 1). This element was specifically present in the strains of *O. meridionalis* (Table 2). These elements supplied additional markers for the classification of rice strains.

Phylogenetic analysis. We carried out a phylogenetic analysis of the 72 cultivated and wild rice strains by constructing a data matrix based on the presence or the absence of *p-SINE1* members at their respective loci in each rice strain. A phylogenetic tree constructed by the NJ method showed that all the strains can be divided into five groups (Fig. 2). The strains of *O. longistaminata*, *O.*

Fig. 2. A phylogenetic tree showing relationships among the 72 strains with the AA genome. All rice strains are listed in Table 2. The tree was constructed by the NJ method based on the pattern of the presence or absence of *p-SINE1* members and three other transposable elements shown in Table 2. All strains were clustered into five groups, corresponding to *O. longistaminata*, *O. meridionalis*, *O. rufipogon/O. sativa*, *O. glumaepatula* and *O. barthii/O. glaberrima*. Note that the strains of *O. rufipogon* (or *O. barthii*) are not distinguishable from those of *O. sativa* (or *O. glaberrima*) in the tree. The scale bar equals a distance of 0.1.

meridionalis, or *O. glumaepatula* were in a cluster forming three distinct groups (Fig. 2). The cultivated rice strains of *O. sativa*, as well as the wild rice strains of *O. rufipogon*, formed the fourth group. The cultivated rice strains of *O. glaberrima* and the wild rice strains of *O. barthii* formed the fifth group (Fig. 2). These findings show that the strains of each species with the AA genome can be distinguished almost exclusively by the presence or absence of *p-SINE1* members and that the present method provides a means of studying the relationships

among these rice strains.

The phylogenetic tree shows that the strains of *O. longistaminata* and *O. meridionalis* are distantly related to each other, as well as to those of the other three wild rice species, *O. rufipogon*, *O. barthii* and *O. glumaepatula* (Fig. 2). The strains of the latter three species are closely related to one another, but those of *O. barthii* and *O. glumaepatula* are more closely related. The perennial strains of *O. longistaminata*, *O. glumaepatula* and *O. rufipogon* and the annual strains of *O. meridionalis*, *O.*

Fig. 3. A phylogenetic tree showing relationships among the rice strains with the AA genome and a hypothetical ancestor. The hypothetical ancestor is defined as the one without all insertions of *p-SINE1* and other transposable elements. Five groups corresponding to the wild and cultivated rice species are shown. The scale bar equals a distance of 0.1.

rufipogon and *O. barthii* are not related to one another (see Table 2 and Fig. 2), showing that there is no correlation between the divergence of species and the divergence of life cycle.

Because all the insertion events could be considered as irreversible events, it is reasonable to assume that the ancestral state would have had no insertion. A hypothetical ancestor with all loci empty for insertions was thus included in the data matrix as an outgroup. The strains of *O. longistaminata* are most closely related to the hypothetical ancestor, with the next closest being *O. meridionalis* and *O. rufipogon/O. sativa* (Fig. 3). The strains of *O. barthii/O. glaberrima* and *O. glumaepatula* are most distantly related to the hypothetical ancestor (Fig. 3).

DISCUSSION

Classification and relationships of rice strains of species with the AA genome. In this study, we have shown that all strains from each of five wild rice species with the AA genome (*O. rufipogon*, *O. barthii*, *O. longistaminata*, *O. meridionalis* and *O. glumaepatula*) exhibit a very similar insertion pattern and thus form a monophyly. This suggests that they evolved independently and is consistent with the conclusion based on the analyses of morphological characters and various molecular markers, such as nuclear RFLP, RAPD, and AFLP, showing that the present method is a powerful alternative tool for classification of rice strains with AA genome.

We have also shown that the Asian cultivated rice strains of *O. sativa* exhibit a very similar insertion pattern to the Asian wild rice strains of *O. rufipogon*, whereas the African cultivated rice strains of *O. glaberrima* exhibit a very similar insertion pattern to the African wild rice strains of *O. barthii*. No *p-SINE1* members can conclusively distinguish the strains of *O. sativa* (or *O. glaberrima*) from those of *O. rufipogon* (or *O. barthii*). These findings support the view that *O. sativa* and *O. rufipogon*, as well as *O. glaberrima* and *O. barthii*, are actually the same biological species, leading to the hypothesis that strains of *O. sativa* and *O. glaberrima* had been domesticated from those of *O. rufipogon* and *O. glaberrima*, respectively (Morishima et al., 1992; Khush, 1997).

We have also shown that strains of *O. longistaminata* and *O. meridionalis* are distantly related to each other, as well as to those of *O. rufipogon/O. sativa*, *O. barthii/O. glaberrima* and *O. glumaepatula*. The strains of the latter species are closely related to one another, but those of *O. barthii/O. glaberrima* and *O. glumaepatula* are more closely related. Based on these results, the relationships among species with the AA genome are shown in Fig. 4. Species relationships based on insertion polymorphisms are largely similar to those based on RFLP and

Fig. 4. A phylogenetic tree showing relationships among species with the AA genome and a hypothetical ancestor. See Fig. 3, from which this figure is derived.

other molecular markers, but the detailed phylogeny is different among these analyses. The relationships among *O. rufipogon/sativa*, *O. glumaepatula* and *O. barthii/O. glaberrima* are the same as those based on nuclear RFLP analysis (Wang et al., 1992; Bautista et al. 2001) and DNA sequence analysis of two nuclear genes (*Adh1* and *Adh2*) (Ge et al., 1999), but are different from those based on the analysis of isozymes (Second, 1985) and other molecular markers, such as AFLP (Aggarwal et al., 1999), RAPD (Ishii et al., 1996), RAPD and NucSSLP (Bautista et al. 2001), which showed that the strains of *O. glumaepatula* or *O. barthii* form a monophyly with those of *O. rufipogon*. Our results suggest that *O. longistaminata* is most distantly related to the other species, which is similar to those obtained by an RFLP analysis (Bautista et al. 2001), but is different from those based on another RFLP (Wang et al., 1992), RAPD (Ishii et al., 1996), and sequence analysis of *Adh* genes (Ge et al., 1999), which show that *O. meridionalis* is most distantly related to the other species. In this study, we have found at least one species-specific insertion for each species, such as r502 in *O. rufipogon* and r801 in *O. glumaepatula* (see Table 2). These species-specific members must have inserted into the genome after their respective host species diverged from their progenitor(s). Also, we have found several *p-SINE1* members specifically inserted in the strains of two species (see Table 2). These members must have been inserted into the genome of a progenitor. *p-SINE1* members specific for one or two species supply an unambiguous tag for species identification and relationship analysis.

On the origin of rice species with AA genome. In this study, the ancestral state of *p-SINE1* insertion can reasonably be inferred to be absent of insertions, because the *p-SINE1* insertion is an irreversible event. A Phylogenetic tree including a hypothetical ancestor with all loci empty for *p-SINE1* insertion showed that the strains of *O. longistaminata* are most closely related to the hypothetical ancestor, with the next closest being *O. meridionalis* and *O. rufipogon/O. sativa* (see Fig. 4). The strains of *O.*

barthii/*O. glaberrima* and *O. glumaepatula* are most distantly related to the hypothetical ancestor (see Fig. 4). These results suggest that *O. longistaminata* diverged earliest in evolutionary time, followed by *O. meridionalis* and *O. rufipogon*, while *O. barthii* and *O. glumaepatula* diverged latest. Our results also suggest that the *Oryza* species with the AA genome may have originated in Africa, where both *O. longistaminata* and *O. barthii*, which respectively diverged earliest and latest, are sympatric. Although the *Oryza* genus has been assumed to originate in Asia (Second, 1985; Wang et al., 1992), previous studies which led this assumption had no reliable information concerning for the probable ancestral state of molecular variations, thus it is difficult to confidently determine the root of a phylogenetic tree.

If the AA genome species originated in Africa as suggested above, *O. longistaminata* may have been derived from a progenitor that originated in Africa very early in evolutionary time, whereas *O. meridionalis* from the progenitor migrated to Oceania very early as well. *O. rufipogon* and *O. glumaepatula* may have been derived from a progenitor(s) that migrated to Asia and South America, respectively, much later than *O. meridionalis*.

O. barthii may have been derived from a progenitor that remained in Africa. It has even been suggested, however, that *O. barthii* (annual) evolved from *O. longistaminata* (perennial) based on the paleo-environment evidence that annual strains are generally derived from perennial strains (Chang, 1976). Our results show that strains of *O. barthii* do not form monophyly with those of *O. longistaminata* (see Fig. 4). It has been demonstrated that there is a differentiation of perennial and annual ecotypes in the wild populations even in a single species *O. rufipogon* (Morishima, 1984). These suggest that *O. barthii* is unlikely to have evolved from *O. longistaminata*. Furthermore, although *O. longistaminata* and *O. barthii* are sympatric in Africa, interspecific introgression does not frequently occur between the two species, as has previously been observed (Chu and Oka, 1970). *O. longistaminata* is supposed to be one of the two earliest diverged species, whereas *O. barthii* is one of the two latest diverged species, as described above, indicating that the period of the divergence between the two species may be long enough to enable a relatively strict crossing barrier to be developed between the two species.

Genetic introgression and ancestral polymorphism. In this study, we showed that *p-SINE1* members exhibit different distribution spectrum among the rice strains with the AA genome. Of the *p-SINE1* members which were present in the strains of a particular species, most do not show intraspecific insertion polymorphism but a few, such as r701 and r901, do (see Table 2). This suggests that a few members might not have been fixed in the population of their respective species,

because they were amplified relatively recently, but that others might have been fixed, because they were amplified earlier.

Some *p-SINE1* members were present in the strains of all the species (see Table 2). Of these, a few members, such as r69, showed intraspecific polymorphism in strains of a particular species (see Table 2). The intraspecific polymorphism shown by these members cannot be interpreted as in the case of r701, because they are present in the strains of all species. We assume that insertion polymorphisms in these members might have been derived from a genetic introgression. In the locus with an insertion of member r69, for example, the empty allele in *O. rufipogon* might have been introgressed from a strain of a non-AA genome species with no r69 insertion, probably not long after speciation.

Similarly, in the loci with insertions of r34 and r803, which are present in the strains of *O. rufipogon*, *O. barthii* and *O. glumaepatula* (see Table 2), the allele with an insertion in *O. rufipogon* might have been introgressed from a strain of a species, such as *O. barthii* or *O. glumaepatula*, with an insertion in the respective locus. Alternatively, the allele with no insertion might have been introgressed from the strains of a species, such as *O. longistaminata* or *O. meridionalis*, with no insertion in the respective locus. The member r6 showed intraspecific polymorphism in the strains of both *O. longistaminata* and *O. barthii* (see Table 2). More complicated introgression might have occurred in the r6 locus.

The intraspecific polymorphism shown by members, such as r69, may, however, also be explained by the unfixed ancestral polymorphism as discussed previously (Wu, 1991; Hamada et al., 1998). Namely, before these *p-SINE1* members were fixed in the population of the ancestral species with the AA genome, speciation occurred and the polymorphic SINEs were inherited by and sorted to populations of the respective species. After speciation, these members became fixed, lost, or polymorphic in each derived species as a result of random genetic drift. There is a possibility that such a situation might have occurred, if two or more successive speciation events occur closely in an evolutionary time, and/or if the population size of the ancestral species is too large, as assumed previously (Wu, 1991). The AA genome species are supposed to be rapidly radiated in the *Oryza* genus (Ge et al., 1999), which makes it possible that ancestral polymorphism might have arisen in the diverged species.

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