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Article in Genome · March 2007
DOI: 10.1139/g06-139 · Source: PubMed

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Genetic variation in the chloroplast genome suggests multiple domestication of cultivated Asian rice (Oryza sativa L.)

Shin-ichi Kawakami, Kaworu Ebana, Tomotaro Nishikawa, Yo-ichiro Sato, Duncan A. Vaughan, and Koh-ichi Kadowaki

Abstract: Two hundred and seventy-five accessions of cultivated Asian rice and 44 accessions of AA genome Oryza species were classified into 8 chloroplast (cp) genome types (A–H) based on insertion–deletion events at 3 regions (8K, 57K, and 76K) of the cp genome. The ancestral cp genome type was determined according to the frequency of occurrence in Oryza species and the likely evolution of the variable 57K region of the cp genome. When 2 nucleotide substitutions (AA or TT) were taken into account, these 8 cp types were subdivided into 11 cp types. Most indica cultivars had 1 of 3 cp genome types that were also identified in the wild relatives of rice, O. nivara and O. rufipogon, suggesting that the 3 indica cp types had evolved from distinct gene pools of the O. rufipogon – O. nivara complex. The majority of japonica cultivars had 1 of 3 different cp genome types. One of these 3 was identified in O. rufipogon, suggesting that at least 1 japonica type is derived from O. rufipogon with the same cp genome type. These results provide evidence to support a polyphyletic origin of cultivated Asian rice from at least 4 principal lineages in the O. rufipogon – O. nivara complex.

Key words: AA genome, chloroplast genome, domestication, genetic diversity, Oryza sativa L.

Introduction

Asian rice, Oryza sativa L., is believed to have been domesticated about 10 000 years ago, and the earliest evidence for this is from China (Crawford and Shen 1998; Zhao 1998). O. sativa is believed to have been domesticated from the gene pool of the wild perennial species O. rufipogon Griff. and (or) the annual species O. nivara Sharma et Shastry (Yamanaka et al. 2003). Various hypotheses have been proposed for the domestication of rice, including single, dual, and multiple domestication hypotheses (Oka 1988; Second 1982; Londo et al. 2006). Rice consists of 2 ecotypes, indica and japonica, and japonica is further classified into tropical (or javanica) and temperate types. Molecular analysis of retrotransposon p-SINE1 members in the nuclear genome have shown that indica germplasm is closely related to O. nivara, whereas japonica germplasm is more closely related to O. rufipogon (Cheng et al. 2003; Ohtsubo et al. 2004). This result supports the view that indica and japonica ecotypes of rice evolved from different wild ancestors.

The chloroplast (cp) and mitochondrial genomes are haploid and essentially maternally inherited, whereas the nuclear genome is biparentally inherited. The uniparental inheritance of cytoplasmic genome markers makes them useful for tracing phylogenetic relations. Phylogenetic analyses based on the nuclear genome are generally more com-
plex than those based on the cytoplasmic genome because gene flow occurs between rice and its wild relatives when they are sympatric (Kuroda et al. 2005). Genetic analyses of the rice mitochondrial genome has revealed that genetic diversity is higher in the indica ecotype than the japonica ecotype (Kadowaki et al. 1988). RFLP analysis of the cp genome has revealed that the japonica ecotype contains 1 type of cp genome, whereas 3 types of cp genome are present in the indica ecotype (Ishii et al. 1988). These results also suggest that rice domestication was polyphyletic. As well, phylogenetic studies of the cp genome support a di- or polyphyletic hypothesis for rice domestication (Chen et al. 1993; Nakamura et al. 1998; Bautista et al. 2001; Park et al. 2003; Vitte et al. 2004; Garris et al. 2005).

The entire cp genome sequence of cultivated rice (O. sativa L. subs. japonica ‘Nipponbare’) has been reported (Hiratsuka et al. 1989). Masood et al. (2004) reported the complete cp genome sequence of O. nivara (Sri Lankan accession ‘SL10’). In the cp genome of O. nivara, compared with that of cultivated rice, there are 57 insertion and 61 deletion (indel) events, in addition to 159 base substitution events. Of the indels, the 3 largest were a 69 bp deletion, a 16 bp deletion, and a 21 bp insertion in O. nivara. Variation in the 69 bp deletion in the open reading frame (ORF) 100 region of rice has been studied in Oryza species (Chen et al. 1993; Kanno et al. 1993).

Rice has been selected for high seed yield and tolerances to biotic and abiotic stresses. To determine genetic relations between cultivated rice and its wild relatives, it is necessary to analyze parts of the genome that are not subject to selection. Here we study the 3 indel regions around the position of the 69 bp and 16 bp deletions and 21 bp insertion in the cp genome of Oryza. They are located in intergenic regions or nonfunctional genes (Masood et al. 2004) and are not believed to be subject to selection. These sequence differences between cultivated rice and a probable progenitor of cultivated rice, O. nivara, provide an opportunity to analyze rice domestication using comparative genome analysis.

The objectives of the study were to analyze and compare the cp genome variation at 3 cp loci in a representative set of Asian rice and related AA genome Oryza species. The results are discussed in relation to the insights they provide into the domestication of Asian rice.

Materials and methods

Plant materials

The 2 cultivated rice species (O. sativa L. (275 accessions) and O. glaberrima Steud. (2 accessions)) and 6 wild diploid AA genome Oryza species, O. rufipogon Griff. (23), O. nivara Sharma et Shastry (8), O. barthii A. Chev. (1), O. glumaepatula Steud. (5), O. longistaminata Chev. et Roehr. (3), and O. meridionalis Ng (2), were used. The origin of 44 accessions of wild Oryza and O. glaberrima species are given in Supplementary Table S1. From the >30 000 cultivated Asian rice accessions deposited in the National Institute of Agrobiological Sciences (NIAS) Genebank, Japan, 275 rice accessions were randomly selected using the random number table from passport data of NIAS Genebank (Kojima et al. 2005). These materials are mainly landraces and come from 16 Asian countries (Supplementary Table S2). The 275 cultivated rice accessions were characterized into indica and japonica ecotypes on the basis of an analysis of 179 nuclear genome RFLP loci and the same accessions as used by Kojima et al. (2005).

DNA extraction, PCR amplification, and DNA sequencing

Total DNA was extracted from seedling and mature rice leaves according to the methods described by Murray and Thompson (1980).

In O. nivara (accession No. SL10) a deletion of 69 bp at ORF100, a deletion of 16 bp, and an insertion of 5 bp in an intergenic region between ORF106 and ORF36, and an insertion of 21 bp in an intergenic region between infA and rps8 were found (Masood et al. 2004) (Fig. 1). These 3 regions were named 8K, 57K, and 76K because their positions were at nucleotide positions 8548–8616, 56948–57077, and 76694–76695, respectively, in the cp DNA sequence of ‘Nipponbare’ (Hiratsuka et al. 1989). The regions were amplified with 3 pairs of oligonucleotide primers: P1 and P2 for the 8K region, P3 and P4 for the 57K region, and P8 and P9 for the 76K region (Fig. 1, Table 1).

The polymerase chain reaction (PCR) amplification was carried out in a 10 μL reaction mixture containing 20 ng of template DNA, 0.4 μmol/L of forward and reverse primer, 0.2 mmol/L of each deoxynucleoside triphosphate, and 0.5 units of KOD Dash DNA Polymerase (TOYobo, Osaka, Japan), using a DNA thermal cycler (Perkin Elmer Co. Ltd., Wellesley, Mass.). The program for PCR was 25 cycles (94 °C for 30 s, 55 °C for 2 s, and 74 °C for 30 s). PCR products were separated on 3% Agarose 21 units of KOD Dash DNA Polymerase (TOYobo, Osaka, Japan) gels. Detailed analyses were conducted using 12% polyacrylamide gel electrophoresis. DNA sequencing was carried out for the 57K region using the CEQ DTCS Quick Start Kit (Beckman Coulter, N. Harbor Boulevard, Calif.) following the manufacturer’s instructions. Three oligonucleotide primers, P5, P6, and P7, were used to read specific sequences between ORF106 and ORF36 in the 57K region (Fig. 1, Table 1). Multiple sequences were aligned using Genetyx v. 6.0 (Genetyx Corp., Tokyo, Japan) software.

BLAST searches for the 3 cp regions (8K, 57K, and 76K) in the ‘Nipponbare’ nuclear genome

BLAST searches were carried out with the 3 cp regions to detect homologous regions in nuclear genome of O. sativa ‘Nipponbare’. The sequences of the 3 regions with their flanking regions (100 bp on both sides) were used for BLAST search in RiceBLAST (http://riceblast.dna.afrc.go.jp/) with default parameters, except “filter off”. Sequences with global homology to the 3 regions were regarded as homologous sequences against the 3 regions. Sequence locations on each chromosome were confirmed by BLAST 2

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2Supplementary data for this article are available on the Web site or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R5, Canada. DUD 5136. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/irm/unpub_eshtml
sequences on the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov/BLAST/) to exclude redundant or unlocated sequences.

Results

Deletion in the 8K region of the cp genome

Agarose gel electrophoreses of PCR products revealed that the 8K region of all accessions belonged to 1 of 2 types: a 69 bp deletion and a nondeletion type (Fig. 2A). Of the 275 rice cultivars, 122 accessions had a deletion in the 8K region, and 153 had no deletion (Table 2). Most japonica accessions (118, 98.3%) had nondeletion type, and most indica accessions had deletion type (121, 76.6%). The 69 bp deletion in the 8K region was found in both O. rufipogon and O. nivara, whereas other AA genome Oryza accessions had the nondeletion type (Table 2; Supplementary Table S1).

Polymorphism in the 57K region of cp genome

The 57K region sequences revealed the presence of various types of deletions across the germplasm analyzed. Neither agarose gel (Fig. 2B) nor polyacrylamide gel electrophoresis (data not shown) enabled fragment sizes to be resolved. Therefore, the 57K region was sequenced in all accessions. The 57K region sequences covering sites of the indel events were aligned and compared (Fig. 3). This enabled various types of indel and nucleotide substitution events to be identified in that region (Table 2), revealing 6 basic sequence types (57K-1 to 57K-6). In addition to the 6 types the following variations were found in the 57K region:

(a) A or G at nucleotide position 64 in 57K-4;
(b) A or T at positions 86 and 87 in 57K-4 and 57K-5;
(c) A or T at positions 102, 103, and 129 in 57K-6;
(d) An inserted A at position 104 in the 57K-4 sequence (Fig. 3, Table 2).

In total, 14 different DNA sequences were obtained for the 57K region. Sequence information is available in the DDBJ/EMBL/GenBank database under the accession numbers AB262305–AB262318.

In the 57K-4 sequence at positions 86 and 87, 18 accessions of 5 widely distributed wild Oryza (O. glumaepatula, O. longistaminata, O. meridionalis, O. nivara, O. rufipogon) had base pairs AA, and 10 accessions of 4 widely distributed wild Oryza (O. barthii, O. glaberrima, O. longistaminata, O. rufipogon) had base pairs TT (Table 2). Among cultivated O. sativa accessions with 57K-4, all 15 japonica accessions had TT at positions 86 and 87, whereas most indica (16 out of the 17 accessions) had AA (Table 2).

Most indica accessions (107 out of the 156 accessions, 68.6%) had 57K-5 with TT at positions 86 and 87. Most japonica accessions had 57K-6 with a similar number of accessions having AA (37 accessions) and TT (54) at positions 102 and 103.

Insertion in the 76K region of the cp genome

Two accessions of O. nivara had an insertion of 21 bp in the 76K region (Fig. 2C; Table 2). No other variation was found in this region.

Eleven types of cp genome in the AA genome of Oryza

Eight types (A–H) of length polymorphism were identified in Oryza AA genome species in the 3 regions of cp genome studied (Table 2). If both the indels in these 3 regions and the nucleotide substitutions (AA or TT) of the 57K region are taken into account (Fig. 3), there are 11 cp major haplotypes (designated as A, B, C, D-AA, D-TT, E-TT, F-AA, F-TT, G-AA, G-TT, and H-TT) (Table 2).

The 7 accessions of O. nivara analyzed had 5 haplotypes (D-AA, E-TT, G-AA, G-TT, and H-TT) (Table 2). For O. rufipogon 23 accessions were analyzed, and this species also had 5 haplotypes, 4 of them in common with O. nivara. Among Oryza species, Type E-TT was found only in O. nivara and O. rufipogon, and H-TT was found only in O. nivara.

Details of the origin of O. sativa accessions with each cp genome type are provided (Supplementary Table S2). Most accessions had cp genome types D-AA or TT, F-AA or TT, and G-AA or TT. Type D-AA was found in indica accessions, whereas Type D-TT was found mainly in tropical japonicas. O. rufipogon had both Type D-AA and Type D-TT.

Type F-AA was found only in O. sativa accessions, in indica (2) and in temperate (28) and tropical (9) japonica. Most temperate japonica accessions with Type F-AA came from Japan (18 out of the 28 accessions, 64.3%). Type F-TT consisted of both indica (15) and japonica (54) accessions. Accessions with Type F-TT were mainly from China.
Table 2. Chloroplast genome types of *Oryza sativa* and other AA genome *Oryza* species based on the polymorphism of 3 regions (8K, 57K, and 76K).

<table>
<thead>
<tr>
<th>Cp type</th>
<th>Length polymorphic region</th>
<th>O. sativa</th>
<th>Other AA genome</th>
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<tbody>
<tr>
<td></td>
<td>8K</td>
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<tr>
<td>A</td>
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</table>

*Cp type, chloroplast genome type.*

*L and S in the 8K and the 76K represent larger and smaller fragments, respectively (See Fig. 2). Numbers (1–6) in the 57K column correspond to the sequences in Fig. 3.*

*Substitution at positions 86 and 87 of 57K-4 or 5, or positions 102 and 103 of 57K-6.*

*tem, temperate japonica; trop, tropical japonica; tem/trop, japonica with both characteristics of temperate and tropical japonica.*

*Type B: 1 accession of *O. glumeatula*; Type D-AA: 4 *O. glumeatula* accessions, 2 *O. longistaminata*, and 2 of *O. meridionalis*; Type D-TT: 1 *O. barthii* accession, 2 *O. glaberrima*, and 1 *O. longistaminata.*

Fig. 2. Examples of cp DNA polymorphism separated by agarose gel electrophoresis. (A, B, and C) Pictures show results from PCR analyses of regions 8K (primer pair: P1 and P2), 57K (primer pair: P5 and P6), and 76K (primer pair: P8 and P9), respectively. The molecular size of DNA is shown at the left with arrowheads. Molecular sizes of PCR products were confirmed by DNA sequence analyses. 1: Tima (JP-81972), 2: Dangrey (JP-84313), 3: Calotoc (JP-12486), 4: Kemasin (JP-37979), 5: *O. rufipogon* (JP-104938), 6: Nipponbare, 7: Bleiyo (JP-81899), and 8: *O. nivara* SL10. M: molecular size marker. L and S represent larger and smaller fragments.

Homologous sequences of the 3 cp regions (8K, 57K, and 76K) in the ‘Nipponbare’ nuclear genome

Using RiceBLAST (http://riceblast.dna.affrc.go.jp), homologous sequences of the 3 cp genomic regions analyzed were found in the nuclear genome of *O. sativa* ‘Nipponbare’. Seven regions homologous to the 8K region were found in chromosomes (chr) 1, 2, 4, 5, and 10 of ‘Nipponbare’. Sequences of the deletion type were found in chr 4 (2 regions) and 10 (1), although the 8K region of the ‘Nipponbare’ cp genome was of the nondeletion type. Sequences of the nondeletion type were found in chr 1, 2, 5, and 10. There were 6 regions homologous to the 57K region on chr 1, 5, 6, 7, 10, and 12. The 57K-4 sequences were found in chr 1 (AA at positions homologous to positions 86 and 87) and chr 6 (TT); the 57K-5 sequences in chr 1 (AA), chr 5 (AA), and chr 12 (TT); and the 57K-6 sequence in chr 7 (AA at positions homologous to positions 102 and 103). Twelve regions homologous to the 76K region were found in chr 1, 3–10, and 12 (2 regions in chr 4 and 5); all sequences were of the noninsertion type. Although the ‘Nipponbare’ cp genome is Type F-AA (57K-6), the nuclear genome of ‘Nipponbare’ includes the deletion type of the 8K sequence and the 57K-5 sequence, both of which are typical of the cp of indica varieties.

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Discussion

Evaluation of DNA length polymorphism at the 8K and 76K regions in the cp genome of Oryza AA genome species

Even though the classification of rice ecotypes analyzed here was based on RFLP analysis of the nuclear genome, the characteristics of the cp genome type are in close agreement with this nuclear genome classification. Chen et al. (1993) proposed that indica cultivars were domesticated from the annual O. nivara with a deletion in ORF100 (the 8K region in this study), whereas japonica cultivars were domesticated from the perennial O. rufipogon with a nondedletion in ORF100. Our results support this relation between ecotypes and ORF100 polymorphism. In most cases the cytoplasmic genome of indica and japonica are genetically distinct from each other. Of the germplasm analyzed, a deletion event at the 8K region was found only in the Asian wild species O. nivara and O. rufipogon, and Asian rice. This suggests that the deletion event at the 8K region happened after Asian AA genome Oryza diverged from AA genome Oryza of other geographical regions.

Polymorphism at the 76K region was restricted to just 2 Sri Lankan accessions of O. nivara (Fig. 2, Table 2), suggesting that this insertion mutation happened during the evolution of O. nivara and was a local event.

Molecular evolution of the indel and the nucleotide substitution events at the 57K region

57K-4

In the 57K-4 sequence, the substitution event at positions 86 and 87 probably occurred in an ancestor of O. rufipogon, because both the AA and TT substitution were found in the 57K-4 sequence of different accessions of O. longistaminata from Africa.

57K-6 and 57K5

In the 57K-6 sequence, the 16 bp insertion seems to have been generated by sequence duplication of 5’-CTTTTTTTTAGAATAC-3’ (positions 84–99), 5’-TTTTTTTTTTAGAATAC-3’ (positions 85–100), or 5’-TTTTTTTTTAGAATAC-3’ (positions 86–101), because the identical sequence is present upstream (5’).

A similar situation was detected in the 57K-5 sequence, in which 5 nucleotides (5’-CTATA-3’, positions 109–113) are also located at position 114–118 (Fig. 3). These sequence duplications most likely occurred during DNA replication. No Asian wild Oryza accession with the 57K-6 sequence was identified. Therefore, either the 16 bp insertion event found in the 57K-6 sequence occurred after domestication of rice or it occurred in a yet unidentified wild Oryza population. Ohtsubo et al. (2004) reported that japonica varieties
and 6 perennial strains of *O. rufipogon* are related. Five out of the 6 perennial *O. rufipogon* accessions used in their study were from China. Since our *O. rufipogon* accessions did not include any from China it will be useful to check whether 56K-6 sequences occur in Chinese wild *Oryza* germplasm.

The occurrence of diversity at the 57K region is mediated by inverted repeats

It is possible that 57K-1, 57K-2, and 57K-3 diverged relatively recently after, or during, the domestication of *O. sativa*. The process by which these 57K types were generated is inferred as follows. The F Type cp genome of ‘Nipponbare’ with the 57K-6(AA) sequence possesses an inverted repeat of 32 bp at the 57K region (Hiratsuka et al. 1989). This inverted repeat showed a 100% complementarity between 2 regions (2 solid arrows in Fig. 3) if position 129 is considered A. It is possible that 57K-2 is derived from the 57K-6 if the palindrome plus 3 nucleotides, CT (5’ side) and T (3’ side), adjacent to this structure are cut off. Such events can occur with transposons (Fedoroff 2000) or be due to skips during DNA replication. An alternative possibility is that 57K-2 is derived from 57K-4, because 57K-4 also has an incomplete inverted repeat (positions 70–87 and 89–106). In the 57K-6 sequence, a shorter inverted repeat (2 broken arrows in Fig. 3) was also found. These sequences show a 100% complementarity if positions 102 and 103 are T, and position 129 is A. Therefore, it is likely that the shorter inverted repeat is involved in the generation process of 57K-3 and possibly 57K-2.

It is difficult to infer the process of 57K-1 generation. The most probable process is that 57K-1 was derived from 57K-4 or 57K-6. There is a T-rich region (positions 10–32) and an A-rich region (positions 96–119 of 57K-4; positions 112–135 of 57K-6), which may have formed a palindrome-like structure with the secondary loop structure being deleted during replication. From this hypothesis, 57K-1 may be derived from 57K-4 or 57K-6.

It is known why there are so many sequence variations in the 57K region in cultivated rice as compared with other *Oryza* species analyzed. The 57K region appears to be a hot spot for sequence polymorphism in the rice cp genome; consequently, it will be a useful region for further evolutionary studies of the rice gene pool.

Insertion of cp sequences into the nuclear genome

Following the scenario presented for the evolution of the 57K region, Type F (57K-6) and Type G (57K-5) seem to be independently derived from Type D (57K-4), which is regarded as the ancestral type because it is common in AA genome *Oryza* species (Fig. 4). Therefore, ‘Nipponbare’ with F Type (57K-6) sequence in the cp genome never possessed the 57K-5 sequence in the cp genome. The 57K-5 sequences found in the nuclear genome of ‘Nipponbare’ are not copied directly from the cp genome. Where did the nuclear genome ‘Nipponbare’ 57K-5 sequences come from? DNA sequence migration among nuclear, plastid, and mitochondrial genomes occurs (Notsu et al. 2002). Plastid DNAs inserted into the nuclear genome are rapidly fragmented and vigorously shuffled, and about 80% are eliminated from the nuclear genome over a period of a million years (Matsuo et al. 2005). Ancestors of *indica* and *japonica* rice are believed to have diverged between 0.2 and 0.44 million years ago (Ma and Bennetzen 2004; Vitte et al. 2004). One hypothesis is that the 57K-5-like sequences were inserted into the nuclear genome of an ancestor of ‘Nipponbare’ by mating. Therefore, the presence of 57K-5 sequences in the nuclear genome...
does not contradict our interpretation of the process of domestication. Rather, it shows the effectiveness of cp analysis for understanding the evolution and domestication of rice.

The domestication process of cultivated Asian rice

This study has revealed cp genome diversity among AA genome *Oryza* species. On the basis of the cp genome diversity revealed here we describe a provisional scenario for the domestication process of cultivated Asian rice (Fig. 4).

*indica*

Most *indica* cultivars have cp Type D-AA, Type G-AA, and Type G-TT. Two independent length mutations at 8K and 57K and 2 substitutions are required to alter Type D-AA to Type G-TT. Therefore, the gene pool of the *O. rufipogon* – *O. nivara* complex that has Type G-TT is distantly related to the wild gene pool with Type D-AA. This suggests that *indica* rice evolved directly from lineages having Type D or Type G of the *O. rufipogon* – *O. nivara* complex with the same cp type.

The origin of *indica* with Type F is unknown, because no wild rice accessions with Type F were identified in this study. One hypothesis is that gene flow from *indica* to *japonica* has occurred. This hypothesis is supported by the fact that the *japonica* cultivar ‘Nipponbare’ with Type F has the 57K-5 sequence in the nuclear genome.

*japonica*

Fourteen accessions of tropical *japonica* had Type D-TT (Table 2). They seem to have evolved from *O. rufipogon* with the same cp type. On the other hand, 47 accessions (Type F-AA, 9; Type F-TT, 38) of tropical *japonica* had Type F (Table 2). It is possible that tropical *japonicas* with Type D-TT are derived from those with Type D-TT. If this hypothesis is true, because all 15 *japonica* accessions with Type D-TT are tropical *japonica*, temperate *japonicas* with Type F-TT are derived from tropical *japonica* with the same cp haplotype. The origin of temperate *japonica* varieties from tropical *japonicas* has also been proposed from other studies (Garris et al. 2005; Londo et al. 2006). Other explanations, such as independent origins of temperate and tropical *japonicas* from as yet unidentified *O. rufipogon* with Type F cp, may be less likely because of the relative lack of diversity in *japonica* varieties compared with *indica* varieties (Ishii et al. 1988; Kadowaki et al. 1988; Nakayama 2005).

Cheng et al. (2002) reported that the 2 ecotypes of *O. sativa*, *indica* and *japonica*, are distinguishable, almost exclusively, by the presence or absence of p-SINE1 members, which are nuclear genome sequences. Their results suggested that *indica* varieties were domesticated from *O. nivara* and *japonica* varieties from *O. rufipogon*. The majority of *japonica* accessions have the Type D-TT, Type F-AA, and Type F-TT. Type D-TT was not found in *O. nivara* but was found in *O. rufipogon*. This supports the view that *O. nivara* was not involved in the domestication of *japonica* rice. Our results suggest that tropical *japonica* with Type D-TT was domesticated directly from a local population of *O. rufipogon*.

In conclusion, on the basis of the results presented here there are at least 4 cp genome types (Type D-AA (*indica*), Type D-TT (*japonica*), Type G-AA (*indica*), and Type G-TT (*indica*)) that link *O. rufipogon* and *O. nivara* and rice (Fig. 4). These results suggest that rice emerged from distinct components of the *O. rufipogon*–*O. nivara* complex and supports a polyphyletic origin of Asian rice.

Acknowledgements

We thank the National Institute of Genetics, Japan, and the International Rice Research Institute, Philippines, for supplying rice seeds or plants, Mr. Y. Kojima for providing rice DNAs, Ms. S. Kuroda for experimental help, and Dr. Y. Kuroda for discussion.

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