

Analysis of genetic diversity in the *Oryza officinalis* complex

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The genetic relationships among 34 accessions of wild rice from Asia, Africa, America and Australia were analysed using RFLP technique. After southern blotting, DNA digestion pattern was hybridised with a highly repetitive DNA sequence of a retrotransposon from a gypsy family of mobile elements. A dendrogram was constructed from RFLP data in which the species clustered according to their genome designation (CC, BB, BBCC and CCDD genomes). Some species did not

appear in the same group, for example, *O. eichingeri* from Africa and Sri Lanka clustered separately from each other. The same situation was observed for the accessions from China of *O. officinalis*, which cluster together showing a close relationship with *O. rhizomatis*, and *O. eichingeri* (both of CC genome). Also, the tetraploid BBCC from India of *O. officinalis* appears in the same cluster of *O. eichingeri* and *O. punctata* (both from Africa) suggesting close phylogenetic relationship

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with the African genomes BB, CC and BBCC.

The genus *Oryza* consists of about 22 species having a pan-tropical distribution (Vaughan, 1994). Taxonomists have divided this genus into several species complexes among which the *O. officinalis* species complex has the most species and greatest number of different genomes (Tateoka, 1962; Vaughan, 1989). Nine species and two distinct chromosome races within two of these species have been described in the *O. officinalis* complex (Table 1). Several traits of potential economic importance to rice improvement have been found in this complex, for example, resistance to brown planthopper which has been incorporated from *O. officinalis* into varieties of rice released in Vietnam (Brar and Khush, 1997).

The *O. officinalis* complex is of particular interest because, in contrast to the single AA genome of all species in the *O. sativa* complex to which the rice cultigens belong, the *O. officinalis* complex has 4 of the 10 genomes so far designated for species in the genus *Oryza*. In addition, the *O. officinalis* complex includes both diploid and allotetraploid species. In the genus *Oryza*, all the other species complexes include either diploid species, such as species in the *O. sativa* complex, or allotetraploid species such as species in the *O. ridleyi* complex. Thus the *O. officinalis* complex not only furnishes broad *Oryza* genomic diversity for study but also may give insights into polyploidy in the genus *Oryza*.

The taxonomy of the *O. officinalis* complex is based mainly on morphological and cytological data. It includes 5 diploid species with the CC, BB and EE genomes and 4 allotetraploid species together with two allotetraploid races of diploid species having BBCC or CCDD genome constitution. The phylogeny within the complex is complicated due to the presence of morphologically similar forms of the same taxa completely isolated from each other such as *O. eichingeri* that occurs in Africa and Asia. In addition, three allotetraploid taxa with the same genome designation, BBCC, are found in widely separated locations in Africa, South Asia and Southeast Asia. Further the allotetraploid taxa of Latin America with CCDD genome are sympatric for part of their range and isolation barriers between them have not been determined yet. Application of different techniques based on DNA markers is necessary to better understanding of phylogenetic relationships within the complex.

Various molecular methods have been used to clarify phylogenetic relationships within the *Oryza* genus, mainly within the *O. sativa* complex, but information and data concerning genome molecular organisation in the *O. officinalis* complex, is still limited. Genetics of A- genome rice has been extensively studied (for review, Oka, 1988) (Kazuyuki et al 2000). RFLP's methodology using single

copy probes from *O. sativa* was previously reported to analyse genetic diversity within Asian A- genome rice species by Nakano et al. 1992 and Kazuyuki et al. 2000.

Molecular approaches such as RFLP's (Wang et al. 1992) and DNA fingerprinting using hypervariable minisatellite probes (Aggarwal et al. 1994) provide genetically interpretable variability with extensive genomic coverage and have thus become immensely important in studies on population biology and systematics. Based on classical isozyme, and RFLP studies, all species in the genus *Oryza* except *O. brachyantha* (FF genome) have been grouped into four main species complexes: (1) *Sativa*, (2) *Officinalis*, (3) *Ridleyi*, and (4) *Meyeriana* (Tateoka, 1962; Vaughan 1989; Second, 1991; Wang et al. 1992; Vaughan 1994). The complexes *Meyeriana* and *Ridleyi* have only 5 species with the recently assigned new genomes, GG and HHJJ (Aggarwal et al. 1997; Aggarwal et al. 1999). Species with CCDD genome of the genus *Oryza* L. were analysed by RFLP markers (Jena and Kochert, 1991) and a very powerful technique, the AFLP molecular markers were applied and revealed phylogenetic relationships among *Oryza* species (Aggarwal et al. 1999).

Repetitive DNA markers are very useful for studying genetic diversity. There are several advantages that make these markers an informative tool for study of genetic diversity. First of all, they represent a largest part of the plant genome (Flavell et al. 1977). They change rapidly compared with single copy sequences (Jelinek and Schmid, 1982). Finally, repetitive DNA sequences show low intraspecific variation so they are suitable to study species divergence and inter specific relationships (Dvorak and Dubcovsky, 1996). Recently, many common and species- or genome- specific repetitive DNA sequences have been isolated from a number of *Oryza* species. These repetitive sequences have been used as markers to study phylogeny within the *Oryza* genus (Zhao et al. 1989; Cordesse et al. 1992; Motohashi et al. 1997; Uozu et al. 1997; Iwamoto et al. 1999; Kumekawa et al. 1998). These sequences represent different types of repetitive DNA including tandem repeats, rRNA genes, short interspersed sequences and LTR retrotransposons.

In the *O. officinalis* complex two sequence families have been studied in detail: tandem repeat TrsC that is specific for the CC- genome and retrotransposon RIRE- 1 highly amplified in the genome of *O. australiensis* (Nakajima et al. 1996; Shcherban et al. 2000). The isolation and characterisation of the pOe.49, a clone isolated from *O. eichingeri*, that represents a part of the integrase domain of a gypsy- like retrotransposon and its use in analysis of diversity within the *Oryza officinalis* complex was reported by Shcherban et al. 2000. Also, a *Tourist* element of the 5'-flanking region of the catalase gene *Cat. A*, reveals evolutionary relationships among *Oryza* species with various genome types (Iwamoto et al. 1999).

In this study, a dispersed repetitive element, a Gypsy- like

retrotransposon (Shcherban, 2000) was used as a probe for the RFLP analysis (restriction fragment length polymorphisms). This technique consists in the digestion of genomic DNA with different restriction enzymes, gel electrophoresis and southern blot hybridisation of fragments with a labeled probe (a repetitive element in this case). In this study detection of polymorphic bands was performed by a process called autoradiography. Specific procedures used will be explained in materials and methods section. Polymorphisms resulted from the presence or absence of the restriction sites (4-8 bp sequences) as well as punctual mutations, and other changes like insertions, deletions, inversions, etc which alter the distance between restriction sites.

This report includes some different accessions than previously mentioned report that represent all *O. officinalis* species and genomes. Also, another set of enzymes was tested. The results obtained confirmed previous studies but also gave new insights into phylogeny in the complex and the origin of some taxa.

Materials and Methods

Plant material, DNA isolation and quantification

Thirty four accessions of different species within the *O. officinalis* complex were used to represent the diversity of germplasm in this species complex. The species names, genome composition, chromosomes, accession numbers, and origin of accession are shown at [Table 1](#).

Preparation of total genomic DNA was carried out according to the following procedure: 1,5 gr of young leaf tissue were frozen in liquid nitrogen, powdered, and then quickly homogenised using a cold mortar and pestle in 3 ml of homogenisation buffer (2X CTAB). The homogenate was incubated at 60°C for 45 min. An equal volume of chloroform/ isoamyl acid was added and mixed gently by a rotor for 45 min. Then, the tubes were centrifuged at 5000 rpm for 15 min and the supernatant was transferred to another tube. A 10% of the total volume of 2X CTAB and equal amount of precipitation buffer were then added. After DNA became visible, samples were centrifuged for 15 min at 12000 rpm. The DNA pellet was dried slightly and resuspended in 500 µl of HSTE (high salt TE buffer) containing RNAase (50 µg/ ml). The samples were incubated overnight at 37°C and DNA was then precipitated by adding two volumes of isopropanol. After centrifugation at 15000 rpm for 5 min, pellet was dried at room temperature and finally resuspended in 500 µl of TE. The DNA samples were stored at 4°C.

DNA was checked for quality in 1-% agarose gel and DNA concentration was estimated by spectrophotometer (absorbance at 260 nm) followed by agarose gel electrophoresis for final estimation against a defined amount of lambda DNA as standard.

RFLP technique

DNA digestion by restriction enzymes and electrophoresis

An amount of 2-5 µg of genomic DNA of each accession was digested with a total of 8 restriction endonucleases (Eco RI, Eco 47I, Eco RV, Hind III, the combination of enzymes Dpn/ Xba I and Bgl II/ Bam HI) for between 12-16 hrs. The reactions were performed in a total volume of 100 µl in the presence of the appropriate buffer with 5 units of endonuclease per 1 µg of DNA. After digestion 10 µl of each sample was electrophoresed in 1- % agarose gel at 75 volts during 6-8 hrs.

Probe labeling

The cloned repetitive DNA sequence pOe.49 (Shcherban et al. 2000) was used as a probe. The DNA of insert was amplified and labeled in 50 µl of reaction mixture containing the unlabelled nucleotides dATP, dGTP, dCTP (2.5 mM of each), labeled DIG- dUTP (10X solution contains 0.125 mM DIG- dUTP, 0.375 mM dTTP), the specific direct and reverse primers for pUC/ M13 (5-10 pg. each), the plasmid DNA of clone (10 ng), buffer (standard PCR buffer containing 1.8 mM MgCl₂), Taq. - DNA polymerase (1 unit). Before addition of Taq. - polymerase the mixture was boiled to denature DNA for 5 minutes.

The PCR program includes 40 cycles consisting of a denaturation step (1-min at 93°C), annealing step (1 min at 55°C) and DNA extension step (0.5 min at 72°C). After PCR the aliquot of labeled DNA (5 µl) was checked by electrophoresis. The rest of DNA was precipitated with ethanol and after centrifugation for 15 min at 12000 rpm the pellet was dissolved in 50 µl of TE.

Southern blot hybridisation

DNA fragments were transferred from the agarose gel to a nylon membrane by capillary blotting overnight. After transfer membranes were rinsed with 2x SSC and baked at 80°C for 1 hr to cross link DNA. Hybridisation was carried out overnight in 5x SSC, 1% Blocking Reagent (Boehringer – Mannheim), 0.1% N-lauryl sarcosine and 0.02% SDS at 65°C (high stringency). The amount of DNA probe added was 1/10 part of the final volume (see Probe labeling) that corresponds to about 50-100 ng of DNA. Before adding, probe was boiled in the water bath for 5 min. After hybridisation membranes were consecutively washed in 2x SSC, 0.1% SDS; 0.5x SSC, 0.1- % SDS for 20 minutes each at 65°C.

Detection procedure

After washing membrane was incubated in the blocking solution (1% of Blocking Reagent dissolved in the AP7.5 buffer containing 0.1 M Tris- HCl, 0.1 M NaCl, 2mM

MgCl₂) for 1-2 hrs at room temperature. Then the membrane was transferred into the same solution containing the antibody conjugate to digoxigenin (anti-DIG-AP) which was previously diluted 100 times in the blocking solution up to 75 mU/ml. After that 2 washing steps of 15 min each were performed by shaking of membrane in the AP 7.5 buffer. After equilibrating of membranes in buffer AP 9.5 (0.1 M TrisHCl, 0.1 M NaCl, 50 mM MgCl₂) it was incubated in 1-2 ml of developing solution within a saran bag for 5 min. Developing solution contains chemoluminescent substrate CSPD (Boehringer – Mannheim) diluted 100 times in buffer AP 9.5. The membrane was rinsed briefly in AP 9.5 buffer to discard the excess of substrate and transferred to a new saran bag. Before autoradiography the membrane was put in the oven at 37°C for 15 min to enhance the luminescent signal. Then, the membrane was exposed up to 20 min to an X-ray film at room temperature.

Genetic analysis

RFLP bands were scored as present (1) and absent (0) and only bands showing unambiguous polymorphism were entered into a data matrix that was used to construct a phylogenetic tree. Genetic similarity was calculated with the simple matching coefficients (Sokal and Michener, 1958) using NTSYS software. Then, the similarity matrices were subjected to cluster analysis by the unweighted pair-group method with the arithmetic averages (UPGMA) cluster method and Plus analysis (Innan et al. 1999).

Results

Genomic organisation of pOe.49 among *O. officinalis* complex species

The repetitive DNA sequence pOe.49 used in this study was taken from a genomic library of *O. eichingeri* (Shcherban et al. 2000). This sequence has a similar very intensive signal of hybridisation with genomic DNA from *O. officinalis*, *O. rhizomatis*, *O. eichingeri*, *O. punctata* (BB) and *O. australiensis*, diploid species of the *O. officinalis* complex. The primary structural analysis of this sequence and database searching revealed it is homologous to different retrotransposon elements of gypsy class widely spread within genomes of many plants (Suoniemi et al. 1998).

The organisation of the pOe.49 repetitive sequence in 34 accessions of different taxa of the *O. officinalis* complex was determined by Southern hybridisation using genomic DNA digested by eight restriction endonucleases (Hind III, Eco47 I, EcoRI, EcoRV, Dnp/ Xba I, Bgl II/ Bam HI). The accessions represent all known species and chromosome races of the complex and each species/chromosome race is represented by 2 – 3 accessions. Each enzyme or combination produced polymorphic patterns containing multiple discrete bands with high intensity of hybridisation to pOe. 49. No obvious variation in total intensity of hybridisation was found between species.

Diversity analysis based on RFLP assay

High polymorphism of the blot-hybridisation patterns was observed between different species of the *O. officinalis* complex. The level of polymorphism was lower within species than between species or between different genomes.

Polymorphism between species with different genomes

All genomes groups including diploid CC, BB, EE and tetraploid BBCC and CCDD could be recognised on the basis of the polymorphism revealed by blot hybridisation. The dendrogram (Figure 1) based on the RFLP data clearly separated the diploid genomes CC (*O. officinalis*, *O. eichingeri* and *O. rhizomatis*), BB (*O. punctata*) and EE (*O. australiensis*). The accessions of *O. minuta* (BBCC) and the BBCC tetraploid accession of *O. punctata* showed closest affinity with the diploid *O. punctata* and were clustered with the latter. The cluster of CCDD species (*O. latifolia*, *O. alta* and *O. grandiglumis*) has a separate position in the dendrogram (Figure 1) and the out-group *O. australiensis* (EE genome) clustered apart, as expected.

Interspecific polymorphism

Each species has specific hybridisation patterns and could be distinguished from one another. However, accessions of *O. eichingeri* from Africa (Uganda) and Asia (Sri Lanka) clustered separately from each other.

The accessions with CC genome of *O. officinalis* and *O. rhizomatis*, together with *O. eichingeri* from Sri Lanka were in the same cluster. Some CC genome accessions (three of *O. eichingeri* from Uganda and one of *O. officinalis* from India) clustered together showing a closer relationship with *O. punctata* diploid accessions than other CC genomes of *O. eichingeri*, *O. officinalis* and *O. rhizomatis*. All *O. punctata* accessions (diploids and tetraploids), cluster together followed by the tetraploid *O. minuta* which show a clear relationship between genomes.

The CCDD cluster contains species that are distant from all species of the complex. *O. grandiglumis* and *O. alta* had almost identical hybridisation patterns and are clearly separated from *O. latifolia*.

Intraspecific polymorphism

A low level of intraspecific variation was found. The highest level of intraspecific polymorphism was found in *O. officinalis* and *O. eichingeri*.

The eco- geographical forms of *O. officinalis* from China clustered together clearly separated from the Myanmar, Brunei, Philippines, Papua New Guinea and India accessions. On the other hand, the *O. officinalis* accession from India clustered separately from all other *O. officinalis* accessions mentioned above, closer to *O. eichingeri* from Uganda and *O. punctata*.

O. eichingeri consists of two clearly distinguished forms, which correspond to accessions from Uganda and Sri Lanka. The accessions from Uganda are clearly divergent from the other CC species while the accessions from Sri Lanka clustered together with *O. rhizomatis*, which is indigenous to Sri Lanka.

Discussion

The phylogenetic tree constructed with RFLP data shows the CC genome species *O. officinalis*, *O. eichingeri* and *O. rhizomatis* clustering together. Another cluster is formed for BB and BBCC genomes of *O. punctata*, *O. minuta*, *O. officinalis* from India and *O. eichingeri* from Uganda followed by a cluster of CCDD genome species (*O. latifolia*, *O. alta* and *O. grandiglumis*). The out group species *O. australiensis* (EE genome) clustered apart from other species, as expected.

Variation in the hybridisation patterns was observed among different accessions of *O. officinalis*. The accessions from Brunei, Philippines and Papua New Guinea formed a separated group. The accessions from China and Myanmar are quite distinct and have diverged from the first group. These 3 groups of accessions probably correspond to distinct eco- geographical forms of *O. officinalis* located in China, South and Southeast Asia (Hu and Chang, 1967).

The most striking divergence was observed within *O. eichingeri*. The taxonomic status of this species has been clearly established (Biswal and Sharma, 1987). However, information presented here suggests that it may be necessary to re-evaluate this species and determine whether infra-specific taxa for the African and Sri Lankan forms are warranted. In this study *O. eichingeri* was represented by six accessions. Four from Africa showed hybridisation patterns quite different from other CC genome species. The hybridisation patterns of the two accessions from Sri Lanka and those of *O. rhizomatis*, reported only from Sri Lanka, are similar implying their close relationships. Such a high level of genetic divergence within one species suggests that *O. eichingeri* is likely to be the ancestral species of the CC genome group which is supported by its wide distribution in West and East Africa and Sri Lanka (Vaughan, 1994).

Diploid *O. punctata*, the only BB genome species, is genetically clearly distinguished from its tetraploid chromosome race. However, based on dendrogram (Figure 1) diploid *O. punctata* is more closely related to its tetraploid race than the other two BBCC taxa. Results presented here, in addition to morphological, ecological and physiological differences between the diploid and tetraploid races of *O. punctata* suggest that these two chromosome races should be considered separated species (Sano, 1980; Sasahara et al. 1982).

There are three tetraploid BBCC taxa, *O. punctata* (4x), *O. officinalis* (4x) and *O. minuta*. The BBCC taxa is not genetically as close to the diploid CC genome species as diploid *O. punctata* (BB genome). Diploid and tetraploid *O.*

officinalis are clearly differentiated and this supports the view that this chromosome race deserves specific rank (Krishnawamy and Chandrasekharan, 1957).

The diploid BB genome species *O. punctata* were found to be genetically closer to the tetraploid *O. punctata* than to either other BBCC taxa.

The putative CC genome donor of the tetraploid *O. punctata* from Africa is *O. eichingeri* from Africa. But the CC donor of *O. officinalis* (4x) and *O. minuta* were not clearly identified because of the similar genetic distances between these tetraploid species and each of the CC diploid species (data not published). The comparatively low values for genetic distance among CC species suggests that they might have evolved from relatively recent hybridisation and polyploidisation events between BB and CC genome species.

The species *O. minuta* was found to be clearly differentiated from both tetraploid *O. punctata* and *O. officinalis*. This suggests that *O. minuta* evolved independently from other BBCC species. The morphology, habit and present distribution of *O. minuta* also clearly separate this species from other in the complex. The species *O. minuta* has closer affinity to diploid *O. punctata* than diploid *O. officinalis*.

The species with the CCDD genome from Latin America are *O. latifolia*, *O. alta* and *O. grandiglumis*. This group is distantly related to other species of the *O. officinalis* complex. This is in agreement with previous data (Aggarwal et al. 1999) and implies that this group of species evolved independently over a much longer period than other genomes within the complex did (with the exception of *O. australiensis*).

Genetic differences among these CCDD species have been clearly demonstrated by total genomic DNA hybridisation and RFLP analysis (Aggarwal et al. 1996). However, other research suggested that the three taxa form a single complex species (Chen and Matsunaka, 1991; Jena and Kochert, 1991). RFLP data presented here shows that *O. latifolia* is clearly distinguished from *O. alta* and *O. grandiglumis*. The species *O. alta* and *O. grandiglumis* are closely related to each other and form a separate cluster within the CCDD group. Observations of *O. alta* and *O. grandiglumis* in their natural habitat, including sympatric populations, have also suggested these two species are very closely related (Morishima and Martins, 1994).

In conclusion, the data presented in this work has demonstrated that the retrotransposon-like sequence pOe.49 is useful for studying phylogenetic relationships within the genus *Oryza*. The results obtained by RFLP markers are generally consistent with previous morphological, cytological, and molecular marker analysis. Besides confirmation of previous results new information on the divergence and differentiation of some species and genomes within the complex has been presented.

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References

- AGGARWAL, R.K.; MAJUMDAR, K.C.; LANG, J.W. and SINGH, L. Generic affinities among crocodylians as revealed by DNA fingerprinting with a Bkm- derived probe. *Proceedings National Academy of Sciences of the United States of America*, 1994, vol. 91, no. 22, p. 10601-10605.
- AGGARWAL, R.K.; BRAR, D.S.; HUANG, N. and KHUSH, G.S. Differentiation within CCDD genome species in the genus *Oryza* as revealed by total genomic hybridisation and RFLP analysis. *Rice Genetic Newsletter*, 1996, vol. 13, p. 54-57.
- AGGARWAL, R.K.; BRAR, D.S. and KHUSH, G.S. Two new genomes in the *Oryza* complex identified on the basis of molecular divergence analysis using total genomic DNA hybridisation. *Molecular General Genetics*, 1997, vol. 254, no. 1, p. 1-12.
- AGGARWAL R.K.; BRAR D.S.; NANDI, S.; HUANG, N. and KHUSH, G.S. Phylogenetic relationships among *Oryza* species revealed by AFLP markers. *Theoretical Applied Genetics*, 1999, vol. 98, no. 8, p. 1320-1328.
- BISWAL, J. and SHARMA, S.D. Taxonomy and phylogeny of *Oryza collina*. *Oryza*, 1987, vol. 24, p. 24-29.
- BRAR, D.S. and KHUSH, G.S. Alien introgression in rice. *Plant Molecular Biology*, 1997, vol. 35, no. 1-2, p. 35-47.
- CHEN, Jian Jun and MATSUNAKA, Shooichi. Correlation of propanil hydrolyzing enzyme activity with leaf morphology in wild rices of genome CCDD. *Pesticide Biochemistry and Physiology*, May 1991, vol. 40, no. 1, p. 80-85.
- CORDESSE, F.; GRELLET, F.; REDDY, A.S. and DELSENY, M. Genome specificity of rDNA spacer fragments from *Oryza sativa* L. *Theoretical Applied Genetics*, 1992, vol. 83, no. 6-7, p. 864-870.
- DVORAK, J. and DUBCOVSKY, J. Genome analysis of polyploid species employing variation in repeated nucleotide sequences. In: JAUHAR, P.P. ed. *Methods of genome analysis of plants*. CRS Press, Boca Raton, 1996, p. 133-145. ISBN 0849394376.
- FLAVELL, R.B.; RIMPAU, J. and SMITH, D.B. Repeated sequence DNA relationships in four cereal genomes. *Chromosoma* 1977, vol. 63, p. 205-222.
- HU, C.H. and CHANG, C.C. Cytogenetic studies of *Oryza officinalis* complex I. F1 hybrid sterility in geographical races of *O. officinalis*. *Botanical Bulletin of Chinese Academy* 1967, no. 8, p. 8-19.
- INNAN, H.; TERAUCHI, R.; KAHL, G. and TAJIMA, F. A method for estimating nucleotide diversity from AFLP Data. *Genetics* 1999, vol. 151, p. 1157- 1164.
- IWAMOTO, M.; NAGASHIMA, H.; NAGAMINE, T.; HIGO, H. and HIGO, K. A tourist element in the 5'-flanking region of the catalase gene Cat A reveals evolutionary relationships among *Oryza* species with various genome types. *Molecular and General Genetics*, 1999, vol. 262, no. 3, p. 493- 500.
- JELINEK, W.R. and SCHMID, C.W. Repetitive sequences in eucariotic DNA and their expression. *Annual Review of Biochemistry* 1982, vol. 51, p. 813-844.
- JENA, K.K. and KOCHERT, G. Restriction fragment length polymorphism analysis of CCDD genome species of the genus *Oryza* L. *Plant Molecular Biology*, May 1991, vol. 16, no. 5, p. 831-839.
- KAZUYUKI D., MUTSUKO NAKANO NONOMURA, ATSUSHI YOSHIMURA, NOBUO IWATA and DUNCAN A. VAUGHAN. RFLP relationships of A-genome species in the genus *Oryza*. *Journal Faculty Agronomy, Kyushu University* 2000, vol. 45, no. 1, p. 83-98.
- KRISHNAWAMY, N. and CHANDRASEKHARAN, P. Note on a naturally occurring tetraploid species of *Oryza*. *Science Culture* 1957, vol. 23, p. 308-310.
- KUMEKAWA, N.; OHTSUBO, H.; HORIUCHI, T. and OHTSUBO, E. Identification and characterisation of novel retrotransposons of the gypsy type in rice. *Molecular and General Genetics*, 1998, vol. 260, no. 6, p. 593- 602.
- MORISHIMA, H. and MARTINS, P.S. Investigations of plant genetic resources in the Amazon basin with the emphasis on the genus *Oryza*. *The Monbusho International Scientific Program Japan and Research Support Foundation of the State of Sao Paulo, Brazil*, 1994.
- MOTOHASHI, R.; MOCHIZUKI, K.; OHTSUBO, E. and OHTSUBO, H. Structures and distribution of p- SINE1 members in rice genomes. *Theoretical Applied Genetics*, 1997, vol. 95, no. 3, p. 359- 368.

- NAKANO, M.; YOSHIMURA, A. and IWATA, N. Phylogenetic study of cultivated rice and its wild relatives by RFLP. *Rice Genetic Newsletter*, 1992, no. 9, p. 132-134.
- NAKAJIMA, R.; NOMA, K.; OHTUSBO, H. and OHTUSBO, E. Identification and characterisation of two tandem repeat sequences (TrsB and TrsC) and a retrotransposon (RIRE- 1) as genome- general sequences in rice. *Genes and Genetics Systems*, 1996, vol. 71, p. 373-382.
- OKA, H.I. Origin of cultivated rice. *Japan Scientific Society Press*, Tokyo, Elsevier, Amsterdam 1988. 254 p.
- SANO, Y. Adaptive strategies compared between the diploid and tetraploid forms of *Oryza punctata*. *Botanical Magazine of Tokyo*, 1980, vol. 93, p. 171-180.
- SASAHARA, T.; SENGOKU, T. and SANO, Y. CO₂ and water vapor exchange as related to shade tolerance of *Oryza punctata*. *Photosynthetica*, 1982, vol. 16, no. 3, p. 356-361.
- SECOND, Gérard. Molecular markers in rice systematics and the evaluation of genetic resources. In: BAJAJ, Y.P.S. ed. *Biotechnology in agriculture and forestry*. Springer, Berlin Heidelberg, New York, 1991, vol. 14, p. 468-494.
- SHCHERBAN, A.B.; VAUGHAN, D.A and TOMOOKA, N. Isolation of a new retrotransposon- like DNA sequence and its use in analysis of diversity within the *Oryza officinalis* complex. *Genetica*, 2000, vol. 108, no. 2, p. 145-154.
- SOKAL, R.R. and MICHENER. A statistical method for evaluating systematic relationships. *Science Bulletin, University of Kansas* 1958, no. 38, p.1409- 1438.
- SUONIEMI, A.; TANSKANEN, J. and SCHULMAN, A.H. Gypsy like retrotransposons are widespread in the plant kingdom. *The Plant Journal*, 1998, vol. 13, no. 5, p. 699-705.
- TATEOKA, T. Taxonomic studies of *Oryza* II. Several species complexes. *Botanical Magazine Tokyo*, 1962, no. 76, p.165-173.
- UOZU, Sakuraku; IKEHASHI, Hiroshi; OHMIDO, Nokubo; OHTSUBO, Hisako; OHTSUBO, Eiichi and FUKUI, Kiichi. Repetitive sequences: cause for variation in genome size and chromosome morphology in the genus *Oryza*. *Plant Molecular Biology*, 1997, vol. 35, no. 6, p. 791- 799.
- VAUGHAN, Duncan A. The wild relatives of rice. *A genetic resources handbook* 1994 IRRI, Manila, Philippines. p. 137.
- VAUGHAN, Duncan A. The genus *Oryza* L.: current status of taxonomy. *IRRI Research Paper Series*, 1989, no.138.
- WANG, Z.Y.; SECOND, G. and TANKSLEY, S.D. Polymorphism and phylogenetic relationships among species in the genus *Oryza* as determined by analysis of nuclear RFLP's. *Theoretical Applied Genetics*, March 1992, vol. 83, no. 5, p. 565-581.
- ZHAO, X.; WU, T.; XIE, Y. and WU, R. Genome specific repetitive sequences in the genus *Oryza*. *Theoretical Applied Genetics* 1989, no.78, p. 201- 209.

APPENDIX

Table

Table 1. Species of the *O. officinalis* complex used, including accession numbers, genomic constitution, chromosome number and origin.

Species (synonym) ¹	Genome	Chromosome Number	Accession number ²	Origin of accessions
<i>O. officinalis</i> Wall ex Watt	CC	24	106520	Papua New Guinea
			106521	Papua New Guinea
			7784	Myanmar
			101151	Brunei
			101412	Philippines
			104314	China
			104618	China
			104619	China
100984	India			
<i>O. eichingeri</i> Peter	CC	24	105407	Sri Lanka
			105412	Sri Lanka
			101429	Uganda
			105161	Uganda
			105160	Uganda
105181	Uganda			
<i>O. rhizomatis</i> Vaughan	CC	24	103417	Sri Lanka
<i>O. australiensis</i> Domin	EE	24	105268	Australia
			105275	Australia
<i>O. punctata</i> Kotschy ex Steud.	BB	24	103887	Tanzania
			103906	Tanzania
			104073	Cameroon
			104174	Kenya
<i>O. punctata</i> (= <i>O. schweinfurthiana</i>)	BBCC	48	105154	Nigeria
			101439	Ghana
<i>O. minuta</i> J. S. Presl ex C. B. Presl.	BBCC	48	104677	Philippines
			101100	Philippines
<i>O. officinalis</i> (= <i>O. Malampuzhaensis</i>)	BBCC	48	100957	South West India
<i>O. grandiglumis</i> (Doell) Prod	CCDD	48	105144	Brazil
			105157	Brazil
<i>O. latifolia</i> Desv	CCDD	48	103787	Colombia
			103808	Argentina
			104985	Cuba
<i>O. alta</i> Swallen	CCDD	48	100952	Brazil
			100161	Brazil

¹ Nomenclature follows Vaughan and Morishima (in press).² All five and six digit numbers are the accession numbers of the International Rice Germplasm Bank, Los Baños, the Philippines. Three and four digit numbers are accession numbers of the Crop Evolution Laboratory, National Institute of Agro- biological Resources.

Figure

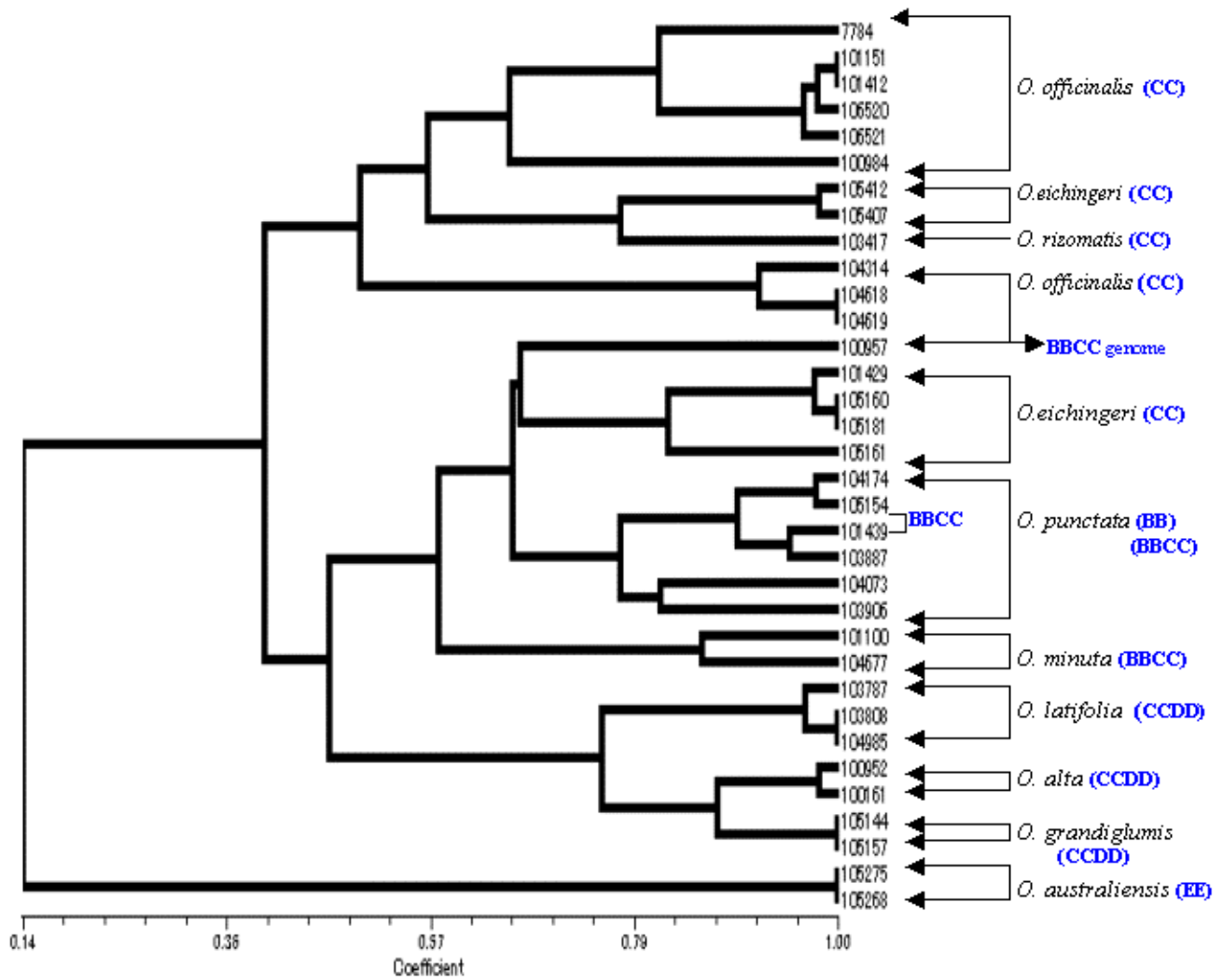


Figure 1. Dendrogram based on RFLP markers.