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Identification of genomic constitutions of *Oryza* species with the B and C genomes by the PCR-RFLP method

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Abstract

Oryza officinalis complex is the largest and the most complicated group in the genus *Oryza* L., consisting of about ten species with the B, C, BC, CD, and E genomes. Taxonomy and identification of the species, particularly those with the B, C and BC genomes, are difficult due to the similar morphology and overlapping distribution of some species. The difference in ploidy levels of some species adds more complexity. In the present study, we surveyed 64 accessions of rice germplasm in the *O. officinalis* complex using RFLP analysis of PCR-amplified *Adh* genes in addition to chromosome counting. The results confirmed that all *O. rhizomatis* accessions are diploids with the C genome, whereas all *O. minuta* accessions are tetraploids having the BC genome. However, both diploid and tetraploid forms were found for the accessions identified in the genebank as *O. officinalis*, *O. punctata* and *O. eichingeri*. The tetraploid form of '*O. officinalis*' with the BC genome is exclusively distributed in India and has been described as *O. malampuzhaensis*. The tetraploid form of *O. punctata* which has been called *O. schweinfurthiana* by some workers was found to be as widely distributed as its diploid form in Africa. It is noteworthy that two accessions that had been determined as tetraploid *O. officinalis* were actually belonging to a species with the CD genome (*O. latifolia*). Our results promote a better understanding of the genomic constitutions of many accessions in the *O. officinalis* complex and correct determination of the genebank material, which serves as an important basis of germplasm cataloguing for further research and utilization.

Introduction

The rice genus (*Oryza* L.) consists of approximately 24 species (Lu 1999) with ten recognized genome types (Khush 1997; Ge et al. 1999, 2001). Among these species, the Asian cultivated rice (*O. sativa* L.) is an economically important crop that serves as the staple food for more than one-half of the world's population. The potential agricultural values of the wild rice species as genetic resources for the improvement of the cultivated rice has been widely appreciated (Brar and Khush 1997; Tanksley and McCouch 1997). For example, many useful genes have been transferred from the wild rice species with various genomes into the cultivated rice, including those for resistance to diseases and insects, and those for tolerance to abiotic stress like unfavourable soil, temperature and water (Brar and Khush 1997). However, efficient utilization of the rice genetic resources and efficient management of the germplasm collections rely on the correct identification of the germplasm (Ge et al. 2001).

Oryza officinalis complex (also referred as *O. latifolia* complex by Tateoka 1962) is the largest

and the most complicated group in the genus, consisting of about ten species with the B, C, BC, CD, and E genomes (Vaughan 1994; Ge et al. 1999). Taxonomy and identification of some species in this complex are not easy due to their similar morphology and overlapping distribution. The difference in ploidy levels of some species, particularly those with the B and C genomes adds further complexity (Nayar 1973; Vaughan 1989, 1994). Based on AFLP analysis, for example, Aggarwal et al. (1999) indicated that two accessions determined as diploid *O. eichingeri* (CC) were actually tetraploids with the BC genome, and one accession as diploid *O. punctata* (BB) was the tetraploid *O. punctata* (BBCC). In recent studies of American wild rice species, Buso et al. (2001) found that 8% of the 230 accessions studied was misidentified as a result of either taxonomic error or contamination. Of these materials, four accessions of *O. punctata* (BBCC) were originally misidentified as *O. eichingeri* (CC) and *O. officinalis* (CC), one accession of *O. eichingeri* (CC) was misidentified as *O. minuta* (BBCC), and one accession of *O. punctata* (BB) was misidentified as *O. rhizomatis* (CC).

Recently, Ge et al. (2001) developed a new PCR-RFLP method, by which all of the ten rice genomes can be identified rapidly and reliably. The objective of this study is to use the PCR-RFLP method to analyse 64 rice accessions belonging to species with the B, C and BC genomes from genebank stored materials. These materials are easy to be misclassified, partly because there is lack of information on their chromosome numbers and genome constitutions in genebank, and partly because some of species (*O. officinalis*, *O. punctata*, *O. eichingeri*) have both dipoid and tetraploid forms. We aimed to identify the genomic constitution of the accessions, to distinguish their diploid and tetraploid forms, and to provide a detailed and corrected catalog of the rice germplasm, which serves as a basis for further research and utilization.

Materials and methods

Materials

Of 64 seed samples used in this study, 60 were kindly provided by the International Rice Genebank at IRRI in the Philippines. These include 24 accessions determined as *O. officinalis*, 16 accessions as *O. punctata*, six accessions as *O. minuta*, five accessions as *O. rhizomatis*, and nine accessions as *O. eichingeri*. The other four samples are either provided by Institute of Genetics, Mishima (Japan) (W067 and W1318) or collected by the authors (C198 and YN2002). The accessions are listed in Table 1 with their respective species names and origins provided by the donors as well as the determined chromosome numbers and genome constitutions in the present study. After 1 week heat shock at 50∼55 °C, seeds were germinated, and the seedlings were maintained in a greenhouse at the Institute of Botany in Beijing, China. When the seedlings were at about 2-months-old, one seedling was randomly chosen from each accession for DNA isolation.

Chromosome determination

Chromosome numbers were determined in meristematic cells of root tips. Fresh roots were collected and fixed in a mixture of acetic acid–absolute alchohol (1 : 3) after pretreated in otta-quinoline for 6 h. The mitotic preparation of the root tips used the acetic orcein squash method described by Lu and von Bothmer (1990). For each accession an average of ten cells with complete chromosomes was scored for the determination of chromosome numbers.

DNA isolation and PCR amplification

Total DNA was isolated from fresh leaves following the procedure described previously by Ge et al. (2001). PCR amplification of *Adh1* and *Adh2* genes was conducted on a Biometra-2000 thermal cycler. Total reaction volume of 25 μL contained 5 pmol each of the primer *AdhF1* and primer *Adh1bR* for amplifying *Adh1*, or 5 pmol each of the primer *AdhF1* and primer *Adh2RR* for *Adh2*; 2.5 μL 20 mmol/L dNTP; 2.5 μL 10× buffer including Tris–HCl 100 mmol/L pH 8.3,10× BSA; 2.5 μL 25 mmol/L $MgCl_2$ and 0.15 μL (5 U/μL) Taq DNA polymerase (Takaya). The primer sequences and thermal cycling procedures are the same to those described by Ge et al. (2001).

Table 1. Accessions used in the present study.

No.	Accession[a]	Original species classification	Origin[b]	Results	
				Chromosome (2n)	Genome constitution
1	C198	*O. officinalis*	China	24	CC
2	80764	*O. officinalis*	India	48	BBCC
3	80765	*O. officinalis*	India	48	BBCC
4	80766	*O. officinalis*	India	48	BBCC
5	80767	*O. officinalis*	India	48	BBCC
6	80768	*O. officinalis*	India	48	BBCC
7	80772	*O. officinalis*	Philippines	24	CC
8	81796	*O. officinalis*	Indonesia	24	CC
9	81972	*O. officinalis*	Thailand	24	CC
10	101152	*O. officinalis*	Brunei	24	CC
11	101412	*O. officinalis*	India	24	CC
12	104708	*O. officinalis*	India	24	CC
13	104972	*O. officinalis*	China	24	CC
14	105080	*O. officinalis*	Vietnam	24	CC
15	105081	*O. officinalis*	Myanmar	24	CC
16	105085	*O. officinalis*	Philippines	–	CC
17	105100	*O. officinalis*	Brunei	24	CC
18	105111	*O. officinalis*	Indonesia	48	CCDD
19	105176	*O. officinalis*	Malaysia	48	CCDD
20	105223	*O. officinalis*	India	48	BBCC
21	105224	*O. officinalis*	India	48	BBCC
22	105328	*O. officinalis*	India	48	BBCC
23	106519	*O. officinalis*	Papua New Guinea	24	CC
24	106520	*O. officinalis*	Papua New Guinea	24	CC
25	106524	*O. officinalis*	Papua New Guinea	24	CC
26	W067	*O. officinalis*	Thailand	–	CC
27	W1318	*O. officinalis*	–	–	CC
28	YN2002	*O. officinalis*	China	–	CC
29	100125	*O. punctata*	–	48	BBCC
30	100937	*O. punctata*	Ghana	48	BBCC
31	101389	*O. punctata*	–	48	BBCC
32	101408	*O. punctata*	Ghana	48	BBCC
33	101439	*O. punctata*	Ghana	48	BBCC
34	103887	*O. punctata*	Tanzania	24	BB
35	103896	*O. punctata*	Tanzania	24	BB
36	104059	*O. punctata*	Nigeria	48	BBCC
37	104067	*O. punctata*	Chad	24	BB
38	104071	*O. punctata*	Cameroon	24	BB
39	104154	*O. punctata*	Cameroon	24	BB
40	105137	*O. punctata*	Zaire	48	BBCC
41	105154	*O. punctata*	Nigeria	48	BBCC
42	105158	*O. punctata*	Kenya	48	BBCC
43	105607	*O. punctata*	Chad	24	BB
44	105984	*O. punctata*	Cameroon	24	BB
45	81803	*O. eichingeri*	Sri Lanka	24	CC
46	100881	*O. eichingeri*	Sri Lanka	–	BBCC
47	101422	*O. eichingeri*	Uganda	–	CC
48	105159	*O. eichingeri*	Uganda	24	CC
49	105160	*O. eichingeri*	Uganda	48	BBCC
50	105181	*O. eichingeri*	Uganda	48	BBCC
51	105182	*O. eichingeri*	Uganda	48	BBCC

Continued on next page

Table 1. Continued.

No.	Accession[a]	Original species classification	Origin[b]	Results	
				Chromosome (2n)	Genome constitution
52	105407	*O. eichingeri*	Sri Lanka	24	CC
53	105413	*O. eichingeri*	Sri Lanka	24	CC
54	103410	*O. rhizomatis*	Sri Lanka	24	CC
55	103417	*O. rhizomatis*	Sri Lanka	24	CC
56	103421	*O. rhizomatis*	Sri Lanka	24	CC
57	105447	*O. rhizomatis*	Sri Lanka	24	CC
58	105448	*O. rhizomatis*	Sri Lanka	24	CC
59	101081	*O. minuta*	–	48	BBCC
60	101082	*O. minuta*	Philippines	48	BBCC
61	101141	*O. minuta*	Philippines	48	BBCC
62	103874	*O. minuta*	–	48	BBCC
63	104674	*O. minuta*	Philippines	48	BBCC
64	105127	*O. minuta*	Philippines	48	BBCC

[a] Accessions W067 and W1318 were originated from Institute of Genetics, Mishima (Japan), and C198 and YN2002 were collected by the authors. The remaining 60 accessions were provided by the International Rice Genebank at IRRI in the Philippines.
[b] Provided by the donors.

Digestion Adh *fragments with restriction enzymes*

Five-μL PCR products were digested in 10 μL reaction containing 1 μL RE buffer, 0.5 μL (10 U/μL) restriction enzyme *Sac*II for *Adh1* or *Eco*NI for *Adh2* at 37 °C for 1 h. Digested PCR products were electrophoresed on 1.5% TBE agarose gels. The gels were stained with ethidium bromide and photo documented under UV light.

Identification of genomic constitutions

Ge et al. (2001) proposed a method to identify rice genomes based on the restriction patterns of PCR-amplified *Adh* genes. Using various combinations of restriction digestion of the two *Adh* genes, all of the ten rice genomes can be identified with high reliability (Ge et al. 2001). In this study, we used two combinations of *Adh* genes and restriction enzymes (*Adh1* + *Sac*II and *Adh2* + *Eco*NI) that could identify specifically the species with the B or C genomes. The combination *Adh1* + *Sac*II can identify the B genome unambiguously because there is one *Sac*II cutting site on *Adh1* gene for the B genome but no cutting site for the other nine genome types, resulting in two bands for the B genome species and only one band for the species with other genome types. Similarly, the combination *Adh2* + *Eco*NI can identify the C genome unambiguously because there is one *Eco*NI cutting site on *Adh2* gene for the C genome but no cutting site for the other nine genome types, resulting in two bands for the C genome species and only one band for species with other genome types (Table 2). When unpredicted restriction profiles were found by using the two combinations, additional combinations of *Adh* genes and restriction enzymes were used. The rationale and utilities of this method were given in Ge et al. (2001).

Table 2. Combinations of two *Adh* genes and restriction enzymes, and their utility in identifying *Oryza* species with the B and C genomes.

Combination	Target genome and its identification	Other genome
Adh1 + *Sac*II	B, two bands (0.66, 1.25)	One band
Adh2 + *Eco*NI	C, two bands (0.66, 1.04)	One band

Numbers in parentheses indicate the reference sizes (kb) of each band.

Results and discussion

Chromosome determination, genomic constitution and detection of misidentification

All the 64 accessions of wild rice germplasm were examined for their chromosome number, and that of 58 accessions was determined. About

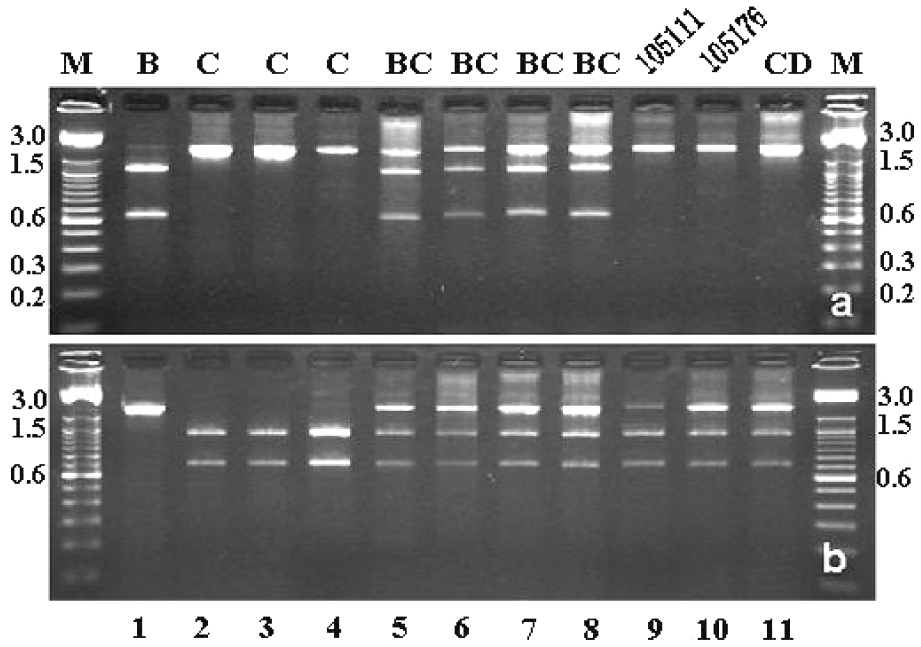

Figure 1. Restriction profiles of the PCR amplification of the *Adh* gene regions followed by digestion with restriction enzyme. (a) *Adh1* + *Sac*II; (b) *Adh2* + *Eco*NI. Lane M is the size marker, and sizes of the fragments (kb) are labeled at the sides. Types of genomes are labeled above the lanes. The species (accession no.) chosen to represent the genomes are as follows: (1) *O. punctata* (104071); (2) *O. officinalis* (198); (3) *O. rhizomatis* (105448); (4) *O. eichingeri* (81803); (5) *O. punctata* (100937); (6) *O. eichingeri* (105160); (7) *O. minuta* (101411); (8) *O. officinalis* (80764); (9 and 10) accessions 105111 and 105176; (11) *O. latifolia* (105141).

ten root–tip observed cells showed consistently $2n = 2x = 24$ for the diploids and $2n = 4x = 48$ for the tetraploids (Table 1).

The 64 accessions were then surveyed by RFLP analysis of PCR-amplified *Adh* genes. As predicted in Table 2, three bands for the BBCC species, two bands for the BB species, and one band for species with other genome were detected, when the *Adh1* gene was digested by *Sac*II (combination *Adh1* + *Sac*II). In contrast, three bands for the BBCC species, two bands for the CC species, and one band for species with other genome were detected, when the *Adh2* gene was digested by *Eco*NI (combination *Adh2* + *Eco*NI). Therefore, every accession can be identified unambiguously based on the restriction profiles of two combinations of *Adh* genes and restriction enzymes. Restriction profiles for the accessions representing species with different genome types are shown in Figure 1a (*Adh1* + *Sac*II) and Figure 1b (*Adh2* + *Eco*NI). It was shown that five accessions originally determined as *O. rhizomatis* were diploids with the C genome, and six accessions determined as *O. Sminuta* were tetraploids with the BC genome (Table 1). For other three species, both diploid and tetraploid were found based on the restriction patterns. Of the nine accessions determined as *O. eichingeri*, five were diploids with the C genome and four were tetraploids with the BC genomes. Of the 16 accessions as *O. punctata*, seven were diploids with the B genome and nine were tetraploids with the BC genomes. Of the 28 accessions determined as *O. officinalis*, 18 were diploids with the C genome, eight were tetraploids with the BC genomes. The genome identification was in agreement with the chromosome counting.

It is noteworthy that the two accessions (105176 and 105111) originally determined in the IRRI genebank as *O. officinalis* showed unexpected restriction profiles. As indicated in Table 2 and Figure 1a, b, the diploid *O. officinalis* showed one band in *Adh1* + *Sac*II and two bands in *Adh2* + *Eco*NI, whereas the tetraploid '*O. officinalis*' showed three bands in both *Adh1* + *Sac*II and *Adh2* + *Eco*NI. However, accessions 105176 and 105111 showed very different restriction profiles

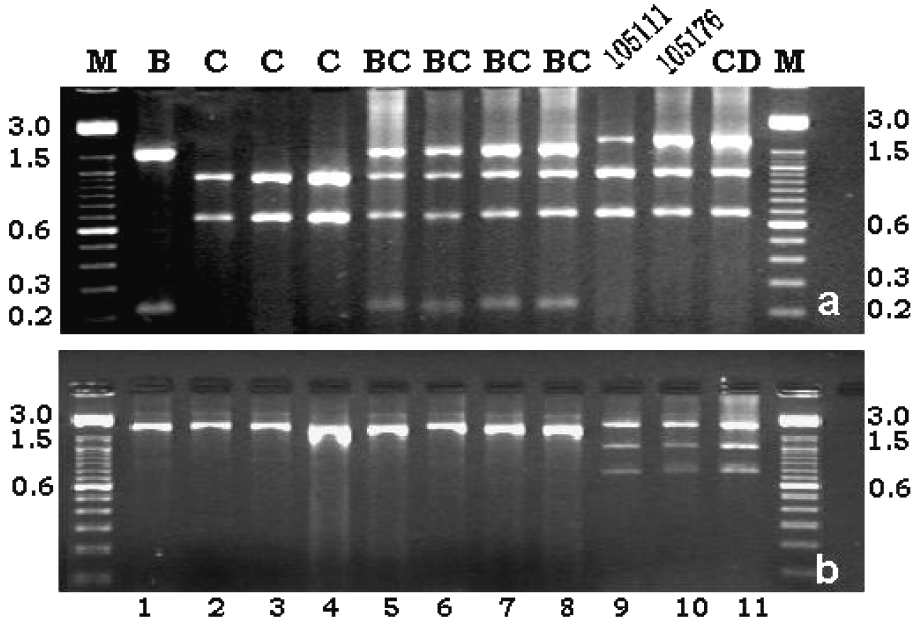

Figure 2. Restriction profiles of the PCR amplification of the *Adh* gene regions followed by digestion with restriction enzyme. (a) *Adh2* + *Eco*RI; (b) *Adh1* + *Afl*II. Lane M is the size marker, and sizes of the fragments (kb) are labeled at the sides. Types of genomes are labeled above the lanes. The species (accession no.) chosen to represent the genomes are as follows: (1) *O. punctata* (104071); (2) *O. officinalis* (198); (3) *O. rhizomatis* (105448); (4) *O. eichingeri* (81803); (5) *O. punctata* (100937); (6) *O. eichingeri* (105160); (7) *O. minuta* (101411); (8) *O. officinalis* (80764); (9 and 10) accessions 105111 and 105176; (11) *O. latifolia* (105141).

from the above patterns, with three bands in *Adh2* + *Eco*NI but one band in *Adh1* + *Sac*II (Figure 1a, b). This result suggests that the two accessions are tetraploids with the C genome, but do not contain the B genomes (see Figure 1a). Interestingly, the restriction profiles of the two accessions are identical to those of the accessions with the CD genomes (see Figure 1a, b). To confirm the identification of the two accessions, we choose additional two combinations (*Adh2* + *Eco*RI and *Adh1* + *Afl*II). These two combinations had the ability to identify accessions with different genome types. As it was expected, the two accessions showed identical restriction profiles as the CCDD species (*O. latifolia*) with three bands in *Adh2* + *Eco*RI (Figure 2a). This identification can be further evidenced by the examination in combination *Adh1* + *Afl*II which identify specifically the D genome (Ge et al. 2001). As shown in Figure 2b, the CCDD species shows three bands, but the BB, CC and BBCC species showed only a single band. Therefore, PCR-RFLPs of *Adh* genes provide strong evidence that the accession 105176 and 105111 were misidentified and should be classified as the CCDD species (Table 1). In order to avoid the potential errors in our sampling and DNA handling, we tried to isolate total DNA from additional plants with the same accession number, and got exactly the same results when they were treated by the above mentioned combinations. Based on our observation on the morphology of these two CD tetraploids, we confirm that they should be treated as *O. latifolia* with the CD genome rather than *O. officinalis* with the BC genome.

As indicated by many authors, the initial designation or field determination of rice germplasm accessions stored in genebanks may not always be reliable for various reasons (Virk et al. 1995; Ge et al. 2001). In their comprehensive study on the phylogenetic relationships of 21 *Oryza* species using nuclear RFLPs, Wang et al. (1992) found that about 13% of the 93 accessions assayed involving species with the A, BC, C, and CD genomes were not correctly determined. Of these studied materials, one Chinese accession (ch83-3) labeled as *O. officinalis* was actually found to be the tetraploid *O. latifolia* with the CD genomes (Wang et al. 1992). Similarly, Aggarwal et al. (1999) indicated

that one Indian accession (105329) labeled as *O. malampuzhanensis* (BBCC) should be a species with the CD genomes. Many other studies also pointed out the misidentifications of *Oryza* species from genebank storage (Martin et al. 1997; Buso et al. 2001). Therefore, misidentification poses a considerable problem for the efficient utilization and management of the wild rice germplasm for breeding and research. Effective identification of the wild rice germplasm stored in genebanks using powerful molecular tools is necessary which will add value to the genebank collections.

Polyploidy variation within species

Three species among the materials that we surveyed in the present study had both diploid and tetraploid forms, which is one of factors leading to misidentification of many accessions. For *O. officinalis*, all the tetraploid accessions were collected from India, whereas the diploids were obtained from a wide distribution in tropical and subtropical Asian countries, including Brunei, China, India, Indonesia, Myanmar, Papua New Guinea, Philippines, Thailand, and Vietnam (Table 1). The tetraploid '*O. officinalis*' was first reported in 1957 from two localities in South India near the town of Malampuzha, and was described as a new species, *O. malampuzhaensis* Krish. et Chand. (Krishnaswamy and Chandrasekharan 1958). The tetraploid form from India was considered as a subspecies or a tetraploid race of *O. officinalis* by Tateoka (1963) or Vaughan (1994), but most authors agreed to retain its original treatment as a separate species, *O. malampuzhaensis* because it has hairy ligules and longer spikelets compared with the diploid *O. officinalis* (Krishnaswamy and Chandrasekharan 1958; Joseph et al. 1999). In addition, the diploid and tetraploid forms of *O. officinalis* differ in patterns of panicle and basal branches, as well as the length of pedicels (Li et al. 2001). Recent molecular and molecular-cytogenetic data also verified its genomic distinction (Aggarwal et al. 1999; Li et al. 2001; Thomas et al. 2001). We strongly support the treatment of *O. malampuzhaensis* as an independent species based on our data.

In Africa, both *O. punctata* and *O. eichingeri* were reported to have diploid and tetraploid forms (Hu 1970), which are easily misidentified with each other (Tateoka 1965b). For example, two tetraploid samples widely used in experimental studies labeled as *O. eichingeri* were identified by Tateoka (1965b) as the tetraploid *O. punctata* (Nayar 1973). It was proposed that diploid and tetraploid forms of *O. punctata* had different habits, with the diploid being annual and the tetraploid being perennial in addition to many different morphological characteristics (Sano 1980; Watanabe et al. 1993). Therefore, some workers (Sharma and Sampath 1985) used the name *O. schweinfurthiana* Prod. referring to the tetraploid *O. punctata* although its ploidy was not known when Prodoehl (1922) published it (Vaughan 1989). Our survey in this study support the previous observation that both diploid and tetraploid forms were widely distributed in Africa (Table 1).

Of the nine accessions determined as *O. eichingeri*, we found through our survey that five accessions (three from Sri Lanka and two from Uganda) were diploids with the C genome and other four (one from Sri Lanka and three from Uganda) were tetraploids (Table 1). It is noteworthy that out of the four tetraploid *O. eichingeri* accessions in this study, three were previously studied and considered to be misidentified (Aggarwal et al. 1999; Buso et al. 2001). Using AFLP markers, Aggarwal et al. (1999) indicated that the two Uganda accessions designed as the tetraploid *O. eichingeri* (Acc. 105181 and 105182) were misclassified because they were clustered closely with tetraploid *O. punctata*. Recently, Buso et al. (2001) also suggested that the tetraploid *O. eichingeri* from Sri Lanka (Acc. 100881) and Uganda (Acc. 105181) should be tetraploid *O. punctata* based on their combined studies of chromosome counting, flow cytometry, as well as total-DNA, cpDNA, and mtDNA analyses. Given the fact that tetraploid *O. punctata* and diploid *O. eichingeri* were easily misidentified with each other (Tateoka 1965a; Vaughan 1994), it is possible that the three accessions from Uganda (105160, 105181, 105182) were misclassified. However, it is difficult to explain the presence of *O. punctata* in Sri Lanka if the accession 100881 was the tetraploid *O. punctata* as revealed by Buso et al. (2001). Although Hu (1970) reported the tetraploid form of *O. eichingeri*, many authors have considered that the tetraploid *O. eichingeri* were either the result of an error or misidentification (Tateoka 1965b; Vaughan 1994). Therefore, further investigations need to be

conducted to clarify these confusions based on additional *O. eichingeri* collections from both Africa and Sri Lanka.

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