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Triploid Wild Rice (BKK) Strain Found in Bangkok Originated from Hybridizations among Three Parental *Oryza* Species

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Abstract

A wild rice (BKK) strain showing sterile spikelet and big leaves inhabited at the basin of the Chao Phraya river of Bangkok city, Thailand. The BKK strain was found as a natural triploid and thus its origin has been interested long time. Three different-sized fragments were amplified in RNA polymerase I largest subunit (*PolA1*) gene, which is a single-copy nuclear gene per haploid genome. Short type (0.14 kb) intron 20 sequence of BKK strain was identical to that of *O. rufipogon* and *O. sativa*. Phylogenetic analysis showed that long type (1.5 kb and 1.8 kb) intron 20 sequences of BKK strain were closely related to that of *O. longistaminata* and *O. officinalis*, respectively. We analyzed protein tag (Ptag) sequence encoded by exons 19 to 21 of *PolA1* gene. Determined three Ptag sequences of BKK strain were identical to that of *O. rufipogon*, *O. longistaminata*, and *O. officinalis*, respectively. Relative DNA content of nuclei in *O. officinalis* and BKK strain was 1.5 and 1.75 times than that in *O. sativa*, respectively. And BKK strain contained CentO-C1 repeats, which were unique to *O. officinalis*. These results indicated that BKK strain comprised three genomes of *O. rufipogon*, *O. longistaminata*, and *O. officinalis*.

Keywords

Oryza rufipogon, Triploid, Flow Cytometry, *PolA1*, CentO-C1 Repeat Sequence

1. Introduction

During several rounds of wild rice surveys, initiated by Drs. Morishima and Oka in 1958, a wild rice (BKK) strain had been found in canals, near the Wat Chalo temple, from the Chao Phraya river in the Bang Kruai district (described as Bangkok Noi), Bangkok, Thailand (Figure 1). The clonally propagated BKK strain with sterile spikelet had unusually big leaves, leaf width: more than 3.5 cm, leaf length: more than 1 m, which are several times bigger than those of *O. rufipogon* (Figure 1). Because W0001 (*O. ridleyi*) and W0002 (*O. officinalis*) strains of wild rice germplasm in the National Institute of Genetics, Japan was obtained in the same place, we are being interested in the origin of BKK strain.

Chu and Oka (1970) reported that hybrids between *O. longistaminata* (formally described as *O. barthii*) and other species showed off-types with big leaves, which they called "Obake" (a monster in Japanese) [1]. In the Mekong Delta of Viet Nam, we found another wild rice strain with big leaves, which was probably a hybrid between *O. rufipogon* and cryptic *O. longistaminata* (Htut *et al.* submitted). The BKK strain, however, had bigger leaves than these wild rice strains mentioned above.

The BKK strain was consistent with *O. rufipogon* and *O. officinalis* in Bangkok in habitat (Figure 1). Although *O. longistaminata* and *O. barthii* are distributed in West Africa, we recently found *O. longistaminata*-like cryptic (MD) strain in the Mekong Delta (Htut *et al.* submitted). Other AA genome wild species, *O. meridionalis* and *O. glumaepatula* are endemic in Australia and Latin America, respectively. Takahashi *et al.* (2009) reported based on sequence analysis

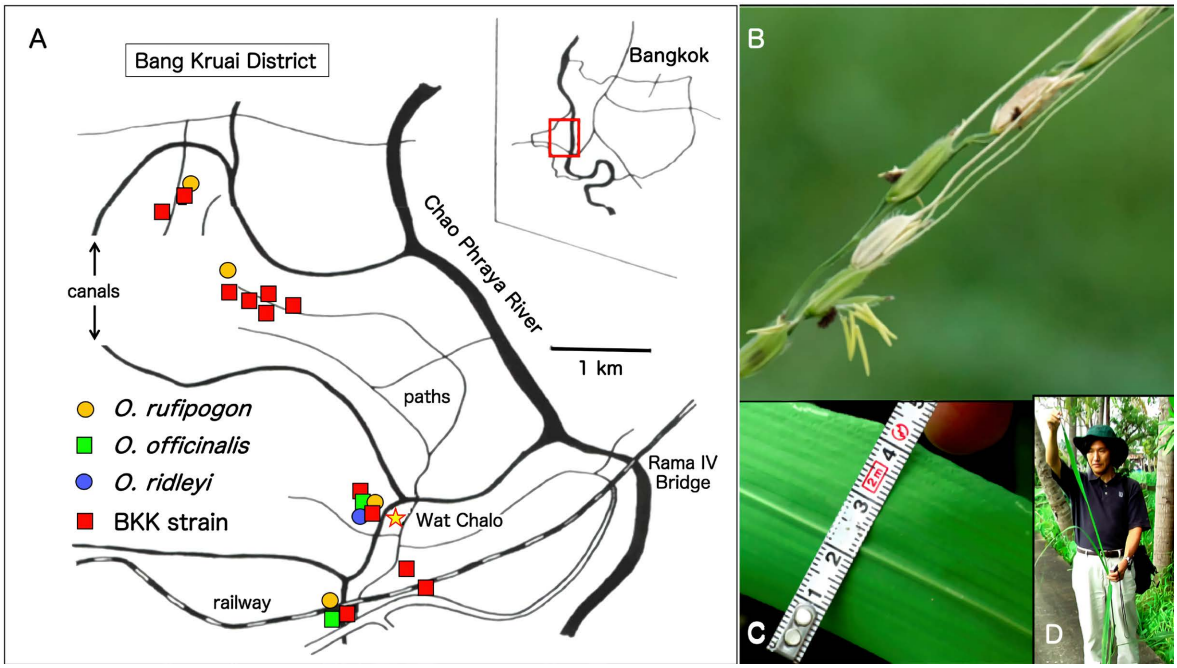

Figure 1. Habitat of BKK strain. (A) localization of three wild rice species (*O. rufipogon*, *O. officinalis* and *O. ridleyi*) and BKK strain near Wat Chalo in Bang Kruai district of Bangkok city in Thailand. Sterile spikelet (B), Leaf width (C), and Leaf length (D) of BKK strain.

of nuclear gene that AA genome species was closely related to BB and CC genome species and distantly related to EE, FF and GG genome species [2]. The same results were obtained by the analysis of retrotransposons [3].

Genome sizes of 14 *Oryza* diploid species containing 6 different genome-types (AA, BB, CC, EE, FF, and GG) were measured using flow cytometry [4]. Except for CC genome, relative DNA content of nuclei corresponding to genome size was conservative characteristics to each genome-type. Thus, the DNA content is helpful to infer genome composition of the BKK strain.

Single-copy nuclear gene, *PolA1*, encodes the largest subunit (194 kDa) of RNA polymerase I which plays an essential role in the synthesis of 45S rRNA precursors [5]. The *PolA1* gene (*ca.* 15 kb) contains 21 exons on chromosome 6 of rice genome [6]. Phylogenetic studies for the intron 19 and exon 20 sequences of *PolA1* gene were useful to analyze relationships in *Petunia* [7], *Aegilops* [8], *Oryza* [2], *Triticum-Aegilops* [9], *Triticum-Hordeum* [10], and *Brassica* [11] [12].

Recently, we found that intron 20 sequence of *PolA1* gene were differentiated into short (S)-type (0.14 kb) and long (L)-type (1.5 kb) in *Oryza* AA-genome species. *O. longistaminata* and *O. meridionalis* had L-type intron 20 while *O. rufipogon*, *O. barthii*, *O. glumaepatule* contained S-type intron 20. Because *O. officinalis* and other *Oryza* species had the L-type, this result provided evidence for the speciation from L-type species to S-type species within AA genome species (Htut *et al.* submitted).

Nakamura (2016) found that a particular protein tag (Ptag) sequence (*ca.* 400 aa) showed a species-specific variation, encoded by exons 19 - 21 of *PolA1* gene in land plants [13]. The Ptag sequence is useful to classify species in *Triticum-Hordeum* [10], *Brassica* [12], and *Trichophyton* fungi [14]. Therefore, Ptag sequences of BKK strain were determined to reveal species, which were involved in the origin of BKK strain.

In this paper, we are interested in the origin of natural triploid BKK strain found in Bangkok city. We found that BKK strain contained three different introns 20 sequences of *PolA1* gene and Ptag-coding sequences, corresponding to those of *O. rufipogon*, *O. longistaminata* and *O. officinalis*, respectively. The results of relative DNA content of leaf cells and Southern blot analyses of CentO-C1 repeats also suggested that *O. officinalis* probably provided its genetic materials to BKK strain.

2. Materials and Methods

2.1. Plant Materials

BKK strain was collected in Bangkok city at 1985 and maintained in Japan (**Figure 1**). Other plant materials were obtained from National Institute of Genetics, Japan (**Table 1**).

2.2. Chromosome Counting

Chromosome number of root-tip cells of BKK strain was counted by enzyme maceration/air drying and Giemsa staining according to Fukui (1996) [15].

Table 1. Materials to analyze the intron 20 sequence of *PolA1* gene in *Oryza* species.

Species	Accession	Description	Intron 20	Genome	A/P
O. sativa	'Nipponbare'	Temperate Japonica	141 bp	AA	
	Ac221	Tropical Japonica	141 bp	AA	
	Ac130	Indica	141 bp	AA	
O. rufipogon	W0106	India	141 bp	AA	A
	W0107	India	141 bp	AA	A
	W1724	India	141 bp	AA	P
	W1956	China	141 bp	AA	P
O. barthii	W0652	Sierra Leone	142 bp	AA	A
	W1416	Sierra Leone	142 bp	AA	A
O. longistaminata	W0643	Gambia	1499 bp	AA	P
	W1232	Tanganyika	1523 bp	AA	P
O. meridionalis	W1297	Australia	1519 bp	AA	A
	W1631	Australia	1523 bp	AA	A
O. glumaepatula	W1169	Cuba	141 bp	AA	P
	W1185	Suriname	141 bp	AA	P
O. punctata	W1514	Kenya	1485 bp	BB	P
O. officinalis	W0002	Thailand	1764 bp	CC	P
O. eichingeri	W1521	Uganda	1767 bp	CC	P
O. rhizomatis	W1808	Sri Lanka	1766 bp	CC	P
O. australiensis	W0008	Australia	1103 bp	EE	P
	W1296	Australia	1100 bp	EE	P
O. brachyantha	W0656	Guinea	2643 bp	FF	P
O. granulata	W0003	India	1634 bp	GG	P
	W0004	India	1634 bp	GG	P
BKK strain		Thailand	141 bp		P
			1519 bp		
			1765 bp		

A/P: annual/perennial.

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2.3. Measurement of Relative DNA Content

Relative nuclear DNA contents of nuclei of leaf cells were determined by flow cytometry using a PA flow cytometer (Partec GmbH, Münster, Germany) on isolated nuclei stained with 4',6-diamidino-2-phenylindole dihydrochloride

(DAPI), according to the method of Mishiba *et al.* (2000) [16].

2.4. DNA Extraction

Total genomic DNA was extracted from 100 mg leaf materials in 2-ml plastic tubes that were frozen with liquid nitrogen and crushed into fine powder using Multi-Bead Shocker (Yasui Kikai Co., Japan). The CTAB method was used for DNA extraction [17].

2.5. PCR Amplification and Direct Sequencing

As shown in **Figure 3(A)**, DNA fragments containing Ptag-coding sequence and intron 20 (S-type and L-type) were amplified by PCR using various pairs of eleven primers (**Table 2**). The primers were designed based on the sequence of rice *PolA1* mRNA (LOC9270399) on japonica rice "Nipponbare". Sequencing primers (5 - 8) was designed based on sequence result of the intron 20 in this study. Two plastid regional sequences, ORF100 (676 bp) and psbZ (792 bp), of BKK strain were determined using primer 11 - 14 shown in **Figure 4(B)** according to Takahashi *et al.* (2009) [18].

The PCR amplification was carried out *ExTaq* DNA polymerase (TaKaRa, Shiga, Japan) according to manufacturer's instruction. The PCR condition were

Table 2. Primers used in this study.

Name	Sequence	Description
1	5'-CTCGCTGGACGGGGTGAGATGAATG-3'	*PolA1*, exon 19 5P
2	5'-CCTTGAGAACTGTTTTTATTGATG-3'	*PolA1*, exon 20 5P1
3	5'-GAGCAACCTCATATTCTGTTAGCC-3'	*PolA1*, exon 20 5P2
4	5'-CTGTGCATACTTCAATTCTCTC-3'	*PolA1*, exon 20 3P1
5	5'-TCTAAACATATACTCCCTCCATCC-3'	*PolA1*, intron 20 5P for AA genome species
6	5'-AACATATACTACACTTATCTTACC-3'	*PolA1*, intron 20 5P for CC genome species
7	5'-ACAACTTCTCCACCAACATTCTCT-3'	*PolA1*, intron 20 3P for AA genome species
8	5'-TAAAGGAATATCATATCAAAACAG-3'	*PolA1*, intron 20 3P for CC genome species
9	5'-CTTACAGGCCTTGACAAAAACAGA-3'	*PolA1*, exon 21 3P2
10	5'-TGAAATCCGCAATCAAGTTCAGATG-3'	*PolA1*, exon 21 3P1
11	5'-GCCGCTTTAGTCCACTCAGCCATC-3'	ORF100 5P
12	5'-TCAATGCCTTTTTTCAATGGTCTC-3'	ORF100 3P
13	5'-TATTTGCTTCTCCTGATGGTTGTT-3'	*psbZ* 5P
14	5'-GAGCGGAGTAGAGCAGTTTGGTAG-3'	*trnM* 3P
15	5'-GTGTAAAAGTTATGTTTCACAAAT-3'	CentO-C1 5P
16	5'-CGGTGTGCCCGCTGGAAAGTTTGT-3'	CentO-C1 3P

40 cycles of 94˚C for 1 min, 58˚C for 1 min for annealing, and 72˚C for 2 min for elongation in a PTC200 thermocycler (MJ Research, Waltham, MA, USA). The amplified PCR products were subjected to 1.5% agarose gel electrophoresis and purified using a PCR purification kit (QIAquick; Qiagen, CA, USA). The purified PCR products of Ptag-coding sequence (1230 bp) were determined by direct sequencing with the same primer as used for PCR amplification in an automated DNA sequencer ABI3100 (Applied Biosystems, CA, USA) with a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, USA).

2.6. Phylogenetic Analysis

The intron 20 sequences of *PolA1* gene were aligned by using CLUSTAW [19]. The alignment was then manually adjusted using Genetyx Software ver. 6.0, Software Development Co., Japan. The phylogenetic tree of the intron 20 sequences were constructed using Neighbor-Joining method with bootstrap estimate from 1000 replicates in the MEGA7 software [20]. The determined intron 20 and Ptag-coding sequences of *PolA1* gene were deposited in DDBJ as accession nos. (LC638415-LC638446).

2.7. Southern Blot Analysis of CentO-C1 Repeats

CentO-C1 repeat sequence (1.2 kb) was amplified by PCR with DNA extracted from *O. officinalis* as a template using primer 15 and 16 (**Table 2**). Primers were designed based on sequence of CentO-C1_11 (Genbank accession DQ058478) [21]. Genomic DNA was extracted from leaves using the cetyl trimethyl ammonium bromide (CTAB) method according to Rogers and Bendich (1985) [22]. Genomic DNAs (15 μg) extracted from leaves of *O. sativa*, *O. officinalis* and BKK strain were digested overnight with *Hin*dIII, *Eco*RI and *Kpn*I at 37˚C.

Restricted DNA fragments were separated on 0.7% agarose gel at 50V for 4 hours and transferred to a nylon membrane overnight by capillary method. Fixing of DNA to membrane was done by exposure under a UV transilluminator for 3 min. CentO-C1 fragment was labelled by PCR reaction incorporating a digoxigenin-labelled nucleotide as in PCR DIG Probe Synthesis Kit (Roche) protocol. Hybridization and stringency washes were carried out following the DIG manual while detection was done by chemiluminescence using CDP-Star, according to manufacturer's instructions.

3. Results

3.1. Habitat of BKK Strain

BKK strain was found in canals near the Wat Chalo temple together with three perennial *Oryza* species, *O. rufipogon*, *O. officinalis* and *O. ridleyi* (**Figure 1(A)**). The BKK strain was clonally propagated by shooting from each node of its column. Because the BKK strain had big stature with completely sterile spikelet (**Figure 1(B)**) and long-wide leaf blades (**Figure 1(C)**, **Figure 1(D)**), it was considered as an interspecific hybrid between *Oryza* species.

3.2. Chromosome Number and Relative DNA Content

Chromosome number of BKK strain was confirmed as 36 using root-tip cell (**Figure 2(A)**). This result clearly indicated that BKK strain was triploid. Relative nuclear DNA content of leaf cells was determined by flow cytometer analysis (**Figure 2(B)**, **Figure 2(C)**). In case of relative DNA content of *O. sativa* was adjusted to 100, *O. officinalis* showed a value of 150 (**Figure 2(B)**). In the same condition, BKK strain showed a value of 175, that was probably corresponded to two haploid A genome (50 + 50) and one haploid C genome (75) (**Figure 2(C)**).

3.3. Analysis of *PolA1* Intron 20 Sequence in BKK Strain

PCR product containing intron 20 of *PolA1* gene was amplified using primer 3 and 10 (**Figure 3(A)**). Short (S)-type intron 20 (0.14 kb) was shared with japonica and indica strains of *O. sativa* and two strains of *O. rufipogon* whereas *O. longistaminata* and *O. officinalis* showed long (L)-type intron 20 (1.5 kb, 1.8 kb) (**Figure 3(B)**). BKK strain contained one S-type intron 20 (rufA) and two L-type intron 20 (lonA, offC) (**Figure 3(B)**). Sequence of the S-type intron 20 was identical to that of *O. rufipogon* and *O. sativa* (Htut *et al.* submitted).

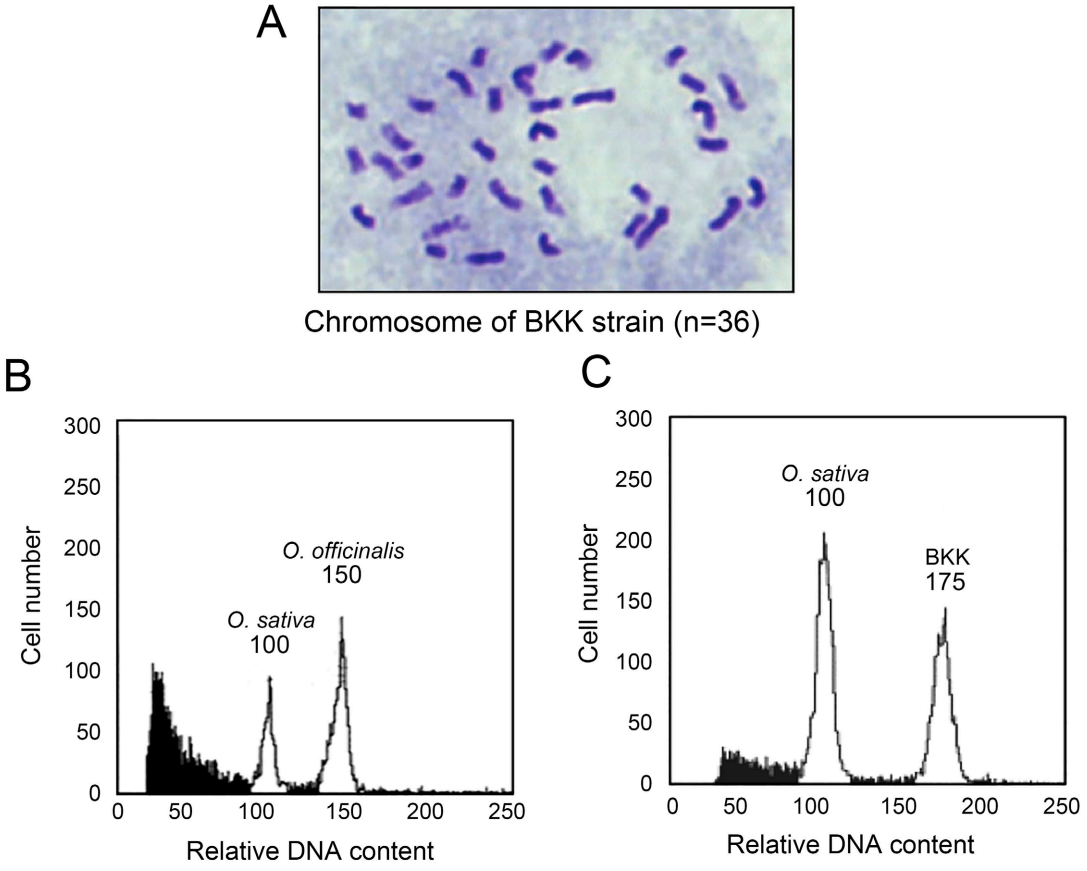


Figure 2. Chromosome number and relative DNA content of BKK strain. (A) Chromosomes (n = 36) of root-tip cell in BKK strain, (B) Relative DNA content of leaf cells in *O. officinalis* was measured using flow cytometry. As internal control, relative DNA content of *O. sativa* was adjusted to 100. (C) Relative DNA content of leaf cells in BKK strain.

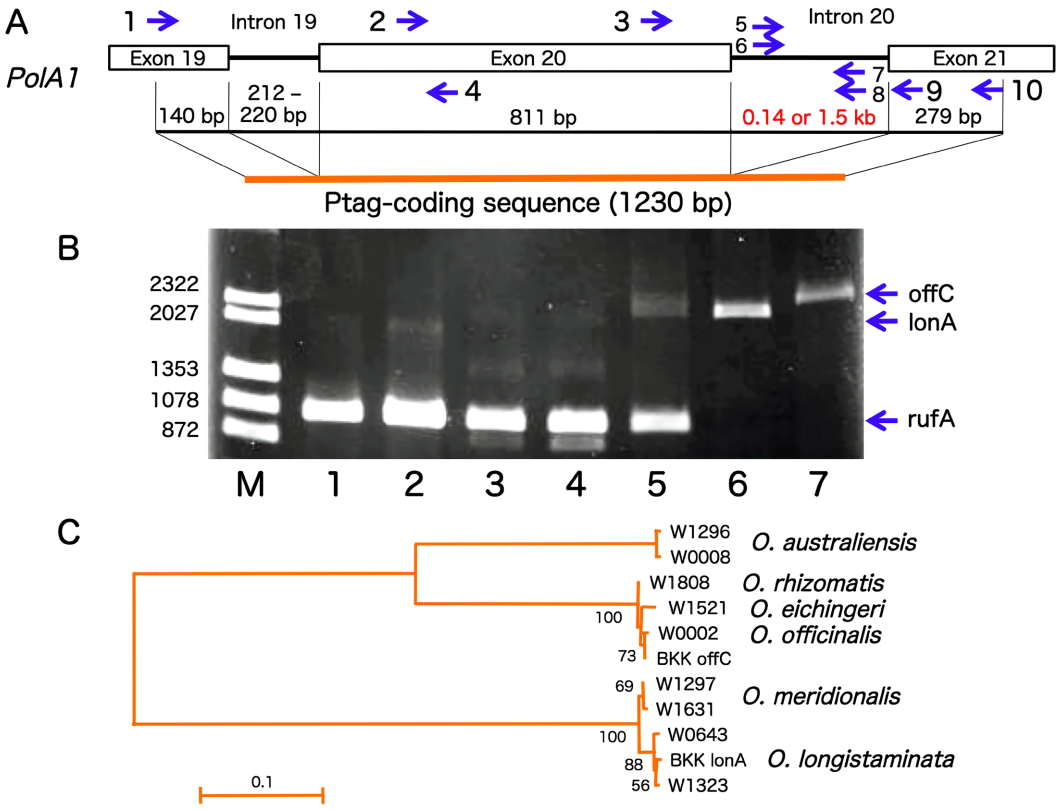


Figure 3. Analysis of intron 20 sequences of *PolA1* gene in BKK strain. (A) Schematic diagram showing the position of primers (1 - 10) used for PCR amplification and sequencing of intron 20 sequence of *PolA1* gene (**Table 2**). Long (L)-type intron 20 sequences (1.5 kb) of AA genome species were determined using a pair of primer 5 and 7, whereas L-type intron 20 sequence (1.8 kb) of CC genome species were determined using a pair of primer 6 and 8. Three Ptag-coding sequences of BKK strain were also analyzed using primers shown in **Figure 3(A)**. (B) PCR amplicons containing the intron 20 sequences in *Oryza* diploid species. M: Size marker λ/*Hin*dIII and φx174/*Hae*III, 1: *O. sativa* subsp. *japonica*, 2: N16 *O. sativa* subsp. *indica*, 3: *O. rufipogon* W0593, 4: *O. rufipogon* W1236, 5: BKK strain showed three bands (offC, lonA, and rufA), 6: *O. longistaminata* IRGC101205, 7: *O. officinalis*. (C) Neighbor-joining phylogenetic tree was constructed by using MEGA7 software based on the L-type intron 20 sequences among two AA genome species, three CC genome species, and BKK strain.

Sequences of two L-type intron 20 of BKK strain were analyzed using sequencing primer 5 and 7 specific to *O. longistaminata* and primer 6 and 8 specific to *O. officinalis* (**Figure 3(A)**). Neighbor-Joining phylogenetic tree of L-type intron 20 sequences was constructed (**Figure 3(C)**). One L-type tntron 20 sequence (lonA) of BKK strain was clustered with those of *O. longistaminata* and *O. meridionalis* (AA genome species) while another L-type intron 20 sequence (offC) was grouped with those of *O. officinalis*, *O. eichingeri* and *O. rhizomatis* (CC genome species).

3.4. Comparison of Ptag-Coding Sequences in BKK Strain

Three Ptag-coding sequences (1230 bp) of BKK strain were determined using primers shown in **Figure 3(A)**. Alignment of three deduced Ptag sequences showed that two amino acid substitutions were found between BKK1 and BKK2

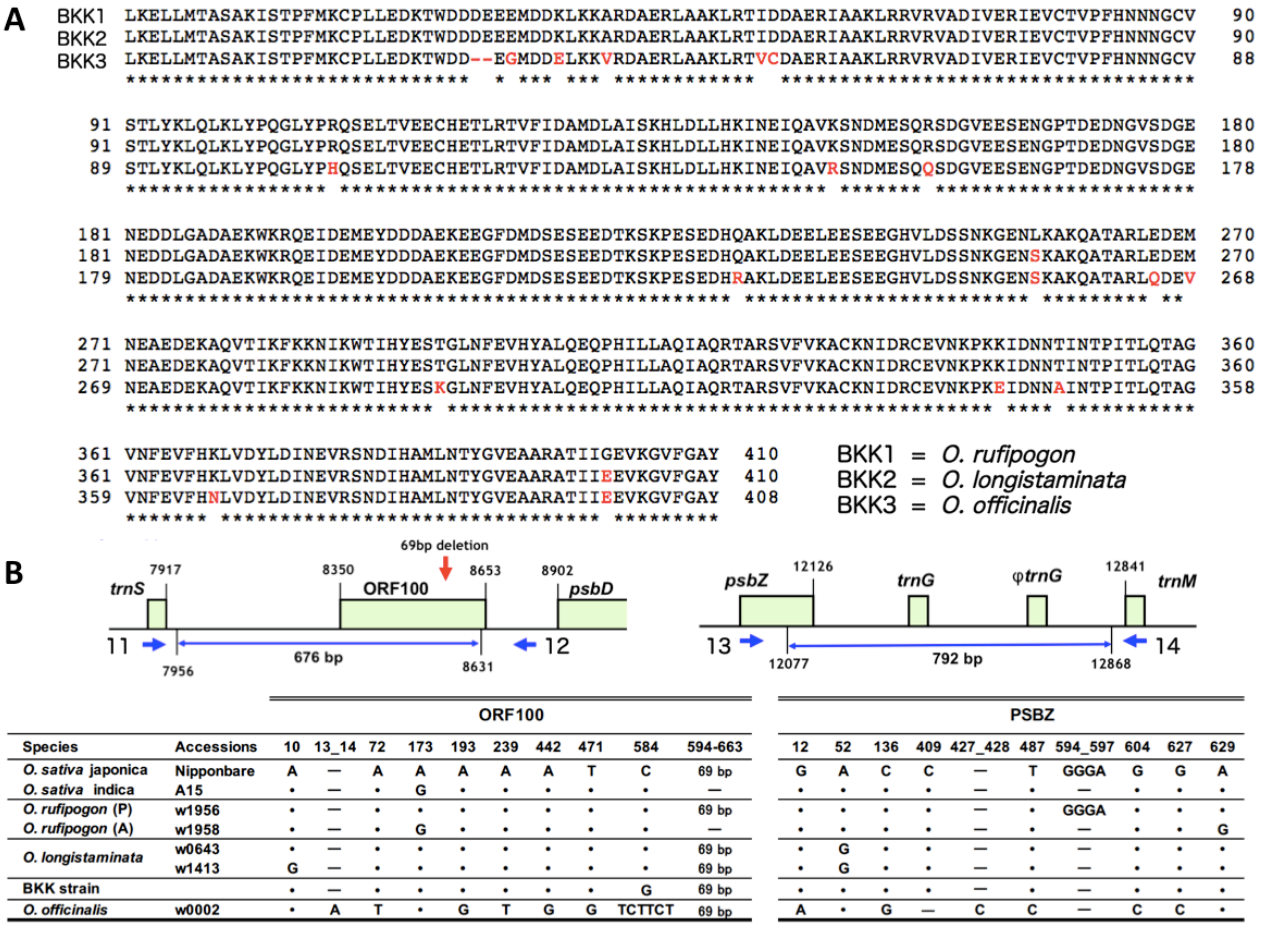
but BKK1 and BKK3 were differed in 16 substitutions (Figure 4(A)). Interestingly, BKK1, BKK2 and BKK3 of BKK strain were identical to that of *O. rufipogon*, *O. longistaminata* and *O. officinalis*, respectively.

3.5. Analysis of Two Plastid Sequences in BKK Strain

Two regional plastid sequences of ORF100 (676 bp) and *psbZ* (792 bp) were compared among BKK strain, *O. longistaminata*, *O. rufipogon* and *O. officinalis* (Figure 4(B)). Sequences of BKK strain were different from those of *O. officinalis* and similar to those of *O. rufipogon* and *O. longistaminata*. Perennial strain of *O. rufipogon* and japonica strain of *O. sativa* contained an unique (GGGA) insertion in *psbZ* sequence.

3.6. Southern Blot Analysis of CentO-C1

CentO-C1 repeat sequences specific to *O. officinalis* were amplified by PCR according to Lee *et al.* (2005) [21]. Genomic DNA isolated from *O. sativa*, *O.*



		ORF100										PSBZ									
Species	Accessions	10	13_14	72	173	193	239	442	471	584	594-663	12	52	136	409	427_428	487	594_597	604	627	629
O. sativa japonica	Nipponbare	A	—	A	A	A	A	A	T	C	69 bp	G	A	C	C	—	T	GGGA	G	G	A
O. sativa indica	A15	•	—	•	G	•	•	•	•	•	—	•	•	•	•	—	•	—	•	•	•
O. rufipogon (P)	w1956	•	—	•	•	•	•	•	•	•	69 bp	•	•	•	•	—	•	GGGA	•	•	•
O. rufipogon (A)	w1958	•	—	•	G	•	•	•	•	•	—	•	•	•	•	—	•	—	•	•	G
O. longistaminata	w0643	•	—	•	•	•	•	•	•	•	69 bp	•	G	•	•	—	•	—	•	•	•
	w1413	G	—	•	•	•	•	•	•	•	69 bp	•	G	•	•	—	•	—	•	•	•
BKK strain		•	—	•	•	•	•	•	•	G	69 bp	•	•	•	•	—	•	—	•	•	•
O. officinalis	w0002	•	A	T	•	G	T	G	G	TCTTCT	69 bp	A	•	G	—	C	C	—	C	C	•

Figure 4. Alignment of three Ptag sequences and plastid DNA sequences in BKK strain. (A) Three Ptag sequences (BKK1, BKK2, and BKK3) of BKK strain were completely identical to that of *O. rufipogon*, *O. longistaminata*, and *O. officinalis*, respectively. (B) Two plastid sequences, ORF100 (676 bp) and *psbZ* (792 bp), were analyzed using primer 11 - 12 and 13 - 14, respectively. Only unique sites among *O. sativa*, *O. rufipogon*, *O. longistaminata*, *O. officinalis* and BKK strain were shown. Dot: same nucleotide, Bar: deletion.

officinalis and BKK strain were digested by three different restriction enzymes and subjected to agarose gel electrophoresis. Southern blot pattern was obtained by hybridization with cloned CentO-C1 fragment as a probe (**Figure 5(A)**). Hybridization signals were clearly detected in *O. officinalis* and BKK strain but there is no signal in *O. sativa.*

4. Discussion

Akimoto *et al.* (1999) reported that presence of unusual big wild rice (named as BKK) strain having completely sterile spikelet and long-wide leaves in canals near the Wat Chalo temple in the Bang Kruai district, Bangkok (**Figure 1**) [23]. Although many plants similar to the BKK strain were grown around there, it considered that they were clonally propagated. Kuroda *et al.* (2003) reported that Chao Phraya basin was one of the most important regions to conserve wild *Oryza* species in mainland Southeast Asia because three *Oryza* species, *O. rufipogon*, *O. officinalis* and *O. ridleyi* habitat together [24].

The BKK strain was confirmed as triploid plant because its chromosome number was 36 while *Oryza* diploid species was $2n = 24$ (**Figure 2(A)**). Relative DNA content of leaf cells of *O. sativa* was compared to that of *O. officinalis* and BKK strain (**Figure 2(B)**, **Figure 2(C)**). When relative DNA content of *O. sativa* was adjusted to 100, *O. officinalis* and BKK strain showed 150 and 175, respectively. Miyabayashi *et al.* (2007) reported that DNA contents of *O. sativa* and other AA genome species were similar and much lower than those of *O. officinalis* and other CC genome species [4]. This result suggested that two AA genome species and one CC genome species were responsible for the origin of BKK strain.

Nakamura (2016) found that protein tag (Ptag) sequence located in the C-terminal region of POLA1 subunit showed species-specific variation in not

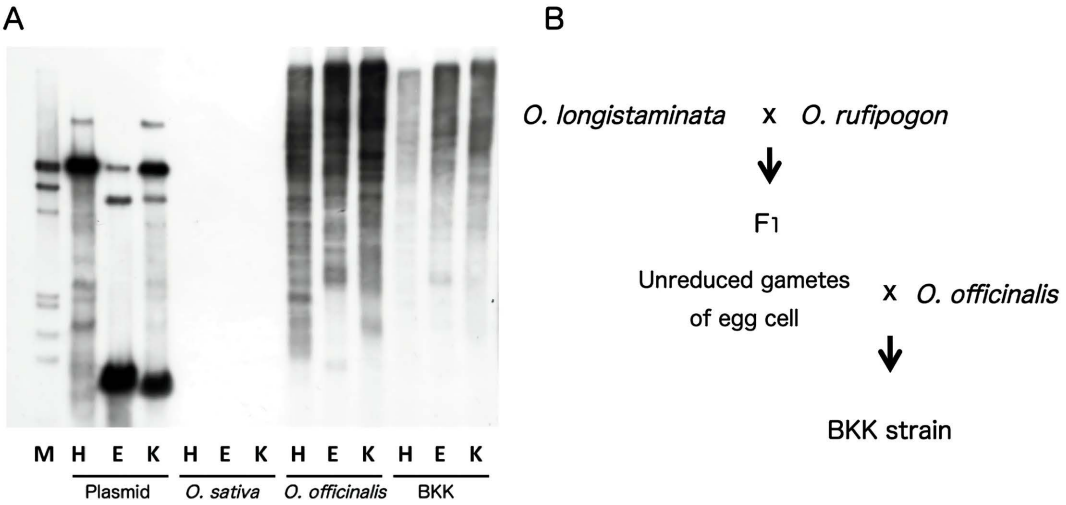


Figure 5. Southern blot hybridization of the CentO-C1 repeat in BKK strain. (A) Genomic DNAs of *O. sativa*, *O. officinalis* and BKK strain were digested by *Hin*dIII (H), *Eco*RI (E), and *Kpn*I (K) and subjected to agarose gel electrophoresis. Blotted nylon membrane was hybridized with labelled CentO-C1 fragment (1.2 kb). (B) Schematic representation for the origin of BKK strain.

only plant but also fungi, animals and protists [13]. The Ptag sequences were highly conservative within a species but differentiated between species [10]. Because intron 19 and 20 sequences of *PolA1* gene were located within Ptag-coding sequence (**Figure 3(A)**), these two intron sequences were also conservative in a species. Therefore, analysis of intron 19 and 20 sequences of *PolA1* gene were useful to reveal ancestral species of polyploid species, such as *Triticum-Aegilops* [9], *Triticum-Hordeum* [10], and *Brassica* [11] [12].

Recent analysis of intron 20 of *PolA1* gene indicated that S-type intron 20 (0.14 kb) was observed in *O. sativa*, *O. rufipogon*, *O. glumaepatula* and *O. barthii* whereas L-type intron 20 (1.5 kb) was found in *O. longistaminata* and *O. meridionalis* (Htut *et al.* submitted). Sequence analysis showed that S-type intron 20 was probably derived from L-type intron 20 due to large deletion by intramolecular homologous recombination between two TTTTGC repeats within L-type intron 20 of *PolA1* gene (Htut *et al.* submitted). Because all *Oryza* species other than AA genome contained the L-type intron 20, this result suggested that *O. rufipogon* was originated from single or few individuals of *O. longistaminata*.

In this study, BKK strain contained three different intron 20 sequences, one S-type intron 20 (rufA 0.14 kb) and two L-type intron 20 (lonA 1.5 kb, offC 1.8 kb) (**Figure 3(B)**). Sequence analysis showed that rufA sequence of BKK strain was identical to S-type intron 20 sequence of *O. rufipogon*. While lonA and offC sequences of BKK strain were grouped with L-type intron 20 sequences of *O. longistaminata* and *O. officinalis*, respectively (**Figure 3(C)**). This result suggested that BKK strain contained genetic materials derived from *O. rufipogon*, *O. longistaminata* and *O. officinalis*. Three Ptag-coding sequences of BKK strain were also determined by direct sequence analysis using various sequencing primers (**Table 2**). Deduced three Ptag sequences (BKK1, BKK2 and BKK3) of BKK strain were identical to that of *O. rufipogon*, *O. longistaminata* and *O. officinalis* (**Figure 4(A)**). This result indicated that these three *Oryza* species were involved in the origin of BKK strain.

Two plastid, ORF100 and *psbZ*, sequences of BKK strain were different from those of *O. officinalis* and similar to those of *O. rufipogon* and *O. longistaminata* (**Figure 4(B)**). Although plastid sequences of BKK strain did not consistent with those of *O. rufipogon* and *O. longistaminata*, cytoplasmic origin of BKK strain might be *O. longistaminata* because *O. rufipogn* perennial strain contained an unique GGGA insertion in the *psbZ* sequence.

Lee *et al.* (2005) reported that CentO-C1 repeats were specific to *O. officinalis* and CC genome species and absent in other *Oryza* species [21]. Southern hybridization showed that CentO-C1 signals were present in *O. officinalis* and BKK strain but absent in *O. sativa* (**Figure 5(A)**). This result indicated that BKK strain contained genetic materials derived from *O. officinalis*.

Although BKK strain inhabited together with *O. rufipogon* and *O. officinalis*, *O. longistaminata* was known to be endemic to Africa. However, we found *O. logistaminata*-like cryptic species in Mekong Delta, Vietnam (Htut *et al.* submit-

ted). And *O. rufipogon* was considered to be originated from *O. longistamina* (Htut *et al.* submitted). Therefore, it is possible that *O. longistaminata* was used to distribute in Southeast Asia.

Taken together of the results in this study, BKK strain probably originated as follow (**Figure 5(B)**). At first, an interspecific hybrid between *O. longistaminata* and *O. rufipogon* was occurred in the past. Then, hybridization between unreduced female gamete produced in the interspecific hybrid and male gamete from *O. officinalis* had produced natural triploid BKK strain. Therefore, it is interested that BKK strain maintained ancient genome of *O. longistaminata*, which was extinct in Southeast Asia.

5. Conclusion

We found wild rice (BKK) strain with big stature and sterile spikelet in Bangkok city of Thailand. The BKK strain is recognized as natural triploid by counting chromosome number and measuring relative DNA content of nuclei. Because Ptag sequence showed species-specific variation in eukaryotic species, Ptag sequence, encoded by exon 19 - 20 of *PolA1* gene, was analyzed. The result indicated that BKK strain contained three Ptag sequences, which was identical to that of *O. longistaminata*, *O. rufipogon* and *O. officinalis*, respectively. Sequence analysis for ORF100 and *psbZ* of plastid DNA showed that *O. longistaminata* was probably maternal parent. Contribution of *O. officinalis* was confirmed by Southern blot analysis using CentO-C1 probe specific to *O. officinalis*. These data indicated that BKK strain was originated by hybridizations among three parental species, *O. longistaminata*, *O. rufipogon* and *O. officinalis*.

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Conflicts of Interest

The authors declare no conflict of interest regarding publication of this manuscript.

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