










# Structural characterization of Brachypodium genome and its syntenic relationship with rice and wheat

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**Abstract** Brachypodium distachyon (Brachypodium) has been recently recognized as an emerging model system for comparative and functional genomics in grass species. In this study, 55,221 repeat masked Brachypodium BAC end sequences (BES) were used for comparative analysis against the 12 rice pseudomolecules. The analysis revealed that \* 26.4% of BES have significant matches with the rice genome and 82.4% of the matches were homologous to known genes. Further analysis of paired-end BES and \* 1.0 Mb sequences from nine selected BACs proved to be useful in revealing conserved regions and regions that have undergone considerable genomic changes. Differential gene amplification, insertions/deletions and inversions appeared to be the common evolutionary events that caused variations of microcolinearity at different orthologous genomic regions. It was found that 17% of genes in the two genomes are not colinear in the orthologous regions. Analysis of BAC sequences also revealed higher gene density (\* 9 kb/gene) and lower repeat DNA content (13.1%) in Brachypodium when compared to the both comparative and functional genomics in grass species. In this study, 55,221 repeat masked Brachypodium BAC end sequences (BES) were used for comparative analysis against the 12 rice pseudomolecules. The analysis revealed that \* 26.4% of BES have significant matches with the rice genome and 82.4% of the matches were homologous to known genes. Further analysis of paired-end BES and \* 1.0 Mb sequences from nine selected BACs proved to be useful in revealing conserved regions and regions that have undergone considerable genomic changes. Differential gene amplification, insertions/deletions and inversions appeared to be the common evolutionary events that caused variations of microcolinearity at different orthologous genomic regions. It was found that 17% of genes in the two genomes are not colinear in the orthologous regions. Analysis of BAC sequences also revealed higher gene density (\* 9 kb/gene) and lower repeat DNA content (13.1%) in Brachypodium when compared to the both comparative and functional genomics in grass species.

**Keywords** Brachypodium distachyon · Comparative genomics · Gene density · Colinearity · Analysis of BAC sequences also revealed higher gene density · Repetitive DNA elements · Genome evolution

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## Introduction

The large grass family (Poaceae), which includes major important cereals such as rice, wheat, and maize, encompasses over 10,000 species (Kellogg 2001). Early comparative genetic mapping based on RFLP markers has revealed considerable synteny between grass species, despite the great variation in genome size and evolutionary divergence times up to 60 million years (Moore et al. 1995; Gale and Devos 1998; Keller and Feuillet 2000). Because of the syntenic relationship among grass species, it is expected that the knowledge gained from an ideal model grass species will greatly facilitate the study of other important cereal crops. Rice was logically chosen as one model system for cereal crop genomics owing to its small

genome size and importance as a staple crop (Goff et al. 2002; Shimamoto and Kyozuka 2002; IRGSP 2005). The completion of the rice genome sequence has fueled its use in comparative genomics to understand the evolution of grass genomes as well as in map-based cloning of important genes in other cereal crops such as wheat (Song et al. 2002; Ling et al. 2003; Lai et al. 2004; Yan et al. 2004; Yan et al. 2006; Bruggmann et al. 2006; Wei et al. 2007).

Despite their close relationship, grass genomes are evolutionarily labile for many characteristics including mapping (Griffiths et al. 2006). Comparative studies on the chromosome number, ploidy, and genome size. In addition, common sequence changes such as insertion, deletion, duplication, and translocation can further complicate the use of many regions of the rice genome for cross-species comparison with other grasses (Sorrells et al. 2003; Bruggmann et al. 2006). Therefore, comparative analysis using more than two grass genomes could allow for elucidation of the nature of sequence changes that occurred in specific lineages during the evolutionary history of grass species (Song et al. 2002; Lai et al. 2004; Wei et al. 2007; Salse et al. 2008). For genome studies in Triticeae species including wheat and barley, a more closely related model grass genome will serve as a better intermediate for comparative and functional analysis.

The genus *Brachypodium* belongs to the Brachypodieae tribe, which is sister group to the four major cool season grass tribes of great economic importance: Triticeae, Aveneae, Poeae, and Bromaeae (Draper et al. 2001; Vogel et al. 2006). Hence, the *Brachypodium* genome is expected to show greater gene colinearity to the genomes of major cool season cereal grain and forage grasses and will be more useful in gene discovery in large Triticeae genomes such as wheat and barley (Garvin et al. 2008; Opanowicz et al. 2008; Ozdemir et al. 2008). In addition, the annual species in the genus *Brachypodium* possess a range of desirable biological features that make them well suited for functional genomics. For example, diploid *Brachypodium* has one of the smallest genomes in grasses (~300 Mb). The plant also has small physical stature, a short life cycle, and undemanding growth requirements, all of which make it amenable to high-throughput genetic screening. Therefore, *Brachypodium* could serve as a utility model system for various types of plant research (Beckmann et al. 2008; Li et al. 2008; Idziak and Hasterok 2008; Parker et al. 2008; Spielmeier et al. 2008).

Despite the growing interest in utilization of *Brachypodium* as a model grass, little is still known about the structure and organization of the *Brachypodium* genome. The utility of *Brachypodium* for grass crop genomics remains to be thoroughly tested. So far, comparative analysis with rice and wheat have been only conducted in a few genetic regions from a perennial, outbreeding species *B. sylvaticum* (Foote et al. 2004; Griffiths et al. 2006; Bossolini et al. 2007; Faris et al. 2008; Spielmeier et al. 2008), which has a genome reported to be ~470 Mb (Foote et al. 2004) with a size similar to that of rice, but larger than that of *distachyon*. *B. sylvaticum* has proved useful in mapping the wheat *Pat1* gene (Griffiths et al. 2006). It was shown that the orthologous sequences from *B. sylvaticum* are more colinear to wheat as compared to those of rice. *B. sylvaticum* and wheat shared such high sequence identity that probes derived from *B. sylvaticum* sequences can be directly used for wheat mapping (Griffiths et al. 2006). Comparative studies on the wheat Lr34 region also indicated that *B. sylvaticum* and wheat showed perfect macro-colinearity of genetic markers, whereas rice contained a 200-kb inversion in the orthologous region (Bossolini et al. 2007). It was estimated that *B. sylvaticum* and wheat diverged 35–40 Mya, significantly more recently than the divergence of rice and wheat (Bossolini et al. 2007). As far as we know, comparative analysis using *B. distachyon* genome has not been reported. A genome study of *B. distachyon* will provide important insights into the gene distribution and evolution of repetitive DNA in a compact grass genome. Previously, we sequenced 65,000 BAC ends from *Brachypodium* BAC libraries to generate a 38 Mb of random short genomic sequence, representing 10% of the *Brachypodium* genome (Huo et al. 2007). Analysis of BES revealed low repetitive DNA content and close phylogenetic relationship with the Triticeae species. In this study, we compared the BES against the rice genome to assess sequence conservation between these two compact grass genomes. To further analyze the colinearity of the orthologous regions, we sequenced nine *Brachypodium* BAC clones selected on basis of BAC-end matches to the rice genome. Our study provides the first comparison of the *Brachypodium* and rice genomes at multiple genetic loci. To evaluate the utility of *Brachypodium* for cereal crops with large genomes, the annotated *Brachypodium* genes were BLASTN searched against the wheat EST database and deletion-bin mapped wheat ESTs. Comparative analysis using the *Brachypodium* genome could offer a potentially useful strategy for the development of wheat genetic and linkage maps.

#### Materials and methods

##### Blast search of *Brachypodium* BES against the rice

To anchor *Brachypodium* BACs onto the rice genome, the 55,221 repeat masked BES were compared to the rice genome sequence using BLASTN. An expectation value (E) of  $e^{-10}$  was used as the significant threshold (www.tigr.org). The BES were assigned to individual rice

chromosomes based on their best match to the rice genome databases of NCBI (March 2008) using BLASTN and BES matching the rice genome were also analyzed by BLASTX. If a hypothetical protein was predicted, the BLASTX against NCBI non-redundant protein databases sequence was searched against UniProt (Ver. 9.7) of European Bioinformatics Institute database (March 2008). Only matches with  $E$  values smaller than  $e^{-6}$  were accepted. The complete annotation of each sequenced BAC clone has been submitted to GenBank (Accession numbers EU730894–EU730902).

#### Sequencing of Brachypodium BAC clones and sequence assembling

BAC DNA was isolated with the Qiagen Large Construct Kit (Qiagen, Valencia, CA) and shotgun libraries were constructed as described previously (Gu et al. 2003). In brief, the purified DNA was sheared and size selected by agarose gel electrophoresis. Fragments with sizes between 3 and 5 kb were ligated into the pCR4TOPO vector using a TOPO cloning kit (Invitrogen, Carlsbad, CA). The ligation mixture was transformed into electrocompetent TOP10 cells. Plasmid DNA was isolated using the PerfectPrep Direct Bind Kit (Eppendorf, Boulder, CO). For each BAC, 768 subclones were sequenced from both ends using T<sub>7</sub> and T<sub>3</sub> primers and BigDye terminator chemistry (Applied Biosystems, Foster City, CA) on an ABI3730XL automated sequencer (Applied Biosystems). Gaps were filled by sequencing PCR products amplified directly from the BAC clones.

The BAC sequences were assembled using the LaserGene SeqMan Module (DNASTAR, [www.DNASTAR.com](http://www.DNASTAR.com)) as described previously (Gu et al. 2003). In this module, we set the stringency for base calling and quality assessment to "high" to generate the most accurate consensus sequence possible. The sequence assembling was performed using a 40-bp window size and a 95% match requirement.

#### Annotation of Brachypodium repetitive elements

To define the Brachypodium repetitive element, a survey of the composition and contents of the Brachypodium repeat element sequences in the sequenced BACs was conducted using the RepeatMasker program (<http://www.repeatmasker.org>). The BAC sequences were also searched using BLASTN against the Triticeae Repeat Sequence (TREP) database (<http://wheat.pw.usda.gov/ITMI/Repeats>) and the local BLAST database containing unique Brachypodium repeat element sequences (Huo et al. 2007).

#### Gene annotation

To annotate coding sequences, a combination of BLASTN and BLASTX against non-redundant nucleotide and protein databases were utilized to identify all putative genes. In addition, coding regions in the BAC sequences were also predicted using FGENESH (<http://www.softberry.com>) set for a monocot model. Predicted genes were then compared to the nonredundant and dbEST databases of NCBI (March 2008) using BLASTN. Brachypodium genes were also compared to NCBI wheat and rice EST databases using BLASTN.

#### Results

##### Anchoring Brachypodium BES onto the rice genome

Analysis of \* 65,000 Brachypodium BES revealed its relatively simple genome with low repetitive DNA content and high gene density (Huo et al. 2007). To provide an initial genome-wide comparison of rice and Brachypodium both with compact genomes, we attempted to anchor Brachypodium BES onto the rice genome. Since the coding sequences of transposable elements (TE) often identify multiple noncolinear matches between the two grass species, it was critical to use repeat-masked BES in the analysis. Our BLASTN analysis showed that 14,547 out of 55,221 repeat-masked BES (26.3%) had matches to the rice genome sequence ( $E < e^{-10}$ ) at the nucleotide level. Further analysis using BLASTX showed that 11,982 (82.4%) out of these 14,547 BES matched to known protein-coding genes at  $E < e^{-10}$ . The nature of the remaining 17.4% of the matches is unclear, however, some of these sequences could represent conserved noncoding sequences as previously suggested (Bossolini et al. 2007).

The matched BES were plotted along the 12 rice chromosomes (Zhao et al. 2001, 2004). According to the positions of the two paired-end matches in the rice conserved sequences, these BACs can be placed into six different classes (Fig. 1b). For Classes I to V, both ends of the BAC aligning to the rest of the chromosomes. There were 2,018 (13.8%), 1,923 (13.2%) and 1,915 (13.2%) BES matched to the sequences on rice chromosome 1, 2 and 3, respectively. The distance separating the two ends ranged from less than 50 kb to over 1 Mb. For Class VI, the best matches of the two BAC ends to the rice sequences are on different chromosomes. The result showed that the number of BAC clones in each category was 116 (6.7%), 734 (42.3%), 58 (3.3%), 20 (1.2%), 153 (8.8%) and 653 (37.7%), respectively (Fig. 1b). We randomly selected 10 BAC clones

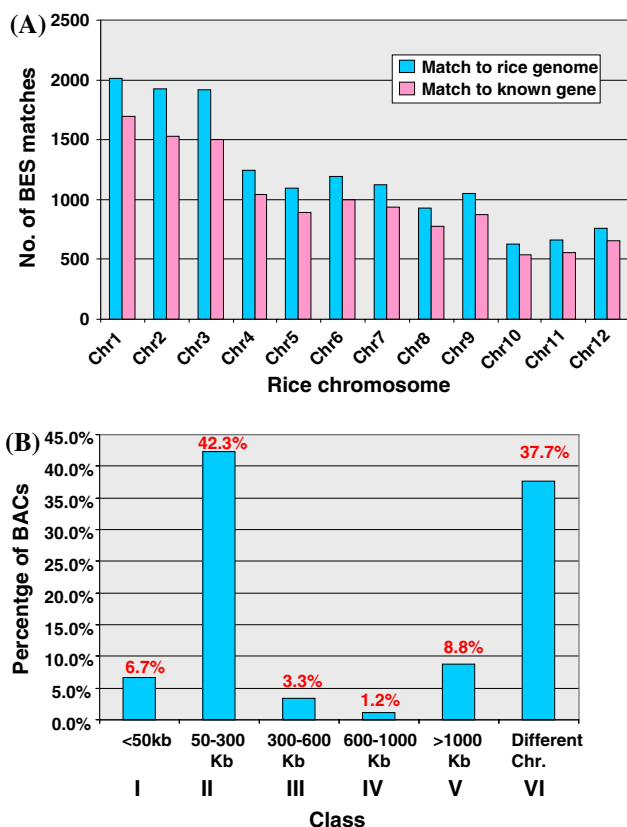
from each category for determination of BAC insert size by CHEF gel electrophoresis. All 10 BAC clones in Class I contained an insert smaller than 50 kb, suggesting that the approximate distance between these matches is known, these BAC clones are more informative in comparative studies to identify putative

reflected the small insert size in Brachypodium BAC clones. The BAC clones in other classes all had an insert with sizes ranging from 80 to 170 kb (data not shown), suggesting that most of the large size difference between a Brachypodium BAC and the orthologous rice region was the result of genomic changes that have occurred since the divergence of the two grass genomes.

#### BAC selection for sequencing

Considering the small insert size of Class I BACs, it is likely that most of the BACs in this category identified a colinear rice region with no major sequence rearrangements. This might be also true for many BACs in the Class II category, although some of the size difference between the Class II BAC insert and the corresponding rice region could be explained by the lower amount of repetitive DNA content in the smaller genome of Brachypodium (Huo et al. 2007). However, the large size differences observed in Class III, IV and V BACs and the scenario in Class VI BACs are likely due to substantial sequence difference between the two genomes. We examined the possibility that the two BAC ends in Class VI might match to the duplicated regions on two different rice chromosomes. 23.3% of these BACs showed that the two ends matched to two different regions originated from ancient genome duplication or chromosomal duplications in rice (Yu et al. 2005).

Dynamic sequence changes such as insertion, deletion, inversion, and translocation, coupled with rapid but differential repetitive DNA amplification and removal, are major evolutionary forces constantly reshaping individual plant genomes (SanMiguel et al. 1996; Vicent et al. 1999; Fu et al. 2002; Jiang et al. 2004; Kazazian 2004; Ma et al. 2004). In order to gain a more detailed view of the sequence changes in the regions represented by these paired BAC clones, we sequenced one or two BAC clones



**Fig. 1** Alignment of Brachypodium BES onto the rice genome. **(A)** Distribution of Brachypodium BES matched on 12 rice chromosomes. Repeat-masked Brachypodium BES was compared against the rice genome using BLASTN and BLASTX. The top match of each BES in BLASTN was used to determine its assignment to the rice chromosome. The number of total matches to each rice chromosome was counted and plotted. **(B)** Percentage of paired BES in each category. 1,734 BAC clones with paired ends both matching to the rice genome were categorized based on the distance of two matches on the same chromosome as indicated. BAC clones with two matches on different chromosomes are classified into category VI

Table 1 Sequence composition of nine *Brachypodium* BACs

Clone ID	Category	Size (kb)	Gene no.	Gene density (kb/gene)	RNA TE (bp)	DNA TE (bp)	Others (bp)	UBRES (bp)	Total repeat
DB009O11	Class II	113.0	16	7.1	950 (0.8%)	2,149 (1.9%)	463 (0.4%)	1,134 (1.0%)	4,696 (4.2%)
DB009L22	Class III	128.8	16	8.1	9,638 (7.5%)	1,400 (1.1%)	929 (0.7%)	7,973 (6.2%)	19,940 (15.5%)
DB038O09	Class IV	102.4	11	9.3	1,994 (1.9%)	2,118 (2.1%)	251 (0.2%)	2,254 (2.2%)	6,617 (6.5%)
DH003L20	Class V	89.3	8	11.2	2,824 (3.2%)	1,091 (1.2%)	199 (0.2%)	1,338 (1.5%)	5,452 (6.1%)
DB031O07	Class V	107.3	9	11.9	11,439 (10.7%)	2,854 (2.7%)	779 (0.7%)	1,170 (1.1%)	16,242 (15.2%)
DH037O21	Class VI	162.5	24	6.8	16,158 (10.0%)	1,812 (1.1%)	1,104 (0.7%)	6,432 (4.0%)	25,506 (15.7%)
DH085B13	Random	138.9	20	6.9	3,625 (2.6%)	1,227 (0.9%)	609 (0.4%)	2,429 (1.8%)	7,890 (5.7%)
DB091J02	Random	100.2	6	16.7	12,942 (12.9%)	987 (1.0%)	551 (0.6%)	8,640 (8.6%)	23,120 (23.1%)
DH002G02	Random	128.6	9	14.3	12,592 (9.8%)	3,921 (3.0%)	1,169 (0.9%)	12,561 (9.8%)	30,243 (23.5%)
Total		1,071	119	9	71,795 (6.7%)	17,559 (1.6%)	5,789 (0.5%)	43,931 (4.1%)	139,706 (13.1%)

<sup>a</sup> Including the known unclassified, small RNA, satellites, simple repeats and low complexity repeat

from different categories (Table 1). Considering the small insert size in Class I and likely colinearity with the corresponding rice region, no BAC clones were selected from this class. Instead, a BAC clone from Class II with an insert size (\* 113 kb) similar to the size in the corresponding rice region (115.8 kb) was selected for sequencing. This BAC spanned a larger colinear region as compared to the BACs in Class I. Three random BACs, DH085B13, DB091J02 and DH002G02 were also chosen for sequencing in order to obtain a random sample sequence. The total assembled sequence of the nine *Brachypodium* BACs was 1,071 kb (Table 1).

#### *Brachypodium* repetitive DNA in sequenced BACs

Transposable elements (TE) are one of the major components of plant genomes (SanMiguel et al. 1996; Meyers et al. 2001; Li et al. 2004; Messing et al. 2004; Paux et al. 2006). To evaluate the TE content in the compact *Brachypodium* genome, the sequences from the nine BAC clones were compared to repetitive DNA databases using the RepeatMasker program (<http://www.repeatmasker.org>). TE content in each BAC also indicates that TEs are not evenly distributed in the genome sequence. This suggests that there may be hot spots for TE insertions (Ma and Benetzen 2004) even in the compact genome of *Brachypodium*. Clearly, there is significant variation of TE content and types among each BAC. The percentage of RNA TE ranges from 0.8% to 12.9% with an average of 6.7%. The percentage of DNA TE ranges from 0.9% to 3.0% with an average of 1.6%. Therefore, the average DNA TE content in the sequenced BACs is comparable to that estimated for the *Brachypodium* genome. Detailed analysis of the BAC end sequences (1.28%); while the average RNA TE content (6.7%) is slightly lower than the previous estimate (7.87%) (Huo et al. 2007).

Previously, we developed a database for the unique *Brachypodium* repetitive element sequences (UBRES) (Bennetzen et al. 2004; Devos et al. 2005; Ma et al. 2005), we used stringent parameters for gene

assignment in *Brachypodium* BACs. We only counted putative genes that had a significant match (BLAST E-value smaller than  $e^{-10}$ ) to a known gene that was not a transposable element (TE). For the nine *Brachypodium* BACs, a total of 196 genes were predicted by FGENESH and 119 of these genes were confirmed by BLASTX search with these criteria. The gene annotations of each BAC are shown in Supplement 1.

The precise borders of each region in the rice genome orthologous to the individual *Brachypodium* BAC were identified by local BLAST of the BAC sequences against the rice genome assembly (TIGR rice pseudomolecule version 5). The detailed information of percentage of annotated repeat DNA and gene sequences in each BAC and its rice orthologous regions are shown in Fig. 2. According to both TIGR and Gramene databases, there were 214 genes annotated in these rice orthologous regions. When we used the same annotation strategy to re-annotate the orthologous rice regions, the gene number in the regions was decreased to 140 (Supplement 2). In general, the number was observed in the *Brachypodium* BACs and the rice orthologous regions (Fig. 2).

The recent estimation of gene number in the rice genome is about 32,000 (Itoh et al. 2007), which is smaller than earlier estimates (Goff et al. 2002; Wu et al. 2002; Yu et al. 2005). It has been noted that many predicted hypothetical genes might be artificial (Itoh et al. 2007). Out of

the originally annotated 214 genes, 75 either matched to the TIGR repeat database or had no match to Arabidopsis protein database. These hypothetical genes were not present in the collinear regions in the *Brachypodium* BACs, providing further support that they were mis-annotated. Meanwhile, on rice chr6, a 3.9-kb non-annotated region (position 10008.6–10012.5 kb) was found to be similar to a gene encoding a serine/threonine kinase in *Brachypodium* BAC DB038O09. The sequence alignment using the VISTA program shows that they have 80% identity (data not shown). The TIGR rice transcript assembly TA64930\_4530 supported our annotation; therefore a serine/threonine kinase gene was assigned to the rice region.

Gene density on selected *Brachypodium* BACs ranged from 6.8 kb to 16.7 kb/gene, with an average of 9.0 kb/gene (Table 1), whereas the gene density in the orthologous rice regions ranged from 6.2 kb up to 26.5 kb/gene with an average of 14.1 kb/gene. This number is much lower than the early estimation of gene density of 9.9 kb/gene in the rice genome (Wu et al. 2002; IRGSP 2005), and is close to the current estimation of 12.2 kb/gene (Itoh et al. 2007).

However, if we consider the 75 hypothetical genes in rice, the average gene density in the rice regions would be 9.2 kb/gene, indicating that annotated gene density changes considerably depending on the gene annotation criterion used. As the same annotation criterion was applied to *Brachypodium* BACs and the orthologous rice regions, our comparison of the gene density in these regions will provide a relatively unbiased result. In the rice regions orthologous to four *Brachypodium* BACs (DB009L22, DB038O09, DH003L20 and DB091J02), the difference in gene density is largely attributable to more repetitive DNA in the corresponding rice regions. However, in the other orthologous regions, the gene density is comparable (Fig. 2). These results suggest that the rice genome might have more regions containing a higher amount of repetitive DNA content. We found that large blocks of repetitive DNA regions were rarely found in *Brachypodium* sequences.

#### Comparison of orthologous regions of *Brachypodium* and rice

We compared the *Brachypodium* BAC sequences to the orthologous regions from rice. BAC DB009011 in Class II is 113-kb in length and contains 16 annotated genes. This region is orthologous to a 115.8-kb region on rice chr2 which contains 15 annotated genes. The VISTA alignments of the orthologous regions identified regions of sequence conservation and divergence (Fig. 3). Two *Brachypodium* genes, a vegetative storage gene 2 and an ATP synthase (Gene 15 gene), are absent in the rice region, while a rice gene (LOC\_Os02g47850) is missing in the *Brachypodium* BAC. Thus, 14 genes are collinear with respect to order and

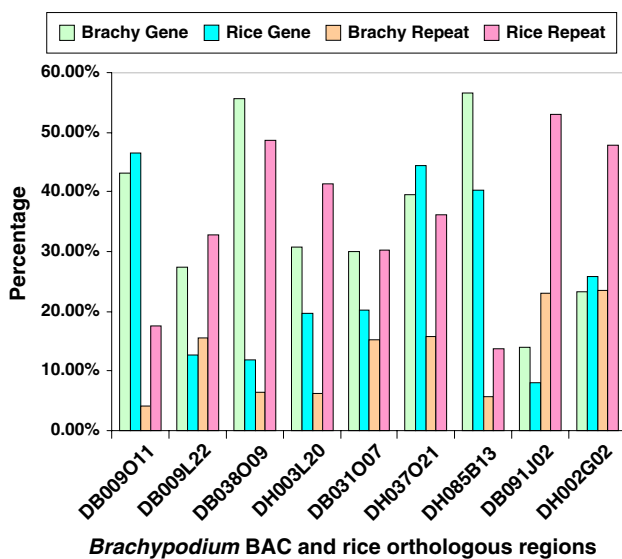


Fig. 2 Comparison of repeat DNA content and gene density in rice and *Brachypodium*. The repeat DNA content and the number of genes in sequence *Brachypodium* BACs and corresponding orthologous regions of rice were analyzed. The sequence percentage taken by repeat DNA and genes was calculated for each orthologous region from rice and *Brachypodium*. The *Brachypodium* BAC clones indicated in x-axis was used to show the analysis results in the orthologous regions

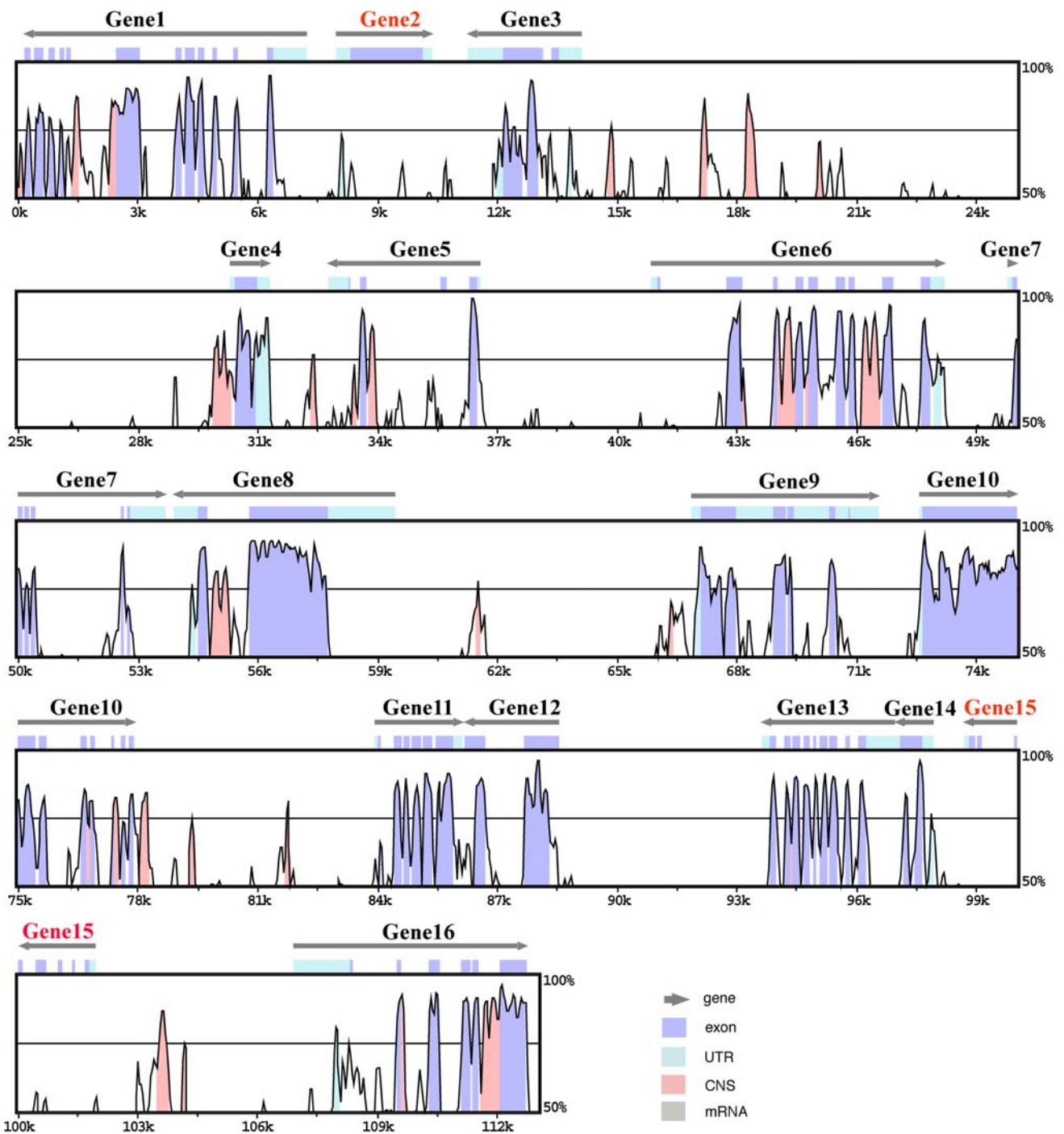
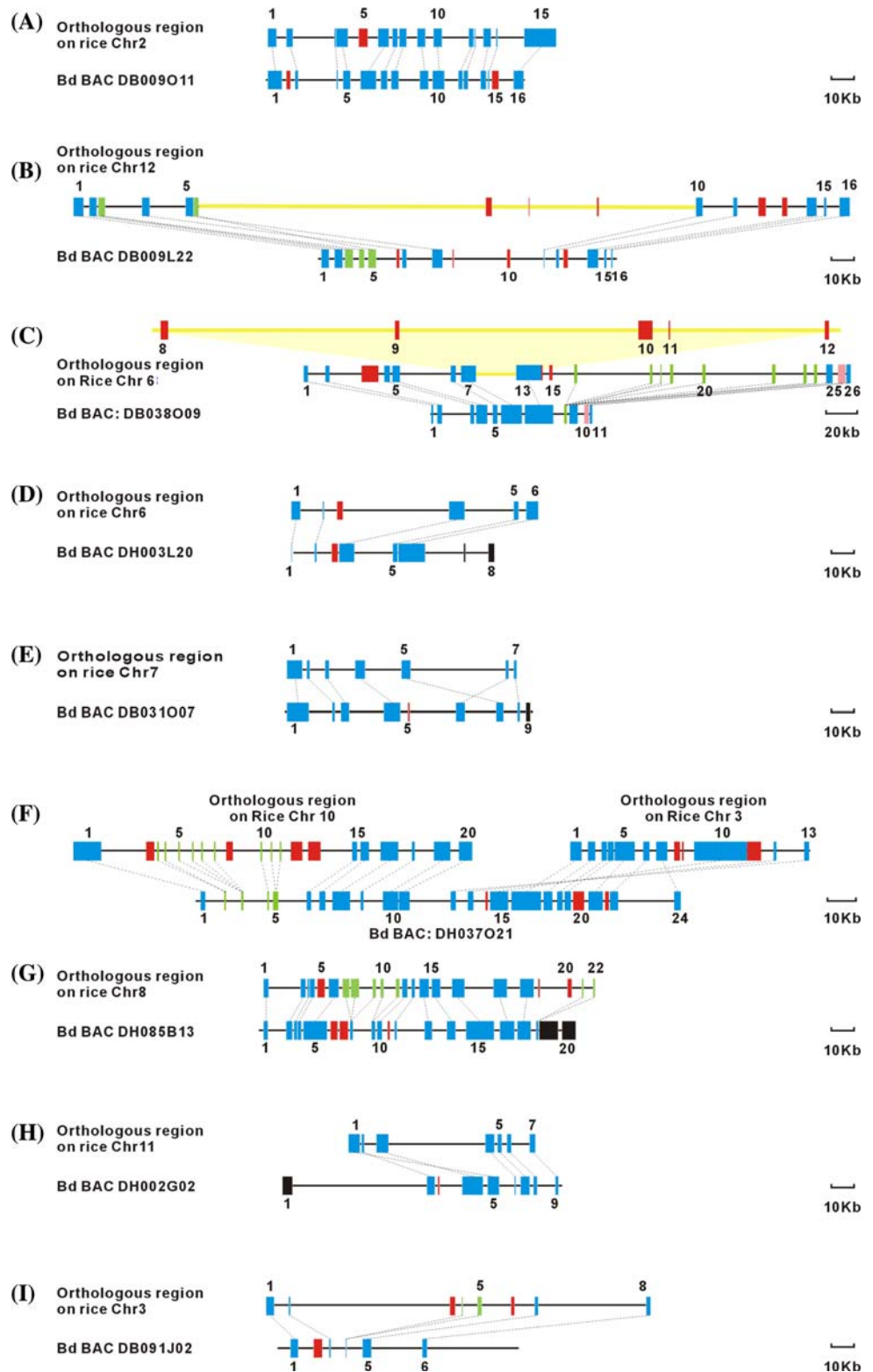


Fig. 3 VISTA analysis of orthologous regions from *Brachypodium* and rice. The *Brachypodium* BAC DB009O11 sequence was used as backbone in comparison with the rice orthologous region. The rice annotation of each gene in *Brachypodium* and rice is provided in Supplement Table 4. Arrows indicate transcription orientation of regions in two compared sequences. Genes annotated in *Brachypodium* BAC are only numbered sequentially in the figure. The rice annotation of each gene in *Brachypodium* and rice is provided in Supplement Table 4. Arrows indicate transcription orientation of regions in two compared sequences. Gene2 and Gene15 indicated in red in the *Brachypodium* BAC are nonshared in the rice orthologous region. The x-axis represents the base sequence and the y-axis represents the percent identity

orientation. Clearly, the most conserved regions are within exons. The intergenic sequences are largely diverged although short regions of conserved non-coding sequences were detected in the colinear regions between the exon regions. The VISTA alignments easily showed the two non-shared genes (Gene2 and Gene15) that

**Fig. 4** Comparison of the sequence Brachypodium BAC regions with the rice orthologous regions. Sequenced Brachypodium BAC regions and the corresponding rice orthologous regions are represented by solid black lines. Genes conserved between Brachypodium and rice are depicted as blue boxes. Non-conserved genes are indicated in red and black boxes. Black boxes represent non-conserved genes at the end of BAC clones. Duplicated genes are labeled as green boxes. Orthologous genes in blue boxes are connected by dotted black lines. Annotated genes from both sequences were numbered sequentially. The annotation of each gene is provided in Supplement 1 and 2. For 4B and 4C, the yellow lines in the middle of the rice orthologous regions represented non-colinear fragments. In 4C, this region is re-drawn in scale on the top with annotated genes indicated. The pink box represents a gene that is missing in the rice genome annotation



are only present in Brachypodium (Fig. 3). The intergenic distances are also similar in size to the orthologous region on rice chr6, respectively. As shown in Fig. 4, the large size difference (587.6 kb) between DB038O09 and the orthologous rice region is primarily due to the presence of a 426-kb fragment containing *Pve* genes in the rice

The Class III BAC DB009L22 (128.8 kb) (Fig. 4b) and Class IV BAC DB038O09 (102.4 kb) (Fig. 4c) are

orthologous to a 341.5-kb region on rice chr12 and 690-kb region on rice chr6, respectively. As shown in Fig. 4, the large size difference (587.6 kb) between DB038O09 and the orthologous rice region is primarily due to the presence of a 426-kb fragment containing *Pve* genes in the rice

genome. The absence of these five genes in Brachypodium region also causes disruption of microcolinearity in respectively. When compared with the rice orthologous the orthologous regions. Another factor causing the large size difference is the multiple duplications of a gene encoding UDP-glycosyltransferase, which resulted in nine copies (Gene16 to Gene24) spanning more than 100-kb in Brachypodium region (Fig. 4c). In addition to the above major sequence changes, there are three genes (Gene4, Gene14 and Gene15) unique to the rice region (Fig. 4c).

The size difference between BAC DB009L22 and the orthologous region on rice chr12 was due to a 178-kb sequence containing three genes that was only present in the rice region (Fig. 4b). The presence of non-colinear 178-kb and 426-kb segments in the two orthologous rice regions could be caused by insertion in the rice genome or deletion in the Brachypodium genome.

Two BAC clones DH003L20 (89.3 kb) (Fig. 4d) and DB031O07 (107.3 kb) (Fig. 4e) were selected from the Class V category. The ends of DH003L20 matched two rice regions separated by 2,089 kb on chr6, while the ends of DB031O07 matched rice regions 7,032 kb apart on chr7. In both cases, it was found that the last genes on one end of the Brachypodium BAC clones were not present in the rice genome; one gene is in colinear positions in rice, instead they matched to regions millions of base pairs away on the same chromosome. The remaining regions in these two BACs were mostly colinear with the rice orthologous regions, except one Brachypodium gene in each BAC was not present in the orthologous rice regions (Supplement 1, 2).

The two ends of BAC DH037O21 (162.5 kb) (Fig. 4f) in Class VI matched to two regions located on different rice chromosomes. Further sequence analysis revealed that the orthologous region containing the first 11 genes is orthologous to a 134.7-kb region on rice chr10, while the region containing the last 13 genes is orthologous to a 86.6-kb region on rice chr3 (Fig. 4f). Rice chr10 is colinear to rice chr3 due to ancient duplication events (Yu et al. 2005). Because of the differential sequence evolution in duplicated regions, it appears that half of Brachypodium became more colinear with a region on rice chr3 and the other half more colinear with the paralogous region on rice chr10. In fact, we were unable to determine the true orthologous region for BAC DH037O21. Sequence changes have also occurred in the two rice regions as compared to the Brachypodium sequence. These include an inversion on chr3 and duplications of a proline-rich protein gene in two regions in rice as compared to one duplication region in Brachypodium (Fig. 4f). Furthermore, there are seven genes unique in the rice regions and three genes unique in the Brachypodium region.

Among the three random BACs, 20, 9, and 6 genes were identified in DH085B13 (138.9 kb) (Fig. 4g), DH002G02

(128.6 kb) (Fig. 4h), and DB091J02 (100.2 kb) (Fig. 4i), respectively. When compared with the rice orthologous regions, major sequence rearrangements were not identified, except a region containing three genes was inverted in BAC DH002G2. In addition, DH085B13 contains three non-colinear genes, DB091J02 and DH002G02 contain one non-colinear gene each (Supplement 1, 2).

#### Analysis of non-colinear genes

Our analysis showed that a total of 17 Brachypodium genes (out of 113 genes; 15%) are not present in the orthologous regions, while 27 rice genes (out of 140 genes; 19%) are absent in the Brachypodium regions. To examine if these non-colinear Brachypodium genes are present somewhere else in the rice genome, we BLASTN compared the non-colinear Brachypodium genes to the rice genome database. The result showed that 15 of the 17 non-colinear Brachypodium genes found matches in the rice genome at a BLAST E value less than  $e^{-10}$ , although it is not clear if these matches represent retrieving of the paralogous genes (Supplement 1, 2). Only three Brachypodium genes were missing from the rice genome; one gene is in colinear positions in rice, instead they matched to regions homologous to a wheat prolamine and the other is a gene similar to wheat and Arabidopsis SKP1 gene.

Among the 27 genes that were not shared between Brachypodium orthologous regions, 26 genes have significant matches in the Brachypodium genome ( $e^{-10}$  or lower). Only one unknown gene LOC\_Os03g26791 was missing from Brachypodium.

Thus, our results are consistent with the previous results showing that most non-colinear genes in the maize and/or sorghum genomes were found in the rice genome at non-orthologous locations (Song et al. 2002; Lai et al. 2004).

Nevertheless, based on the comparative sequence analysis, only 17% of the genes in the two genomes are not colinear in the orthologous regions. It is worth noting that the degree of microsynteny based on sequence comparison in orthologous regions appeared to be different from that of Brachypodium macro-synteny estimated on BES alignment to the rice genome (Fig. 1b). Although the reason causing the difference in microsynteny and macro-synteny levels is unclear, Gaut (2002) estimated that the macro-synteny probability of any given marker based on loss rate of syntenic genes during genome evolution is about 50% between two grass species with the divergence time of 50 Mya, which is similar to the result observed in BES alignment to the rice genome. One possible explanation for the difference in our results could be attributable to the different analysis methods used. Detailed comparative sequence analysis in the orthologous regions allows identifying colinear genes that might have been dramatically changed due to high sequence divergence, rearrangement or partial deletion. In

our BES alignment analysis, only the top match was counted. Therefore, the top match might be a paralogous gene on different chromosomes if the orthologous genes have been significantly changed, resulting in lower degree of synteny.

#### Sequence comparison among Brachypodium, rice and wheat

Several phylogenetic studies have indicated that Brachypodium and the Triticeae (wheat and barley) are more closely related to each other than to rice (Vogel et al. 2006; Bossolini et al. 2007; Huo et al. 2007). This close relationship suggests that Brachypodium may serve as a better model for Triticeae crop research. To evaluate the utility of Brachypodium as a model for wheat, we first compared all annotated Brachypodium genes to the wheat EST database. For the nine individual BACs, the percentages of genes with significant matches (P value less than  $10^{-5}$ ) ranged from 44.4% to 100%. Overall, out of the 119 annotated genes, 91 (76.5%) had significant matches to the wheat EST database (P value less than  $10^{-5}$ ). Since only two Brachypodium genes were missing from the rice genome, the Brachypodium genes that were not present in the rice EST database must either be underrepresented in the rice tissues sampled for EST sequencing, or be pseudogenes. Since a similar number of wheat (1,051,465) and rice (1,220,261) ESTs were compared to the Brachypodium genes, most of the Brachypodium genes that did not match wheat EST are likely contained in the wheat genome.

To further evaluate the degree of divergence among Brachypodium, wheat, and rice, the number of synonymous substitutions (Ks) and nonsynonymous substitutions (Ka) per site between orthologous genes in Brachypodium, wheat, and rice were determined. To do so, only the coding

**Table 2** The number and percentage of Brachypodium genes matched to wheat and rice EST

Clone ID	Gene no.	No. of rice EST hit	No. of wheat EST hit	No. of mapped wheat EST hit
DB009O11	16	11 (68.8%)	13 (81.3%)	3
DB009L22	16	7 (43.8%)	10 (62.5%)	0
DB038O09	11	9 (81.8%)	11 (100.0%)	0
DH003L20	8	6 (75%)	7 (87.5%)	0
DB031O07	9	4 (44.4%)	4 (44.4%)	0
DH037O21	24	14 (58.3%)	17 (70.8%)	4
DH085B13	20	15 (75%)	16 (80.0%)	1
DB091J02	6	4 (66.7%)	5 (83.3%)	2
DH002G02	9	6 (66.7%)	8 (88.9%)	1
Total	119	76 (63.9%)	91 (76.5%)	11

Table 3 Estimates of divergence time and the rates of synonymous (Ks) and nonsynonymous (Ka) substitutions

BAC	Gene index	Wheat				Rice			
		Ka	Ks	Ka/Ks	Divergence (Mya)	Ka	Ks	Ka/Ks	Divergence (Mya)
DH002G02	5	0.005	0.229	0.022	35.2	0.008	0.299	0.027	46.0
DH085B13	15	0.051	0.285	0.179	43.8	0.093	0.280	0.332	43.1
DB038O09	4	0.064	0.305	0.210	46.9	0.112	0.435	0.257	66.9
DB009L22	2	0.025	0.209	0.120	32.2	0.071	0.232	0.306	35.7
DB038O09	11	0.059	0.279	0.211	42.9	0.061	0.380	0.161	58.5
DH037O21	12	0.033	0.131	0.252	20.2	0.078	0.219	0.356	33.7
DH037O21	5	0.088	0.276	0.319	42.5	0.105	0.313	0.335	48.2
DH037O21	23	0.031	0.249	0.124	38.3	0.033	0.403	0.082	62.0
Average		0.045	0.245	0.181	37.8	0.070	0.320	0.219	49.3

6D, respectively (Qi et al. 2004; Randhawa et al. 2004). Song and coworkers (2002) hypothesized that these regions were potential hotspots for chromosome changes. The order of bin-mapped ESTs is usually unknown. However, given the sequence conservation represented by BAC DB009O11 in *Brachypodium* and rice, the order of DB091J02 and the corresponding wheat region. The three mapped wheat ESTs can now be tentatively assigned.

Four annotated genes (Gene5, Gene7, Gene12 and Gene19) from BAC DH037O21 matched to bin-mapped wheat ESTs (BI480570, BF482960, BE424589 and BG604404), respectively. Three of these ESTs (BI480570, BF482960, and BG604404) have map positions on wheat Chr4. The other EST BF424589 corresponding to Gene12 has been mapped to the short arm of Chr7 (7AS8-0.45–0.89, 7BS1-0.27–1.00). BAC DH037O21 represents a Class I cluster that about 26.4% of repeat-masked BES matched to the VI clone with its paired BES matched two rice regions (rice genome and 82.4% of the matches (11,982 BES) were from different chromosomes (Fig. 3b). However, considering that Gene5, Gene7 and Gene19 in the *Brachypodium* BAC DH037O21 are mapped to the same wheat chromosome (Chr4), but are located in different rice chromosomes (BACs and three random BACs provides the first snapshot (Fig. 4f), it is likely that *Brachypodium* and wheat will share more colinearity in this region.

The two wheat ESTs (BG274272 and BE517956) corresponding to the two annotated *Brachypodium* genes (Gene1 and Gene6) in BAC DB091J02, were mapped to deletion bins on different wheat chromosomes (C-4DL9-0.31 and C-5AS1-0.40). Clearly, colinearity is not retained based on these two mapped wheat ESTs. However, translocations involving wheat chromosomes 4A, 5A, and 7B, have a compact genome and represent different lineages in paracentric inversion on chromosome 4A and a small pericentric inversion in centromeric bins on 5AS, 5BL and 5DL have been reported several times (Nelson et al. 2005; Linkiewicz et al. 2004; Qi et al. 2004). It was reported that part of the rice chromosome 3S is colinear with wheat 4BL, while the rest is colinear with wheat 5AL and 4AS (Buell et al. 2005). It seems that these regions were conserved junctions that interrupt synteny blocks in each genome. The same conserved junction was reported in a maize/sorghum/rice comparison (Bruggmann et al. 2006). Rice and *Brachypodium* diverged about 50 Mya, both have a compact genome and represent different lineages in the evolutionary path of grass species (Kelloff 2001). However, analysis of BES revealed that 12,113 BES had BLASTX matches to the non-redundant protein database at  $e^{-10}$  or smaller (Huo et al. 2007). Among them, 11,982 BES ( $< e^{-10}$ ) to the rice protein database, suggesting that over 82.6% of the protein-encoding genes are shared between rice and *Brachypodium*. A comparison of the rice and *Arabidopsis* protein sets revealed that 5,663 proteins are rice-specific and 3,402 are *Arabidopsis*-specific (Itoh et al. 2007). The *Brachypodium*

genome will allow us to determine which rice-specific genes are shared with Brachypodium and therefore, how many of these genes are monocot-specific genes.

Our analysis using paired BES was useful in identifying of the draft genome sequence ([www.jgi.doe.gov](http://www.jgi.doe.gov)). Genome size can vary considerably, even within the same genus. For example, two diploid *Oryza* species (*Oryza sativa* and *Oryza australiensis*) have genome size that differs by 2.7-fold (\* 390 Mb and \* 975 Mb respectively). This larger size of *Oryza australiensis* genome is largely due to the rapid amplification of three LTR-retrotransposon families (Piegu et al. 2006). Thus, it is worth noting that Brachypodium sequenced genome is an effective method to identify divergent regions. Comparative syntenic maps among grass species often only reveal large regions that are conserved on the basis of gene content and order with less emphasis on individual non-colinear genes. Our result showed that 15% of Brachypodium genes may fail to find their rice orthologs in the colinear rice regions and that 19% of rice genes are absent from the colinear position. Among the non-colinear genes, over 90% had at least one match in another part of the rice genome. However, it is difficult to determine if these genes moved to different locations or were lost in a genome-specific manner due to the presence of paralogous copies in the genome. In maize at least 50% of the duplicated genes have been lost over a short period of time (Lai et al. 2004). Despite the considerable loss of duplicated genes, transposon-mediated gene movements have also been observed (Lal et al. 2003; Jiang et al. 2004; Lai et al. 2005), however, the importance of gene movements to the decay microcolinearity is not clearly understood.

The small genome of diploid Brachypodium is one of the important characteristics that make it an ideal model for large-genome grass species. The authoritative estimate of diploid Brachypodium is 0.36–0.39 (Bennett and Leitch 2005), and five different diploid accessions of Brachypodium have been identified with comparable value (Vogel et al. 2006). Comparison to rice, with a value of 0.51 pg (Bennett and Leitch 2005) and an accurate genome size of 389 Mb as determined by map-based sequencing (IRGSP 2005), yields an estimated genome size between 300 and 320 Mb for diploid Brachypodium. Our study further supports that the genome size of diploid Brachypodium is considerably smaller than that of rice. The repetitive DNA content observed in BES and the BAC sequences indicate that Brachypodium has less than 20% repetitive DNA, less than the rice genome (35%) (IRGSP 2005). The gene density observed in Brachypodium BACs (\* 9.0 kb/gene) was higher than the gene density that rice and Brachypodium have the same number of genes (estimated 32,000), the estimated genome size of Brachypodium would be slightly less than 300 Mb, much

these hypothetical genes in the rice genome. In this study, we mapped the complex Ph1 locus region in wheat (Griffiths et al. 2006). We identified 14 hypothetical proteins that were conserved in Brachypodium and rice. Among these fourteen genes, three (21.4%) had no homolog in Arabidopsis. On the other hand, Bossolini and coworkers (2007) have found that the percentage of the conserved genes increased when they re-annotated the rice region orthologous to wheat Ph1 locus region. They concluded that the apparent degree of conservation or colinearity of two compared genomes depends, in part, on the correct annotation of the compared sequence. In this study, 75 nonshared rice genes were removed as they matched TE or no Arabidopsis protein hit. We also observed that the increasing percentage of the conserved genes between two genomes.

#### Potential utility of Brachypodium for wheat genomics

Brachypodium has been proposed as a new model for the large-genome temperate grass crops because of its numerous desirable attributes, including a close relationship with Triticeae species. Several studies have shown that the relationship between Brachypodium and wheat is much closer than rice and wheat (Draper et al. 2001; Griffiths et al. 2006; Vogel et al. 2006; Bossolini et al. 2007; Huo et al. 2007). However, can Brachypodium really serve as a model for wheat? Bossolini et al. (2007) have doubted this because they found that only two-thirds of the genes from Pve wheat BACs on Lr34 locus were colinear with Brachypodium and relatively lower in gene density than that in the rice orthologous region. Conversely, Griffiths et al. (2006) in the course of mapping the wheat Ph1 candidate gene found that wheat and Brachypodium are more conserved, and that markers derived from Brachypodium sequences gave clear southern hybridization signals in wheat whereas markers made from rice sequence often failed.

We found that 77% of Brachypodium genes have strong Triticeae EST matches (Table 2), and when matches were identified in wheat and rice EST databases, a higher matching score and lower E value to a wheat EST was usually found. These results suggest that Brachypodium sequences would be more useful for developing cross-species markers than the rice sequences. One potential strategy to improve wheat mapping is to identify wheat ESTs based on the annotation of colinear Brachypodium regions and to assess if they can be mapped onto the corresponding wheat genetic regions, thereby increasing the marker density. Furthermore, although 20% of the annotated Brachypodium genes have no matches in the Triticeae EST database, we can still confirm their genetic/physical locations in the wheat genome by directly using Brachypodium markers as has been demonstrated in the Pve

The ideal model for wheat should share perfect microcolinearity with regard to gene content and order within a much compact genome. A few studies have shown the evolution of microcolinearity between Brachypodium and wheat, and rice at local genomic regions. We can expect that the level of colinearity will not be homogenous along the chromosomes. For example, many resistance gene homologs are clustered in plant genome, and regions containing clusters of disease resistance sequences evolve more rapidly due to the frequent sequence exchanges than other regions containing house-keeping genes (Michelmore and Meyers 1998; Hulbert et al. 2001). Our result also indicated the translocation events specific in several wheat chromosome regions could have resulted in the disruption of colinearity between Brachypodium and wheat. The extent that Brachypodium can serve as a model species for genomics research on large genome grasses such as wheat is still unknown. Comparative analysis using the complete sequence of Brachypodium genome in the near future will provide an unprecedented view regarding the evolution of the grass genomes.

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