

Global genomic diversity of *Oryza sativa* varieties revealed by comparative physical  
mapping

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The BES sequence data reported in this article have been deposited in the GenBank  
with accession numbers: KG702200-KG737748 for Minghui 63 and  
KG737749-KG771717 for Zhenshan 97.

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## Rice comparative physical mapping

Key Words: Minghui 63; Zhenshan 97; BAC library; Oryza; Physical map

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**ABSTRACT** BAC physical maps embedding a large number of BAC end sequences (BESs) were generated for *Oryza sativa* ssp. *indica* varieties Minghui 63 (MH63) and Zhenshan 97 (ZS97), and were compared with the genome sequences of *O. sativa* spp. *japonica* cv. Nipponbare and *O. sativa* ssp. *indica* cv. 93-11. The comparisons exhibited substantial diversities in terms of large structural variations, and small substitutions and indels. Genome-wide BAC-sized and contig-sized structural variations were detected, and the shared variations were analyzed. In the expansion regions of the Nipponbare reference sequence, in comparison to the MH63 and ZS97 physical maps, as well as to the previously constructed 93-11 physical map, the

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amounts and types of the repeat contents, and the outputs of gene ontology (GO) analysis, were significantly different from those of the whole genome. Using the physical maps of four wild *Oryza* species from OMAP (<http://www.omap.org>) as control, we detected many conserved and divergent regions related to the evolution process of *O. sativa*. Between the BESs of MH63 and ZS97 and the two reference sequences, a total of 1,532 polymorphic simple sequence repeats (SSRs), 71,383 SNPs, 1,767 MNPs, 6,340 insertions and 9,137 deletions were identified. This study provides independent whole genome resources for intra- and inter-subspecies comparisons and functional genomics studies in *O. sativa*. Both the comparative physical maps and the GBrowse, which integrated the QTL and molecular markers from GRAMENE (<http://www.gramene.org>) with our physical maps and analysis results, are open to public through our website (<http://gresource.hzau.edu.cn/resource/resource.html>).

## Introduction

Rice is a staple food crop worldwide and a model organism for the study of monocots (LI *et al.* 2007). The genus *Oryza* contains 21 wild and 2 cultivated species (*O. sativa* and *O. glaberrima*) with 10 genome types (AMMIRAJU *et al.* 2006; JACQUEMIN *et al.* 2013). *O. sativa* is composed of two subspecies: *japonica* and *indica* (HAN and XUE 2003). The *indica* varieties Minghui 63 (MH63) and Zhenshan 97 (ZS97) contain a

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number of important agronomic traits and are the parents of Shanyou 63 which is the most widely cultivated hybrid rice in China (XIE *et al.* 2010). Recombinant inbred line (RIL) populations, derived from a cross between MH63 and ZS97, have made great contributions for identifying and analyzing yield related quantitative trait loci (QTL) (LI *et al.* 2000; XING *et al.* 2002; FAN *et al.* 2006; XUE *et al.* 2008).

Comparative analysis is an important tool for structural, functional and evolutionary genomics studies. Whole genome sequences of *japonica* variety Nipponbare and *indica* variety 93-11 have been released and updated (YU *et al.* 2002; IRGSP 2005; YU *et al.* 2005; GAO *et al.* 2013; KAWAHARA *et al.* 2013), and massive comparisons between these two genomes have been carried out to reveal the genetic variations (HAN and XUE 2003; FELTUS *et al.* 2004; MA and BENNETZEN 2004; YU *et al.* 2005; DING *et al.* 2007; HUANG *et al.* 2008). The *Oryza* Map Alignment Project (OMAP) has constructed bacterial artificial chromosome (BAC) libraries and BAC-based physical maps for 17 of 23 *Oryza* species representing all the 10 genome types (AMMIRAJU *et al.* 2006; KIM *et al.* 2008; AMMIRAJU *et al.* 2010b; JACQUEMIN *et al.* 2013). These BAC-based resources make whole or targeted genomic comparisons available and shed light on the genomic variation and evolution (KIM *et al.* 2007; ZHANG *et al.* 2007; AMMIRAJU *et al.* 2008; FENG *et al.* 2009; LU *et al.* 2009; AMMIRAJU *et al.* 2010a; HURWITZ *et al.* 2010). With the development of next generation sequencing technology, it is now cost effective and possible to extensively

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detect small nucleotide variations, such as SNP and indels (HUANG *et al.* 2010; HUANG *et al.* 2012).

Although many studies have been made for comparisons within *O. sativa*, those comparative genomics studies were mainly focused on the small nucleotide variations. Genome-wide comparisons at the structural level (such as expansion/contraction, inversion etc.) were limited due to the lack of suitable resources. Previously we made a physical mapping comparison of two *japonica* varieties, Nipponbare and Zhonghua 11 (ZH11), that revealed substantial variations (SNP, indels and expansions/contractions) (LIN *et al.* 2012). Subsequently we constructed a BAC-based physical map of 93-11 that provided a resource for completion of the 93-11 reference sequence and robust inter-subspecies comparisons (PAN *et al.* 2013). Although the varieties in both subspecies display high genetic diversity, especially in *indica* groups (GARRIS *et al.* 2005; HUANG *et al.* 2012), to date, no suitable genomic resources of other *indica* varieties are available to carry out intra-subspecies comparisons with 93-11 and differentiate the real inter-subspecies variations from the variety-specific variations between 93-11 and Nipponbare.

MH63 and ZS97 are typical *indica* varieties and important materials in functional genomics studies. Here we report the generation and application of the BAC libraries, BESs and BAC-based physical maps of MH63 and ZS97. With these new resources, we detected inter-subspecies BAC-sized and contig-sized structural variations by

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comparing these two physical maps with the Nipponbare reference sequence, and inter-subspecies and intra-*indica* subspecies polymorphic simple sequence repeats (SSRs), SNPs, MNPs, indels by comparing these two BES datasets with the Nipponbare and 93-11 reference sequences. Using the physical maps of *O. glaberrima* (Accession number: IRGC 96717), *Oryza rufipogon* (OR105491) (Accession number: IRGC 105491), *Oryza rufipogon* (OR106424) (Accession number IRGC 106424) and *Oryza nivara* (Accession number: IRGC 100897, W0106) from OMAP (<http://www.omap.org>) as control, we identified many conserved and divergent regions related to the evolution and domestication processes. Both the comparative physical maps and the GBrowse, which integrated the QTL and molecular markers from GRAMENE (<http://www.gramene.org>) with our physical maps and analysis results, are open to public through our website (<http://gresource.hzau.edu.cn/resource/resource.html>).

## Materials and Methods

### **BAC library construction, end sequencing, fingerprinting and contig assembly**

The BAC libraries of MH63 and ZS97 were constructed as previously described. (LUO *et al.* 2001; LUO and WING 2003; WANG *et al.* 2013). The Megabase genomic DNA was isolated from young seedlings. The vector, pAGIBAC1 with *Hind*III, was

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prepared as previously described (LUO *et al.* 2006). The *E coli* DH10B T1-resistant competent cells were used as host, and the clones were arranged into 384-well plates. Copies of the two BAC libraries were stored at both the Arizona Genomics Institute ([www.genome.arizona.edu](http://www.genome.arizona.edu)) and the Genome Resource Laboratory of Huazhong Agricultural University (<http://gresource.hzau.edu.cn>). For insert sizing, 220 clones of each library were randomly selected, and the plasmids were restricted with *NotI* and analyzed on 1% agarose CHEF gels (Bio-Rad) with a 5–15 sec linear ramp time at 6 V/cm and 14 °C in 0.5X TBE buffer for 16 h. BAC end sequencing and fingerprinting were performed following previous protocols (LUO *et al.* 2003; KIM *et al.* 2007; LIN *et al.* 2012; WANG *et al.* 2013). BAC end sequences (BESs) with a quality score less than Phred 16 or total length less than 100 bp were removed. All of the BESs are deposited in GenBank [KG702200-KG737748 for MH63 and KG737749-KG771717 for ZS97]. The clones which produced fewer than 25 or more than 180 fingerprint bands were excluded in contig assembly. The clones were assembled into contigs with the program FPC (SODERLUND *et al.* 2000).

### **Contig alignment and manual editing**

The Os-Nipponbare-Reference-IRGSP-1.0 and updated 93-11 whole genome sequences were used as reference sequences (GAO *et al.* 2013; KAWAHARA *et al.* 2013). The physical maps of MH63 and ZS97 were aligned to reference sequences

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and displayed by SyMAP (SODERLUND *et al.* 2006). The contigs and alignments were manually edited and improved against the Nipponbare reference sequence as described by Kim *et al.* (KIM *et al.* 2007) and Lin *et al.* (LIN *et al.* 2012).

### **Comparative analysis of physical maps and integration of resources**

The BAC-sized structural variations were detected as described by Hurwitz *et al.* (HURWITZ *et al.* 2010) with some modifications. We used a more conservative cutoff value (insert size range: 75-190 Kb for MH63 and ZS97, 70-190 Kb for 93-11) to eliminate false positives. Also, we added the following criteria to acquire more precise results: 1) the expansion/contraction clones, with the differences of less than 20 Kb between the length on contig according to average CB (consensus band) size and its hit length on reference sequence, were excluded; 2) The hit location of a discordant clone on the reference sequence was restricted to within the hit location of the corresponding contig on reference sequence (defined by SyMAP on comparative physical maps). The two independent alignments (BESs alignment and SyMAP alignment) ensured that the BES could be mapped to the correct location on reference sequence; 3) The clones whose hit regions spanned the gaps on the reference sequence were removed from the results considering the imprecise sizes of the gaps; 4) The latest edition Os-Nipponbare-Reference-IRGSP-1.0 (KAWAHARA *et al.* 2013) was used for the Nipponbare reference sequence. The clones selected for expansion/contraction verifications were digested by *NotI* and separated on CHEF

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gels. The reference sequences of Maize, Sorghum and *Brachypodium distachyon* were downloaded from NCBI database (<http://www.ncbi.nlm.nih.gov>), and the contigs of MH63 and ZS97 were aligned to these reference sequences by SyMAP (SODERLUND *et al.* 2006). GBrowse 2.0 (STEIN *et al.* 2002) was used to integrate the genomic data.

### **BES analysis**

SSRs in the BESs were identified by SciRoko3.4 (KOFLEER *et al.* 2007), with the criteria of a minimum repeat number of 3 and a minimum total length of 15 bp. The primers for SSR were designed by primer3 (ROZEN and SKALETSKY 2000). Bowtie2 (LANGMEAD and SALZBERG 2012) was used to map the SSR primers to reference sequences, and the primers which could be mapped to their original locations in BESs were considered as effective ones. If the two regions in reference sequence and BES, selected by a pair of SSR primers, contained the same SSR motif but had a different length, then the SSR locus was defined as a polymorphism between the reference sequences and BES. To verify the SSR loci, a total of 21 pairs of primers were randomly selected from the above Primer3-designed primers and used for PCR with the MH63 and ZS97 genomic DNA as templates. Before performing the comparisons of SSR contents and distributions, the BESs of each species were clustered by CAP3 (HUANG and MADAN 1999) to reduce the redundancy.

The known repetitive sequences (homologous with the identified rice repetitive

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sequences) within BESs were identified by RepeatMasker (<http://repeatmasker.org>) using the Rebase of rice (JURKA *et al.* 2005) combined with the rice repetitive sequences downloaded from the Michigan State University Rice Genome Annotation Project (MSU: [ftp://ftp.plantbiology.msu.edu/pub/data/TIGR\\_Plant\\_Repeats/](ftp://ftp.plantbiology.msu.edu/pub/data/TIGR_Plant_Repeats/)). As described by Lin *et al.* (LIN *et al.* 2012) and Aggarwal *et al.* (AGGARWAL *et al.* 2009), we manually detected the variety-specific repeat sequences in MH63 and ZS97 genomes, and annotated them with Blast2GO (GOTZ *et al.* 2008). The BESs and physical maps of wild rice were downloaded from OMAP (<http://www.omap.org>). The small variations (SNP, MNP, insertion and deletion) between the repeat/organelle sequences-masked BESs and reference sequences were detected with the method and standard of Feltus *et al.* (FELTUS *et al.* 2004).

## Results

### **Generation of MH63 and ZS97 BAC libraries, BAC end sequences and fingerprints**

To obtain basic genomic resources of MH63 and ZS97, we constructed high quality and deep coverage BAC libraries for both varieties. Each library contained 36,864 clones arrayed in 96 384-well plates, and the average insert sizes for the MH63 and ZS97 libraries were 125 Kb and 117 Kb, covering ~10.7-fold and ~10.0-fold genome,

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respectively, based on the 430 Mb genome size.

The 18,432 clones from the first 48 384-well plates of each library were end-sequenced bi-directionally and fingerprinted. After quality trimming, 35,549 MH63 BESs with an average length of 659 bp and a cumulative length of 23,421,124 bp, and 33,969 ZS97 BESs with an average length of 666 bp and a cumulative length of 22,623,600 bp were successfully generated. In terms of fingerprinting, we successfully generated 17,310 BAC fingerprints for MH63 with an average CB unit size of 1,078 bp, and 16,580 BAC fingerprints for ZS97 with an average CB unit size of 1,114 bp (Table S1).

### **Construction of the MH63 and ZS97 physical maps**

With the tolerance of 4 and the cutoff of  $10^{-25}$ , 16,580 and 12,965 clones were assembled into 1,230 and 1,929 contigs, and 730 and 3,615 clones were left as singletons for MH63 and ZS97, respectively. Based on the average CB unit size, the total and average contig sizes are 353,085 Kb and 287 Kb, and 393,765 Kb and 204 Kb for MH63 and ZS97, respectively (Table S1). The primary FPC assembly for each map was referred to as phase I physical map.

The two phase I physical maps were manually edited referring to the Nipponbare reference sequence. The resulting phase II physical maps of MH63 and ZS97

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consisted of 574 and 1,228 contigs, containing 16,735 and 13,972 clones, and leave 575 and 2608 clones as singletons, respectively. The total and average contig sizes are 304,227 Kb and 530 Kb for MH63, and 353,177 Kb and 288 Kb for ZS97. In contigs, a total of 15,823 clones for MH63 and 12,118 clones for ZS97 have paired-end sequences that are invaluable resources for comparative genomics (Table S1). The phase II physical maps of MH63 and ZS97 could be downloaded from our website.

### **Comparison of physical maps with the rice reference sequences**

**Genome-wide BAC-sized structural variations:** The genome structural variations could be detected based on the information of paired-end BES hits and the physical maps (HURWITZ *et al.* 2010). We aligned 9,563 clones of MH63, 7,243 clones of ZS97 and 15,142 clones of 93-11 to the Nipponbare reference sequence through BESs. In Nipponbare, 33 contractions (83 clones) and 140 expansions (464 clones), 69 contractions (201 clones) and 31 expansions (84 clones), and 46 contractions (124 clones) and 180 expansions (726 clones) were identified when MH63, ZS97 and 93-11 BAC clones were aligned to Nipponbare reference sequence, respectively (Table 1). The structural variations were widely dispersed across the Nipponbare genome, except for the middle region of chromosome 5 in which no expansion was found (Figure 1). The detailed information of structural variations was shown in File S1.

Table 1 Computationally detected structural variations in Nipponbare relative to three *indica* varieties

species	Total BESs	Total clones with paired-end BESs	Total BES hits <sup>a</sup>	Clones aligned with paired-end BES hits	Clones aligned and in FPC contig (with paired-end BES hits)	Structural variations in the Nipponbare reference sequence					
						Contraction		Expansion		Inversion	
						Total clones	Cluster (clones in cluster)	Total clones	Cluster (clones in cluster)	Total clones	Cluster (clones in cluster)
MH63	35550	17336	26796	10332	15185 (9563)	402	33 (83)	983	140 (464)	199	18 (45)
ZS97	33970	15953	24965	9256	12223 (7243)	623	69 (201)	393	31 (84)	171	18 (44)
93-11	65140	29315	47716	16906	27244 (15142)	481	46 (124)	1219	180 (726)	551	50 (143)

<sup>a</sup>The BESs were aligned to reference sequences by blat and processed by PslReps program. The matches were removed if the hit had gap larger than 40 bp, less than 90% coverage, or map to three or more locations.

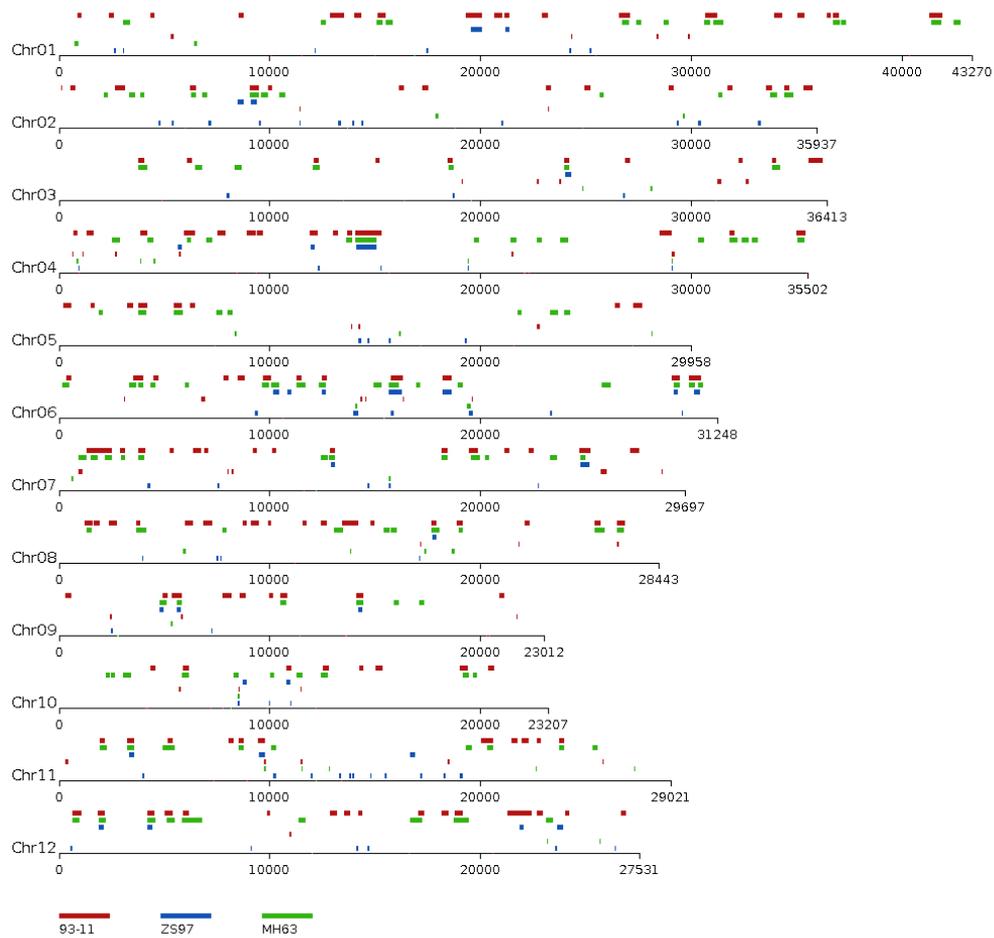


Figure 1 The expansions and contractions in *japonica* variety Nipponbare detected by the genome-wide comparisons with *indica* varieties MH63, ZS97 and 93-11. For each chromosome, lines 1-3 from top to bottom show expansions, and lines 4-6 show contractions.

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To validate the putative expansions and contractions, we compared the hit distance of paired-end BESs on the Nipponbare reference sequence with the actual size of each BAC clone measured on CHEF gel. A total of 186 MH63 clones (from 14 contractions and 50 expansions) and 77 ZS97 clones (from 12 contractions and 18 expansions) were randomly selected and experimentally analyzed. The differences between the actual sizes of all clones and their hit lengths on Nipponbare were confirmed and were more than 20 Kb, except for the 4 clones of one MH63 contraction (File S2).

The regions, which were identified as expansions in Nipponbare genome relative to one or more of the three *indica* varieties, have higher than average repeat contents (48% in expansions vs. 42.73% in the whole genome). The repeats in the expansion regions were mainly LTR Gypsy/DIRS1 (55.98% of expansion repeats vs. 48.98% of whole genome repeats). However, the ratio of DNA transposons in expansion (29.40% of expansion repeats) was lower than average of the whole genome (34.64% of whole genome repeats). It is interesting that the gene density of the expansion regions is approximately equal to the value of the whole genome. By analyzing the GO terms in these expansions, we found a statistically significant ( $P < 0.05$ ) over-representation of genes related to response to stimulus in biological processes, especially the genes related to response to stress and abiotic stimulus. We also found an over-representation of GO terms of macromolecule biosynthetic process, gene expression and photosynthesis of metabolic process, under biological processes. The

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nucleic acid binding and transcription regulator activity categories under molecular function and the nitrogen compound metabolic process under biological processes were found to be under-represented (File S3). A total of 14 expansion regions and 1 contraction region were shared when the three *indica* varieties were compared with the Nipponbare reference sequence. Of the above shared expansion regions, most were also shared in comparisons of *ORI05491*, *ORI06424* and *O. nivara* to the Nipponbare reference sequence (File S4). In these shared expansion regions, 54.93% of the sequences were classified as repeat elements, in which the ratio of Gypsy/DIRS1 was 58.40% and that of DNA transposons was 25.69%, although the gene density was still approximately equal to the value of the whole genome.

**Genome-wide contig-sized structural variations:** We constructed the inter-subspecies comparative physical maps by aligning the MH63 and ZS97 physical map contigs onto the Nipponbare reference sequence using the BESs embedded in the contigs as bridges (Figure 2). The anchored contigs accounted for 85% and 75% of the total contigs, and the percentage of CB units of anchored contigs to total contigs was 95% and 86% in the comparisons of MH63 and ZS97 to the Nipponbare reference sequence, respectively. A total of 16,356 MH63 clones and 12,845 ZS97 clones were anchored to reference sequence, of which 13,456 and 10,654 clones were directly anchored by BES hits (6,258 and 4,707 clones with paired-end BES hits), respectively (Table 2). The unanchored contigs either were unique regions in MH63

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and ZS97 genomes, relative to reference sequences, or corresponded to the gap regions of reference sequence. We also aligned the MH63 and ZS97 physical map contigs onto the 93-11 reference sequence. However, considering that the 93-11 reference sequence contains many gaps and assembly errors (PAN *et al.* 2013) that would affect the outputs of the comparisons, we only displayed the comparative results on our website, and did not describe them here.

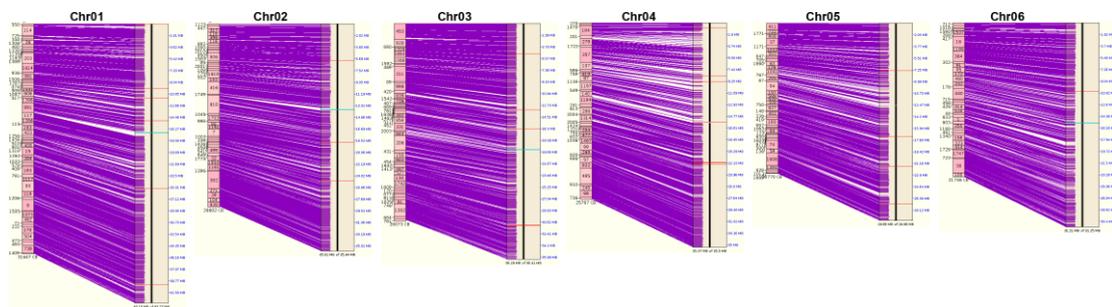
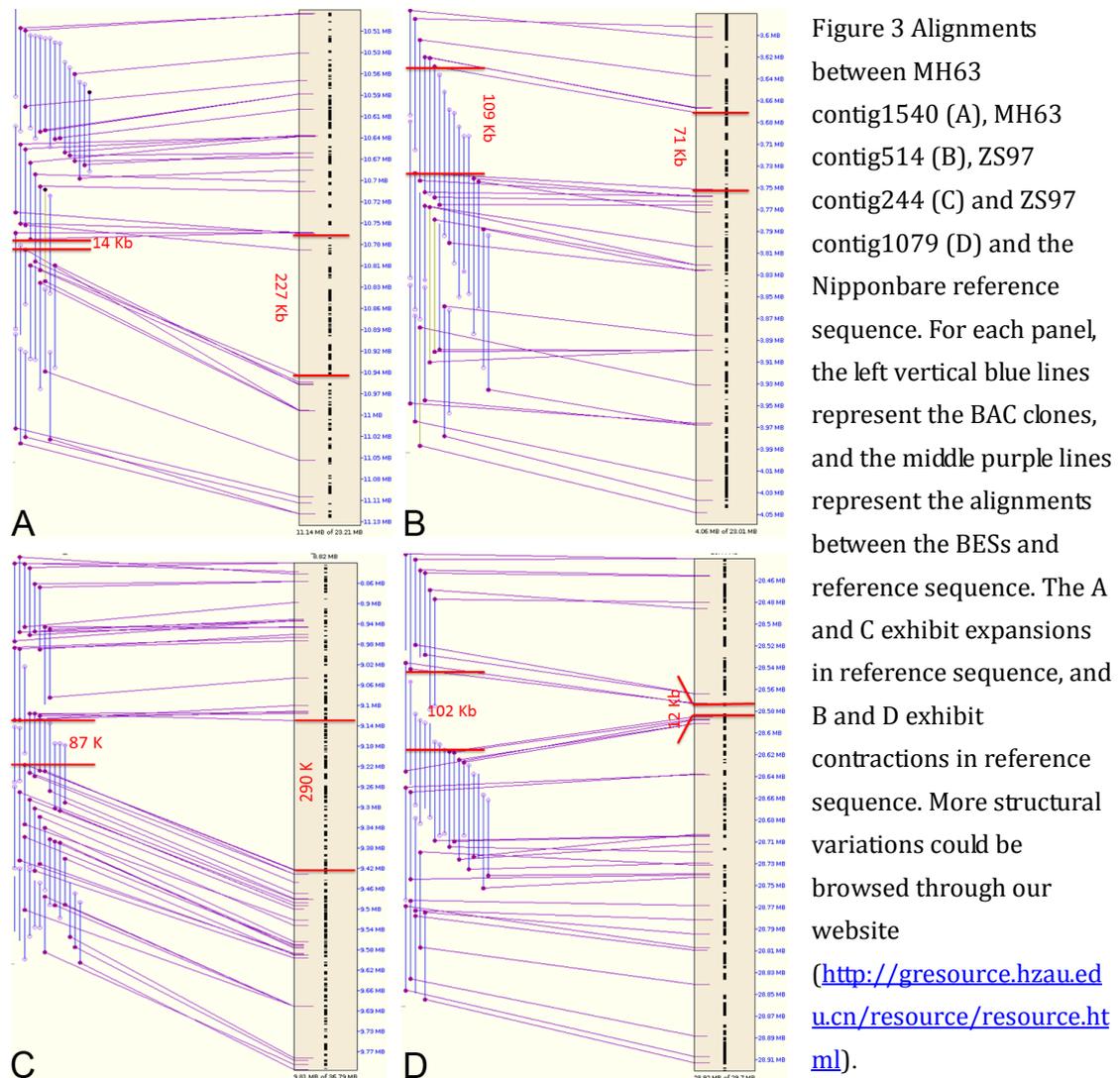


Figure 2 The SyMAP alignments of the MH63 Phase II physical contigs to the Nipponbare reference sequence Chr1-Chr6. For each panel, the left boxes represent the contigs of MH63 and the right bar represents the Nipponbare chromosome. The green and red lines on the Nipponbare chromosomes indicate the centromere and gap locations, and the vertical black lines represent the gene locations. The centromere locations of chromosome 4 and 5 were not available. The purple lines represent the alignments between BESs and reference sequences. The alignments for the remaining six chromosomes are available through our website (<http://gresource.hzau.edu.cn/resource/resource.html>).

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In anchored contigs, the single-end BES hits were as invaluable resources as paired-end BES hits for detecting the structural variations. Based on the CB unit size and distance of BES hits, the expansions and contractions could be visualized by non-parallel alignment lines on comparative physical maps (LIN *et al.* 2012). Figure 3 presented two expansions and two contractions on the Nipponbare reference sequence relative to MH63 and ZS97 contigs. The estimated sizes of the two expansion regions were 213 Kb and 203 Kb, and the two contraction regions were 38 Kb and 90 Kb, respectively. A user friendly visualization of the expansions/contractions, with zoom features, may be browsed from our website. We also found many inversely matched BESs in the two alignment projects. Using the alignment of MH63 contigs to the Nipponbare reference sequence as example, we detected 1,225 inversely matched BESs, and 705 were clustered into 260 groups of 2 or more BES hits (Table 2). The clusters of inversely matched BESs on comparative maps (viewed as cross alignment lines) may reflect genetic or spurious inversions (LIN *et al.* 2012; DENG *et al.* 2013; PAN *et al.* 2013). Figure 4 illustrated a 6,844 bp region on the Nipponbare reference sequence (Chr5:3550117-3556961) inversely matched by 5 BESs of MH63 contig37. Because the same region was normally matched by BESs of Nipponbare physical contig41 (downloaded from OMAP), and MH63 contig37 has a high quality (with deep coverage and high stringency), this region could most probably contain a genetic inversion between MH63 and Nipponbare. We did PCR experiments to verify this

speculation and the results showed that the OSIABa0011B13.r was on the left of the OSIABa0041A04.r in the MH63 contig37. Because the hit location of the OSIABa0011B13.r on the Nipponbare reference sequence was on the right of the hit location of the OSIABa0041A04.r, the genetic inversion between MH63 and Nipponbare was confirmed (Figure 4). All detailed information of the inversely matched BESs has been listed in File S5.



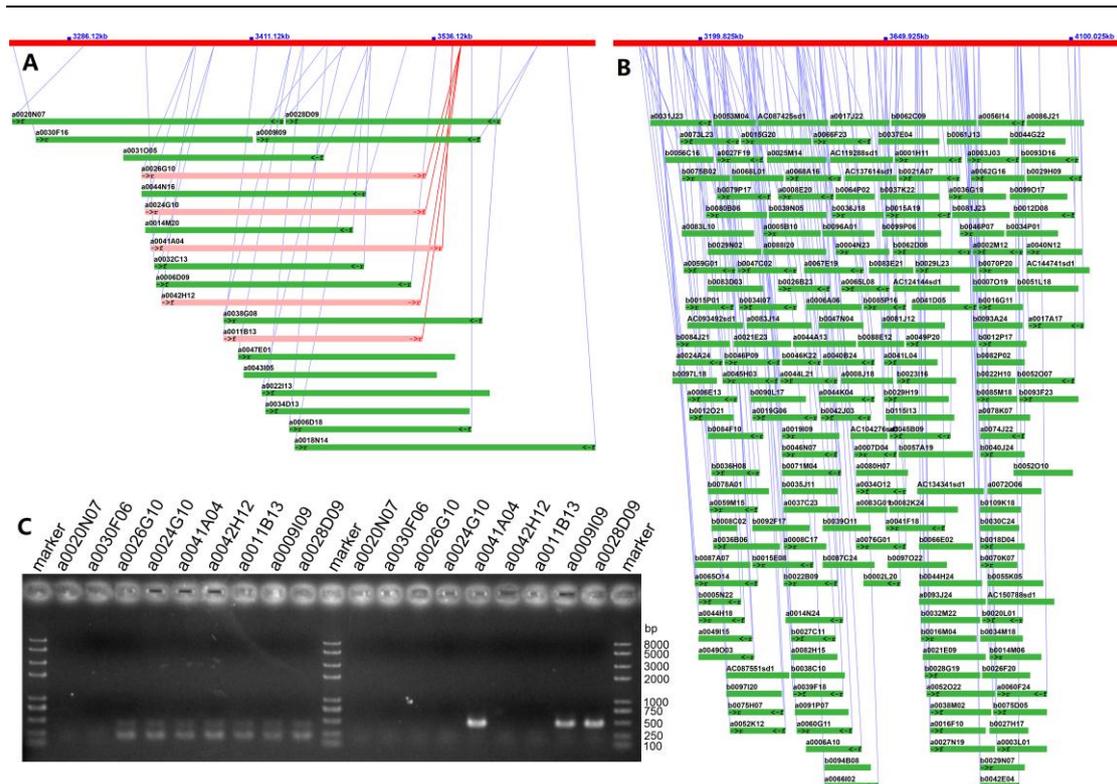


Figure 4 Discovery and confirmation of the inversions by BES alignments. (A) The alignments between the BESs of partial MH63 contig37 and the Nipponbare reference sequence. (B) The alignments between the BESs of the Nipponbare contig41 and the same region of the Nipponbare reference sequence. The top red bars represent the Nipponbare reference sequence, and the blue lines represent the alignments of BESs to reference sequence. The green bars represent the normally matched clones and the pink bars represent the inversely matched clones. (C) Experimental validation of the genetic inversion. In the left panel, the pair of primers was designed from the BES OSIABa0011B13.r. of the clone a0011B13 in the MH63 contig37. In the right panel, the pair of primers was designed from the BES OSIABa0041A04.r. of the clone a0041A04 in the MH63 contig37.

Table 2 Summary of the SyMAP alignments between phase II physical maps of MH63 and ZS97 and the Nipponbare reference sequence

Catalog	MH63	ZS97
Anchored contigs with one placements	489	925
Percentage to total contigs	85%	75%
Clones in anchored contigs	16356	12845
With paired-end BESs	15477	11178
With single-end BESs	758	1415
Total BESs with hits	19714	15361
Percentage to total BESs	55.5%	45.2%
with inversely matched BES hits	1225	1589
Hits in cluster (cluster number)	705 (260)	949 (348)
Clones anchored by BESs	13456	10654
With paired-end BES hits	6258	4707
With single-end BES hits	7198	5947
Total CB units of anchored contigs	268620	273316
Percentage to total CB units	95%	86%
Total length of anchored contigs (Kb)	289572	304474
Covered length of reference sequence (Kb)	330350 (88%)	285800 (76%)
Unanchored contigs	85	303
Clones in unanchored contigs	379	1127
With paired-end BESs	347	940
With single-end BESs	22	154
Total CB units of unanchored contigs	13641	43719
Percentage to total CB units	5%	14%
Total length of unanchored contigs (Kb)	14705	48703

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### **Contigs spanning the remaining physical gaps of the Nipponbare reference**

**sequence:** Though the Nipponbare reference sequence is well known for its high quality, it still contains 44 physical gaps in the latest version (KAWAHARA *et al.* 2013).

Of these, a total of 41 physical gaps can be spanned by contigs from one to all of our four physical maps (ZH11, 93-11, MH63 and ZS97 physical maps). One gap only could be spanned by MH63 contig and two gaps only could be spanned by ZS97 contigs. The BAC sequences of the gap-corresponding contigs could provide an initial insight into the contents of the gaps and help to close these gaps. Three gaps cannot be spanned by any contigs of the four physical maps (File S6). These three gap-corresponding regions may have complex structures and seem to be conserved in *O. sativa*. Of these three gaps, the seventh physical gap on chromosome 4 also cannot be spanned by any physical map contigs of three ancestors of *O. sativa* (*O. nivara*, *ORI05491* and *ORI06424*). However, this gap can be spanned by a physical map contig of *O. glaberrima* which has a farther evolutionary distance with the *O. sativa* and the above three mentioned *O. sativa* ancestors (KOVACH *et al.* 2007; HUANG *et al.* 2012). This result indicates that this gap-corresponding region was conserved in evolution and domestication of *O. sativa*. The other two gaps (the sixth physical gap on chromosome 3 and the eighth physical gap on chromosome 4) can be spanned by physical map contigs of all the three wild rice except for the sixth physical gap on chromosome 3 that cannot be spanned by the physical map contig of *ORI06424*.

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## Analyses of MH63 and ZS97 BESs

**Simple sequence repeats in BESs:** SSRs are effective and robust molecular markers in genetic and genomic analyses. We identified 3,781 SSR loci and designed 3,157 pairs of primers from 3,165 MH63 BESs, and 3,750 SSR loci and designed 3,163 pairs of primers from 3,129 ZS97 BESs. After mapping these primers to the Nipponbare and 93-11 reference sequences, we identified 415 (18%) and 383 (15.9%) polymorphic SSR loci from MH63, and 409 (17.3%) and 325 (12.9%) polymorphic SSR loci from ZS97, respectively (File S7). Ten pairs of MH63 and 11 pairs of ZS97 primers were randomly selected to experimentally verify the SSR loci and all of these SSR loci were confirmed (File S8). The SSRs from BESs of *japonica* variety ZH11, *O. glaberrima*, *OR105491*, *OR106424* and *O. nivara* were also identified, and the ratio of polymorphic SSRs to the Nipponbare and 93-11 reference sequences were 6.7% and 20.6%, 20.9% and 26%, 19.8% and 22.3%, 19.5% and 22.6%, and 20.7% and 22.8%, respectively (File S7). The detailed information of SSRs and primers are available from our website.

We compared the SSR contents and distributions among the above analyzed species. The results showed that all of the species have similar SSR densities and motif types. For example, the CCG, AG and AT motifs were predominant in all of the species and accounted for between 27% in *O. glaberrima* and 32% in MH63 (Figure S1). The SSRs composed of AT or AAT motifs showed the most variable repeat length, with

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an average of  $49.5 \pm 46.1$  bp and  $31.6 \pm 27.2$  bp in MH63, and  $56.9 \pm 63$  bp and  $34 \pm 24.5$  bp in ZS97. Similar to the results for wild rice (KIM *et al.* 2008), A/T rich di-nucleotide and tri-nucleotide repeat motifs have longer average and variable repeat lengths in cultivated rice than other motifs.

**Repeat sequences in BESs:** A total of 8,781,060 bp (37.49%) of MH63 BESs and 8,936,689 bp (39.5%) of ZS97 BESs were homologous with the repeat sequences deposited in databases. In terms of repeat category, the retroelement Gypsy/DIRS1 was most common in these two species, comprising 19.93% and 20.95% of total BESs of MH63 and ZS97, respectively (Table S2).

To search variety-specific repeat sequences and reveal their functions, the known repeat/organelle sequences-masked BESs were self-blasted and the high score pairings (HSPs) sequences were further analyzed (AGGARWAL *et al.* 2009; LIN *et al.* 2012). A total of 119 sequences (49,197 bp) in MH63 BESs and 84 sequences (34,813 bp) in ZS97 had at least six HSPs in BESs (25.62 presentation times in 100 Mb MH63 sequence and 26.52 presentation times in 100 Mb ZS97 sequence) and were identified as new repeat sequences by this method. Of these sequences, 38 from MH63 and 29 from ZS97 were considered as specific repeats in *indica* varieties that have less than or equal to five HSPs in whole Nipponbare reference sequence. Under this standard, a total of 12 sequences from MH63 and 15 sequences from ZS97 were regarded as MH63- and ZS97-specific repeats, respectively. Of the specific repeats in *indica*

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varieties, 22 sequences from MH63 and 18 sequences from ZS97 contained coding regions, most of which were annotated as proteins containing the NBS-LRR domain, a typical domain related to disease-resistance in plants. This result indicates that repeat sequences may play an important role in the evolution process of disease-resistance related genes. The BESs of other *Oryza* species from OMAP were also used to estimate the frequencies of these specific sequences in wild rice (the presentation times per every 100 Mb BESs was used because the wild rice have different number of BESs and genome size), and the result indicates that most of the specific sequences have high frequencies in *OR105491*, *OR106424*, *O. nivara* and *O. glaberrima*, which have closer distances with *O. sativa* (MA and BENNETZEN 2004; KOVACH *et al.* 2007) (File S9).

**Substitutions and indels between BESs and the reference sequences:** To identify small genetic variations, we compared the repeat-masked BESs of MH63 and ZS97 with their orthologous regions in Nipponbare and 93-11 genomes. A total of 46,349 SNPs, 1,007 MNPs, 3,806 insertions and 5,134 deletions were identified by the comparisons of MH63 and ZS97 BESs to the Nipponbare reference sequence, and 49.16% of SNPs, 44.59% of MNPs, 39.99% of insertions and 45.62% deletions were located in Nipponbare gene regions. A total of 25,034 SNPs, 760 MNPs, 2,534 insertions and 4,003 deletions were identified by the comparisons of MH63 and ZS97 BESs to the 93-11 reference sequence, and 34.35% of SNPs, 27.5% of MNPs, 29.08%

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of insertions and 35.12% deletions were located in 93-11 gene regions (File S10). Compared to other variation types, the SNP, followed by the deletion has the highest frequency of occurring in exon regions. The BES regions encompassed by the 21 pairs of primers, which were used to experimentally verify the SSR loci above, contained 14 SNPs, 5 insertions and 8 deletions. All these variations were confirmed by sequencing of the PCR products (File S8). The masked BESs of wild species were also used to identify the genetic variations (File S10). The detailed variations are available from our website.

In agreement with the findings of Nasu *et al.* and Feltus *et al.* (NASU *et al.* 2002; FELTUS *et al.* 2004), the SNPs that we found were not evenly distributed across the Nipponbare chromosomes, and many low and high SNP frequency regions could be observed which represent the low and high divergent regions (Figure 5). The average SNP frequencies of each Nipponbare chromosome were obviously different and chromosome 4 had the highest SNP frequency (Table S3). A total of 3,943 SNPs and 75 MNPs were shared when the three *indica* varieties were compared with the Nipponbare reference sequence, and these variations were putative inter-subspecies variations. In these variations, 1,111 (28.18%) SNPs and 10 MNPs were located in exon regions, 949 SNPs and 22 MNPs in intron regions and 490 SNPs and 23 MNPs in 1 Kb upstream regions of genes. Of the 3,943 SNPs and 75 MNPs shared by the three *indica* varieties, the 1,668 SNP and 32 MNP loci were also shared in comparison

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between *O. nivara* and Nipponbare, and the 1,230 SNP and 21 MNP loci in comparison between *OR106424* and Nipponbare. The results indicate that these variations may have occurred in Nipponbare or represent the genetic diversity between the ancestors of the *japonica* and *indica*.

### **Resource integration and application**

To facilitate functional and comparative genomics studies, we integrated physical maps (MH63, ZS97, 93-11, ZH11, *O. nivara*, *OR105491*, *OR106424* and *O. glaberrima*) and analysis results with the information of QTL and molecular markers from GRAMENE into GBrowse. The contigs, clones, SNPs, MNPs, indels and polymorphic SSRs were linked with molecular markers, QTL and gene models, and were aligned to the Nipponbare reference sequence. The discordantly matched clones, from the cultivated and wild rice, visualized the high divergent regions in the Nipponbare genome and the regions corresponding to the discordantly matched clones could be investigated. According to the high collinearity in grasses, we aligned the above eight physical maps to the genome sequences of Maize, Sorghum and *Brachypodium distachyon* for searching the collinear regions and carrying out comparative genomic studies. The GBrowse and comparative physical maps are available to the public at our website.

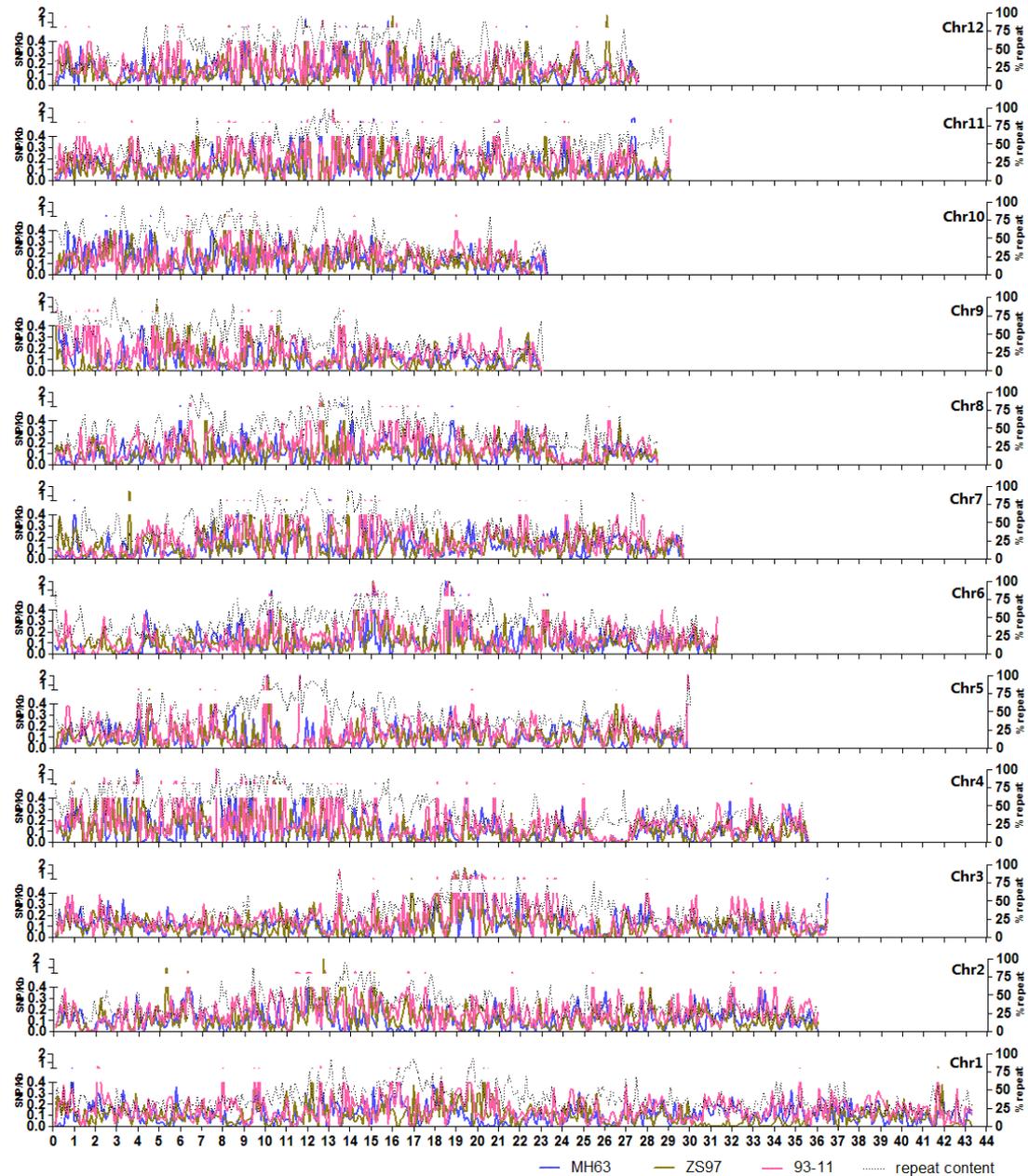


Figure 5 SNP distributions across the Nipponbare chromosomes. The SNPs were identified from the comparisons of repeat-masked BESs of three *indica* varieties: MH63, ZS97 and 93-11 with the Nipponbare reference sequence. The frequency was defined as the number of SNPs divided by the length of repeat-masked reference sequence within 100 Kb window. The x-axis shows the chromosome position in which each unit is a megabase. The left y-axis shows SNP frequency. The right y-axis shows the percentage of repeat sequences in 100-kb window (dotted black lines).

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## Discussion

The *O. sativa* ssp. *indica* cv. MH63 line, which was bred from a cross between IR30 and Gui630, is an elite restoring line and has the following characters: strong ability and broad spectrum in restoration, resistance to rice blast and high production yields. The *indica* cv. ZS97 is one of the most widely used sterile lines. Together, MH63 and ZS97 are the parents of Shanyou 63 which is the most widely cultivated hybrid rice in China (XIE *et al.* 2010). Based on the RIL populations of MH63 and ZS97, Xing *et al.* constructed a genetic linkage map (XING *et al.* 2002) and Xie *et al.* constructed an ultra high-density linkage map (XIE *et al.* 2010). With these RIL populations as materials, many important agronomic trait-related genes and QTL's have been identified and analyzed, such as *GS3* (FAN *et al.* 2006; MAO *et al.* 2010), *Ghd7* (XUE *et al.* 2008), and *Xa26* (SUN *et al.* 2004). The integrated genomic and genetic resources reported here provided a platform for identifying and analyzing other important agronomic traits possessed by the two varieties.

Genome-wide comparative analysis at the structural level would facilitate the understanding of the evolution and domestication processes of cultivated rice. In other model systems, analyses of genome-wide structural variations have proven to be an effective strategy for uncovering the evolution process and relating the genetic diversity to the given phenotypes (ZHAO *et al.* 2004; TUZUN *et al.* 2005; KIDD *et al.* 2008; NICHOLAS *et al.* 2009; SPRINGER *et al.* 2009; ZHANG *et al.* 2011). The BAC

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libraries, BESs, physical maps of MH63 and ZS97, and the integrated information provided here are invaluable resources for inter- and intra-subspecies genome-wide comparative analysis in *O. sativa*.

The studies from OMAP shed light on the genome-wide structural variations between Nipponbare and wild rice species (KIM *et al.* 2007; KIM *et al.* 2008; HURWITZ *et al.* 2010). Previously we constructed the physical maps of ZH11 and 93-11, and compared them with the Nipponbare and 93-11 reference sequences (LIN *et al.* 2012; PAN *et al.* 2013). Here we identified BAC-sized and contig-sized structural variations in *O. sativa* by comparisons of the physical maps of MH63 and ZS97 to the Nipponbare reference sequence. The shared structural variations between the Nipponbare genome and the three *indica* varieties were putative inter-subspecies genetic diversities that may have occurred before divergence of these *indica* varieties. Huang *et al.* provided the model that *japonica* rice was first domesticated and then *indica* rice was generated from the cross between *japonica* and wild rice (HUANG *et al.* 2012). Among the above shared regions, some could be conserved between *indica* group and its wild ancestors (the regions which were also shared in one or more of three relative wild species) and not interrupted by the gene introgression of early *japonica*, whereas others could be diverged immediately after the cross between *japonica* and wild rice (the regions which were not shared with wild species).

Transposable elements (TEs) are one of the major contributing genomic features that

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relate to an organism's genome size and its course of evolution (MA and BENNETZEN 2004). In our analysis, the expansion regions contained more repeat content, especially the LTR element Gypsy/DIRS1, but the gene density in these regions was approximately the same as the average value of the whole genome. By skimming the gene annotations, we observed a number of LTR retrotransposon-related genes in the expansion regions and a statistically significant difference of GO term representations, such as the genes related with response to stress and abiotic stimulus. Most of the unique repeat sequences in the *indica* group contained the motif of NBS-LRR, which has been reported to be a typical domain related with disease resistance. These results further demonstrate that TEs, especially LTR retrotransposons, may contribute largely to gene formation, genome evolution, genetic diversity and asymmetry in *O. sativa*.

Our results exhibited that the Nipponbare reference sequence contained more expansions than contractions of BAC- and contig-sizes relative to MH63 and 93-11 physical maps. Han *et al.* (HAN and XUE 2003) and Ma *et al.* (MA and BENNETZEN 2004) also found more insertions in Nipponbare than in *indica* variety GLA4 and proposed that the Nipponbare genome was expanded relative to *indica*. In *japonica* group, Lin *et al.* reported that the Nipponbare genome is ~7% larger than ZH11. The larger genome size of Nipponbare could be mainly due to the amplification of LTR retrotransposons, especially the Gypsy/DIRS1 elements. However, more contractions of BAC-sized were detected on the Nipponbare reference sequence relative to the

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ZS97 physical map. This could be caused by the smaller average contig size of ZS97 than MH63 and 93-11 physical maps because the structural variations were restricted to contig hit regions.

Small nucleotide variations played an important role in the rice domestication process, (FAN *et al.* 2006; KONISHI *et al.* 2006; LI *et al.* 2006; SWEENEY *et al.* 2006; JIN *et al.* 2008; SHOMURA *et al.* 2008). Feltus *et al.* (FELTUS *et al.* 2004) identified many SNPs and indels between 93-11 and Nipponbare. Xie *et al.* (XIE *et al.* 2010) identified many SNPs by resequencing 238 RILs derived from a cross between MH63 and ZS97, and Huang *et al.* (HUANG *et al.* 2010) identified many SNPs by sequencing 517 rice landraces at low-coverage. Our SNPs, MNPs and indels increased the nucleotide variation densities and provided the materials for identifying and analyzing important agronomic trait-related genes in MH63 and ZS97. Similar to the distribution of SNPs between Nipponbare and 93-11 (FELTUS *et al.* 2004), the SNPs between Nipponbare and MH63 or ZS97 were also not evenly distributed. According to the domestication model of Huang *et al.*, as mentioned above (HUANG *et al.* 2012), the low frequency regions could be from the exchanged regions between *japonica* and the wild rice. The shared variations in *indica* varieties relative to Nipponbare could be *indica*-specific gene candidates, possibly for domestication and adaptation. The repeat-masked BESs of *O. nivara*, *OR106424*, and *OR105491* were also used to identify variations relative to Nipponbare and 93-11, and the shared variations relative to *O. sativa* could be

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putative domestication loci.

In conclusion, we generated the BAC libraries, BESs and physical maps for MH63 and ZS97. Compared with the reference sequences of Nipponbare and 93-11, we detected a number of polymorphic SSRs, SNPs, indels and structural variations. The physical maps, comparative analysis results, molecular markers and QTL from GRAMENE and gene models were integrated and aligned to the Nipponbare genome in Gbrowse. These invaluable resources provide a platform for comparative genomics, positional cloning, whole genome sequencing, functional genomics research and crop improvement in *O. sativa*. Both the comparative physical maps and the GBrowse, are open to public through our website. The BAC-based whole genome sequencing of MH63 and ZS97 are now underway. Once whole genome reference sequences become available, more genetic diversity and important agronomic traits-related genes will be revealed and understood clearly.

### **Acknowledgments**

We thank So-Jeong Lee, Nicholas B. Sisneros, Fusheng Wei, HyeRan Kim, Yeisoo Yu, Jetty S.S. Ammiraju, and other members of the Arizona Genomics Institute for production of the BAC library, fingerprints, and BESs. This work was supported by the National Natural Science Foundation of China (grant no. 30971748) and the Chinese 111 Project (grant no. B07041).

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## Supporting Information

Figure S1: The compositions of the ten most frequent SSR motifs in eight *Oryza* species. The x-axis present the varieties, and the y-axis present the ratio of relative SSRs to total SSRs.

File S1: Detailed information of all expansion and contraction regions in the Nipponbare genome.

File S2: Validation of expansions and contractions.

File S3: Over- and under-representation of gene ontology (GO) terms in the expanded regions of the Nipponbare.

File S4: The shared expansion and contraction regions in the Nipponbare.

File S5: The clusters of inversely matched BESs between the Nipponbare reference sequence and six physical maps (MH63, ZS97, *O.nivara*, *OR105491*, *OR106424* and *O.glaberrima*).

File S6: The spanned gaps on the Nipponbare reference sequence.

File S7: The detailed information of the verified SSRs and variations.

File S8: Statistics of SSR information in BESs of eight *Oryza* species.

File S9: The frequencies of specific repeat sequences in BESs of *Oryza* species.

File S10: Summary of the small variations identified by the comparisons between repeat-masked BESs and reference sequences.

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Table S1: Statistics of the BESs and fingerprinting information.

Table S2: Repeat contents in the BESs of *O. sativa* and related *Oryza* species.

Table S3: The SNP frequencies on each Nipponbare chromosome.