Reference-Guided De Novo Genome Assembly to Dissect a QTL Region for Submergence Tolerance Derived from Ciherang-Sub1

Yuya Liang 1,†, Shichen Wang 2, Chersty L. Harper 3, Nithya K. Subramanian 1, Rodante E. Tabien 3, Charles D. Johnson 2, Julia Bailey-Serres 4 and Endang M. Septiningsih 1,*

1 Department of Soil and Crop Sciences, Texas A&M University, College Station, TX 77843, USA; liangy27@msu.edu (Y.L.); nithya@tamu.edu (N.K.S.)
2 Genomics and Bioinformatics Service, Texas A&M AgriLife Research, College Station, TX 77843, USA; shichen.wang@gmail.com (S.W.); charlie@ag.tamu.edu (C.D.J.)
3 Texas A&M AgriLife Research Center, Beaumont, TX 77713, USA; c-harper@aesrg.tamu.edu (C.L.H.); RETabien@ag.tamu.edu (R.E.T.)
4 Center for Plant Cell Biology, Department of Botany and Plant Sciences, University of California Riverside, Riverside, CA 92521, USA; serres@ucr.edu

* Correspondence: eseptiningsih@tamu.edu
† Present address: Department of Plant Biology, Michigan State University, East Lansing, MI 48823, USA.

Abstract: Global climate change has increased the number of severe flooding events that affect agriculture, including rice production in the U.S. and internationally. Heavy rainfall can cause rice plants to be completely submerged, which can significantly affect grain yield or completely destroy the plants. Recently, a major effect submergence tolerance QTL during the vegetative stage, qSub8.1, which originated from Ciherang-Sub1, was identified in a mapping population derived from a cross between Ciherang-Sub1 and IR10F365. Ciherang-Sub1 was, in turn, derived from a cross between Ciherang and IR64-Sub1. Here, we characterize the qSub8.1 region by analyzing the sequence information of Ciherang-Sub1 and its two parents (Ciherang and IR64-Sub1) and compare the whole genome profile of these varieties with the Nipponbare and Minghui 63 (MH63) reference genomes. The three rice varieties were sequenced with 150 bp pair-end whole-genome shotgun sequencing (Illumina HiSeq4000), followed by performing the Trimmmomatic-SOAPdenovo2-MUMmer3 pipeline for genome assembly, resulting in approximate genome sizes of 354.4, 343.7, and 344.7 Mb, with N50 values of 25.1, 25.4, and 26.1 kb, respectively. The results showed that the Ciherang-Sub1 genome is composed of 59–63% Ciherang, 22–24% of IR64-Sub1, and 15–17% of unknown sources. The genome profile revealed a more detailed genomic composition than previous marker-assisted breeding and showed that the qSub8.1 region is mostly from Ciherang, with some introgressed segments from IR64-Sub1 and currently unknown source(s).

Keywords: submergence; Ciherang-Sub1; genome assembly; genome; rice

1. Introduction

Rice (Oryza sativa L.) is a staple food that feeds nearly half of the world’s population [1]. Complete submergence of rice reduces the availability of carbon dioxide and oxygen, reducing photosynthesis and limiting the aerobic metabolism, which together can dramatically reduce crop productivity [2]. In the U.S., for example, hurricane Harvey brought heavy rain and flooding that caused USD 7.5 million in losses for rice and soybean in Texas in 2017 [3]. Due to climate change, extreme weather events have increased the frequency of severe flooding, which significantly affects agriculture, including rice production in the U.S. and globally. A major submergence tolerance locus, Submergence 1 (Sub1) from the landrace FR13A, was identified and introgressed into several popular rice varieties [4–12]. The key gene of this locus is Sub1A-1, encoding an ethylene-responsive factor (ERF) in
subgroup VII, which is activated by ethylene that accumulates in submerged tissues [13]. The linked Sub1B and Sub1C genes encode the most related ERFs in the genome, indicating this locus arose through tandem duplication [14] prior to domestication [15].

Complete submergence during the vegetative stage is a polygenic trait. Efforts to identify additional resources for submergence tolerance include germplasm screening [16–18], quantitative trait loci (QTL) identification [19–21], and assessment of allelic sequence variation of Sub1A and the related Sub1B and Sub1C within the Sub1 locus [14,15,22]. One of such efforts mapped a major effect submergence tolerance QTL (qSub8.1) on chromosome 8 using recombinant inbred lines derived from a cross between Ciherang-Sub1 and IR10F365 [20]. Both parents possessed the Sub1A-1 gene, which provides about two weeks of tolerance to complete submergence during the vegetative growth phase [4,13]. The qSub8.1 tolerant allele was derived from Ciherang-Sub1, and it accounts for about 28% of the phenotypic variance, with a heritability of 23.3% [20].

The International Rice Research Institute (IRRI) has worked closely with its national partners to introgress Sub1A-1, a submergence tolerance gene contributing the largest effect identified to date, into several popular rice varieties. There were six Sub1 lines developed in the first stage: Swarna-Sub1, Samba Mahsuri-Sub1, IR64-Sub1, TDK1-Sub1, CR1009-Sub1, and BR11-Sub1 [5,6,10]. Another two Sub1 lines (Ciherang-Sub1 and PSB Rc18-Sub1) were developed in the second stage [8]. In addition to providing tolerance to submergence, this gene also enhanced survival of rapid dehydration following desubmergence and water deficit under drought [23]. Previous reports also showed that Sub1 lines were more tolerant to leaf blast and bacterial blight under normal or submerged conditions [24,25]. The level of tolerance of these eight Sub1-lines under submergence stress is affected by genetic backgrounds and environments. Gene-to-gene interactions, including epistasis, may play a role in this case [26], and additional QTL regions may also contribute to the phenotypic variations. However, generally, we could still see the clear differences between the Sub1 lines and their original susceptible parents [5,6,8]. Additionally, thus far, there were no yield penalties reported with the introgression of Sub1 alone or Sub1 combined with other major effect QTL associated with drought tolerance or anaerobic germination [8,27,28].

Many of these lines have been released in various countries, especially in South Asia and South East Asia [7,27].

One of the parents used to map qSub8.1 was Ciherang-Sub1, which was developed from a cross between Ciherang, a popular indica variety from Indonesia [8], and IR64-Sub1 [6] using just one backcross and one selfing generation (derived from a BC1F2 individual plant). After being completely submerged for 25 days, Ciherang-Sub1 had a survival rate of 89.9% compared to a survival rate of 3.7% for Ciherang. Ciherang-Sub1 also had a higher survival rate compared to other Sub1-introgressed rice varieties such as Swarna-Sub1 (77.9%), BR11-Sub1 (79.0%), PSB Rc18-Sub (58.2), and SambaMahsuri-Sub1 (65.8%) [8]. This improved variety has been released in several countries and has also been used in various genetic and molecular studies as an elite cultivar [7,20,28–31].

The second parent used to map qSub8.1 was IR10F365, which is an advanced breeding line with Sub1A introgressed through conventional breeding methods. It was hypothesized that qSub8.1 might have a complementary effect with Sub1A in the event of submergence during the vegetative stage [20]. A variety with prolonged submergence tolerance, beyond what Sub1A provides alone, would be beneficial to maintain rice production when we face the increasing effects of climate change. The objectives of the present study are to (a) characterize the region of qSub8.1 by analyzing sequence information of the three associated cultivars (Ciherang-Sub1, Ciherang, and IR64-Sub1) that facilitates further downstream investigation, and (b) compare the whole genome profile of Ciherang-Sub1 with its parental genome sequences using whole-genome shotgun sequencing and reference-guided de novo genome assembly.
2. Results and Discussion
2.1. Genome Assembly

The Illumina sequencing results produced more than 100 million raw reads in each cultivar, of which more than 93% of the raw read-pairs were of sufficient quality for use in the de novo assembly (Table S1). A total of 102,213,313 reads from the Ciherang-Sub1 genome, corresponding to 15,331,996,950 bp, were generated, representing 38X sequencing depth and covering 92.6% of the Nipponbare (Oryza sativa L. ssp. japonica) reference genome and 91.6% of the Minghui 63 (MH63) (O. sativa L. ssp. indica) reference genome (Table 1). Reference-guided de novo assembly of the three cultivars was combined using the de novo assembler SOAPdenovo2 with a reference genome alignment (Figure 1). In the first step, reads were independently de novo assembled with two different functions, 63mer and 127mer. All the three cultivars had better assembly performances with the 63mer function (Table S2). The average scaffold length of Ciherang-Sub1, Ciherang, and IR64-Sub1 were 6907, 7456, and 7521 bp with 127mer and 7819, 8115, and 8321 bp with 63mer, respectively. The longest scaffolds of Ciherang-Sub1, Ciherang, and IR64-Sub1 were 80,935, 101,859, and 120,411 bp with 127mer and 212,257, 210,201, and 210,221 bp with 63mer, respectively. Lastly, the N50 values, which demonstrate the length of the 50th percentile of scaffolds, were also measured. In this case, the N50 values of Ciherang-Sub1, Ciherang, and IR64-Sub1 were 80,935, 101,859, and 120,411 bp with 127mer and 212,257, 210,201, and 210,221 bp with 63mer, respectively. Lastly, the N50 values, which demonstrate the length of the 50th percentile of scaffolds, were also measured. In this case, the N50 values of Ciherang-Sub1, Ciherang, and IR64-Sub1 were 11,298, 12,635, and 12,524 bp with 127mer, respectively, which doubled using the 63mer function to 25,138, 25,390, and 26,139 bp, respectively. Therefore, the scaffolds assembled with 63mer were used in the next step.

All scaffolds were aligned to the Nipponbare reference genome and the Minghui 63 (MH63) reference genome separately with the nucmer function. The scaffolds were then connected based on their orders to become a complete genome. The results showed that Ciherang-Sub1, Ciherang, and IR64-Sub1 had genome sizes of 345.4, 343.7, and 344.7 Mb, which correspond to 92.2–92.6% coverage on the Nipponbare reference genome (Table 1). For alignment based on the MH63 reference genome, the three cultivars had genome sizes of 353.9–354.9 Mb, which correspond to 91.3–91.6% of the MH63 genome.

2.2. Validation of Three Assembled Genomes

The qualities of the three genome assemblies were validated by the position of five known genes, which included GW5 (Os05g0187500), SD1 (Os01g0883800), Sub1A (DQ011598), Sub1B (Os09g0287000), and Sub1C (Os09g0286600). Results showed that the positions of the best BLAST results, with the highest similarity and coverage (top match, thereafter) of SD1 and GW5, were correct in all three cultivars and aligned well to both Nipponbare (Table 2) and MH63 (Table 3). Previous research revealed that the physical distance between Sub1A and Sub1C is around 61 kb in FR13A, and the physical distance between Sub1B and Sub1C is around 15.5 kb in Nipponbare [4]. Our results showed that the top matches of Sub1B and Sub1C for Ciherang-Sub1 and IR64-Sub1 have similar physical distances with the Nipponbare reference genome. The physical distances between Sub1B and Sub1C in both Ciherang-Sub1 and IR64-Sub1 are 17.0 and 17.3 kb, respectively. However, for Ciherang, the top match of Sub1B is located on chromosome 1, while the top match of Sub1C is on chromosome 4. Previous research has indicated that the Sub1A gene is absent in japonica rice, including Nipponbare, and some other Oryza species such as O. rhizomatis and O. eichingeri [4,15]. Due to this variation in gene presence, Sub1A does not have a position in the Nipponbare reference genome. However, we reported the Sub1A positions in the three genomes using the MH63 reference genome (Table 3).

![Reference-guided de novo assembly pipeline](https://example.com/f1.png)

**Figure 1.** Reference-guided de novo assembly pipeline. The blue, orange, red, and green lines denote raw sequences, clean sequences, the reference sequence, and ordered-contigs/scaffolds, respectively.
Table 1. General sequencing statistics and summary of assembly.

| Variety         | Number of Reads | Total Read Length (bp) | Sequencing Depth (X) | Genome Size (bp)          |
|-----------------|-----------------|------------------------|----------------------|---------------------------|
|                 |                 |                        |                      | Nipponbare Reference *    |
| Ciherang-Sub1   | 102,213,313     | 15,331,996,950         | 38                   | 345,442,284 (92.6%)       |
| Ciherang        | 115,172,273     | 17,275,840,950         | 43                   | 343,737,849 (92.2%)       |
| IR64-Sub1       | 114,085,140     | 17,112,771,000         | 43                   | 344,678,967 (92.4%)       |
|                 |                 |                        |                      | MH63 Reference *          |
|                 |                 |                        |                      | 354,934,762 (91.6%)       |
|                 |                 |                        |                      | 353,859,778 (91.3%)       |
|                 |                 |                        |                      | 353,859,751 (91.3%)       |

* The Nipponbare and MH63 reference genome sizes are 373 and 387 Mb, respectively.

2.2. Validation of Three Assembled Genomes

The qualities of the three genome assemblies were validated by the position of five known genes, which included GW5 (Os05g0187500), SD1 (Os01g0883800), Sub1A (DQ011598), Sub1B (Os09g0287000), and Sub1C (Os09g0286600). Results showed that the positions of the best BLAST results, with the highest similarity and coverage (top match, thereafter) of SD1 and GW5, were correct in all three cultivars and aligned well to both Nipponbare (Table 2) and MH63 (Table 3). Previous research revealed that the physical distance between Sub1A and Sub1C is around 61 kb in FR13A, and the physical distance between Sub1B and Sub1C is around 15.5 kb in Nipponbare [4]. Our results showed that the top matches of Sub1B and Sub1C for Ciherang-Sub1 and IR64-Sub1 have similar physical distances with the Nipponbare reference genome. The physical distances between Sub1B and Sub1C in both Ciherang-Sub1 and IR64-Sub1 are 17.0 and 17.3 kb, respectively. However, for Ciherang, the top match of Sub1B is located on chromosome 1, while the top match of Sub1C is on chromosome 4. Previous research has indicated that the Sub1A gene is absent in japonica rice, including Nipponbare, and some other Oryza species such as O. rhizomatis and O. eichingeri [4,15]. Due to this variation in gene presence, Sub1A does not have a position in the Nipponbare reference genome. However, we reported the Sub1A positions in the three genomes using the MH63 reference genome (Table 3).

Table 2. Summary of the position of the top match of known genes in the three genomes based on the Nipponbare reference genome.

| Gene     | Position of the Top Match in Each Genome Assembly (bp) |
|----------|--------------------------------------------------------|
|          | Nipponbare | Ciherang-Sub1 | Ciherang | IR64-Sub1 |
| SD1 (Os01g0883800) | chr01:38382385-38385469 | chr01:42499233-42501392 | chr01:43076922-43079161 | chr01:42597830-42599591 |
| GW5 (Os05g0187500) | chr05:3566701-3566701 | chr05:4832476-4834024 | chr05:4873155-4874734 | chr05:5262475-5264054 |
| Sub1B (DQ011598) | | chr09:6440482-6400093 | chr09:525965-5252048 | |
| Sub1C (Os09g0286600) | chr09:6387891-639798 | | | |

Table 3. Summary of the position of the top match of known genes in the three genomes based on the MH63 reference genome.

| Gene     | Position of the Top Match in Each Genome Assembly (bp) |
|----------|--------------------------------------------------------|
|          | MH63 | Ciherang-Sub1 | Ciherang | IR64-Sub1 |
| SD1 (Os01g0883800) | chr01:39643093-39644426 | chr01:42732034-42734193 | chr01:42313535-42313794 | chr01:42124122-42126361 |
| GW5 (Os05g0187500) | chr05:5428353-5430112 | chr05:5278241-5279789 | chr05:5178118-5179697 | chr05:4864937-4866218 |
| Sub1A (DQ011598) | chr06:22422489-22419199 | chr11:17912307-17916149 | chr11:8553735-8556945 | chr11:18314723-18318566 |
| Sub1B       | chr09:7179132-7180335 | chr09:4262487-4264027 | chr12:7911412-7912963 | chr09:5209175-5210715 |
| Sub1C (No annotation) | chr09:7162325-7164223 | chr10:10781009-10782407 | chr8:20306150-20308046 | chr09:5193229-5195103 |

Table 3 summarizes the position of five genes in the three genomes based on the MH63 reference. The top match of SD1 and GW5 for the three cultivars are located in similar positions on chromosomes 1 and 5, respectively. For Sub1B, the top matches for Ciherang-Sub1 and IR64-Sub1 have similar positions with the MH63 reference, both
Plants 2021, 10, 2740 located on chromosome 9. For Ciherang, however, the top match of Sub1B is positioned on chromosome 12. For Sub1C, the top match for IR64-Sub1 has a similar position with MH63. However, the top matches are on chromosome 10 for Ciherang-Sub1 and chromosome 8 for Ciherang. Interestingly, the top match of Sub1A in MH63 itself is on chromosome 6, while for the three cultivars, the top match for Sub1A is on chromosome 11.

The results showed that the top match of some Sub1 genes could have different chromosomal locations in the assembled genome than chromosome 9 reported for FR13A, the donor of Sub1 [4]. Sub1B and Sub1C are on the short arm of chromosome 9 in Ciherang-Sub1 and IR64-Sub1 in the Nipponbare reference genome (Table 2). This result reveals that Ciherang-Sub1 and IR64-Sub1 have high similarities in the Sub1B and Sub1C regions. Marker-assisted breeding depends on either shared relatedness due to recent co-ancestry or linkage disequilibrium (LD) between DNA markers and the genetic variants associated with the phenotypic traits [32]. Indeed, Ciherang-Sub1 was derived from Ciherang and IR64-Sub1, and IR64-Sub1 was the Sub1 donor. When Sub1 was introgressed into Ciherang, it included Sub1A, Sub1B, and Sub1C. Therefore, Ciherang-Sub1 not only has Sub1A from IR64-Sub1 but also Sub1B and Sub1C [8]. The positioning of the top matches of Sub1B and Sub1C on different chromosomes in Ciherang might be due to the scaffold size that is relatively shorter compared to those of Ciherang-Sub1 and IR64-Sub1; therefore, the top matches may have recognized an ERF-VII related to Sub1B or Sub1C on different chromosomes.

Sub1B and Sub1C are located on the short arm of chromosome 9 in IR64-Sub1 using the MH63 reference genome. Sub1B is also positioned on the short arm of chromosome 9 in Ciherang-Sub1; however, Sub1C is located on chromosome 10. On the other hand, Ciherang has the top match for Sub1B on chromosome 12 and Sub1C on chromosome 8 (Table 3). The Sub1A gene was not annotated in the MH63 genome [33]. Therefore, we searched for Sub1A in MH63. The top match of the complete sequence of Sub1A was found at the distal end of chromosome 6 in MH63 and on chromosome 11 in the three cultivars (Table 3). Previous research has indicated that some indica varieties might also lack the Sub1A locus, such as IR24, Swarna, IR50, and Habiganj Aman [4]. The unexpected location of Sub1A in the MH63 genome and its dissociation from Sub1B and Sub1C could be due to variations in genetic background or an assembling error in MH63. Hence, for our purpose, we used Nipponbare as a reference genome in the variant calling process even though all three cultivars belong to the indica rice group.

2.3. Genome Profile of Ciherang-Sub1

The genome profile of the three cultivars was examined to identify and characterize SNPs. A total of 590,664 SNPs were detected among Ciherang-Sub1, Ciherang, and IR64-Sub1. Based on the assessment of SNPs, 515,698 (87%) were a Ciherang-like genotype, 65,426 (11%) were an IR64-Sub1-like genotype, and 9540 (2%) were neither Ciherang- nor IR64-Sub1-like (Table 4). We identified a total of 78,190 SNPs on chromosome 8, of which 21,833 SNPs were located within the qSub8.1 physical interval that varied from 12.4 to 24.1 Mb.

**Table 4.** Genome profile of Ciherang-Sub1 with different window sizes.

|                      | All SNPs | 50 kb Window Size | 100 kb Window Size |
|----------------------|----------|-------------------|--------------------|
| Ciherang-like genotype | 515,698 (87%) | 4406 (59%)         | 2328 (63%)         |
| IR64-Sub1-like genotype | 65,426 (11%) | 1791 (24%)         | 832 (22%)          |
| Other                | 9540 (2%) | 1256 (17%)         | 564 (15%)          |
| Total SNP number     | 590,664  | 7453              | 3724               |

SNPs may cluster on certain chromosome regions (i.e., not evenly distributed along chromosomes), which have important implications for genetic mapping and crop improvement [34]. Therefore, we further examined the genome profile of Ciherang-Sub1 by grouping SNPs into blocks (windows) every 50,000 bp (50 kb) or 100 kb based on their physical positions (Figure S1). A total of 7453 SNPs were identified using a window size of.
50 kb, of which 4406 (59%) SNPs were Ciherang-alike and 1791 (24%) SNPs were in the IR64-Sub1-like genotype. The remaining 1256 (17%) SNPs were neither Ciherang- nor IR64-Sub1-like (Table 4). Using a 100kb window size, we identified 3724 SNPs that consisted of 2383 (63%) SNPs that were Ciherang-like, 832 (22%) SNPs that were IR64-Sub1-like, and 564 (15%) SNPs that were neither Ciherang- nor IR64-Sub1-like. In summary, Ciherang-Sub1 has an estimated 59–63% genetic background from Ciherang, 22–24% from IR64-Sub1, and 15–17% from unknown sources. To better visualize the genome profile of Ciherang-Sub1, SNPs from 50 and 100 kb windows were plotted according to their physical position with different colors that represented the genetic backgrounds (Figure 2).

The 50 kb window and 100 kb window analyses reveal similar genome profiles, confirming the accuracy of the genome composition (Figure 2). Both graphs illustrated that most parts of chromosomes 2, 5, 6, 7, 8, 10, and 12 of Ciherang-Sub1 originated from Ciherang, whereas chromosomes 1, 3, 4, and 11 were mosaics of homozygous loci from both Ciherang and IR64-Sub1. The recombination rates, especially around the centromeric regions of some chromosomes (e.g., Chrs 2, 5–8, 10), were very low. Different studies have reported that the distribution of recombination events in diverse crops is often skewed toward the distal ends of the chromosomes, with little to no crossovers near the centromere [35]. This includes chromosome 6A in bread wheat [36] and chromosome 9A in peanut [37]. The genomic landscape could be influenced by several factors, including external processes such as gene flow, background selection, and divergent selection. Background selection in the genomic regions with a reduced recombination rate and share relatedness can generate peaks of relative differentiation, along with gene flow. Additionally, the inherent properties of the genome, such as gene density, recombination rate variation, and mutation rate variation, also play significant roles [38]. Although the majority of the Ciherang-Sub1 genome originated from the recurrent Ciherang parent, almost the entire short arm of chromosome 9 originated from IR64-Sub1, which agrees with the history of Ciherang-Sub1 breeding. Ciherang-Sub1 was developed after just one generation of backcrossing (BC$_1$F$_2$) using 2 Sub1-flanking microsatellite markers (ART5 and RM8300) for foreground selection and 27 SSR markers for background selection [8]. Although the use of molecular markers for background selection facilitates the recovery of the recurrent parent genome with maximum homozygosity and minimal introgression from the donor parents, at least three generations of backcrossing are required for optimal results [39].

![Figure 2. Genome profile of Ciherang-Sub1 with two window sizes: (a) 7453 SNPs based on a 50 kb window; (b) 3724 SNPs based on a 100 kb window. The blue and red colors represent the Ciherang-like genotype and IR64-Sub1-like genotype; the green color indicates neither Ciherang nor IR64-Sub1. The physical map position (Mb) is shown on the far left. CEN indicates the approximate centromere region according to the Nipponbare reference genome; the orange vertical line indicates the qSub8.1 QTL region; the positions of Sub1A, Sub1B, Sub1C, SD1, and GW5 are shown. SUB1A is represented by a Sub1 flanking SSR marker, RM8300 (since Sub1A is absent in the Nipponbarre genome).](image-url)
Our analysis found other chromosomal regions that are mosaics of both parents. This may partly be due to recombination events but may also be partly due to the impurity of Ciherang itself since this variety was released about two decades ago [40]. There could be some slight differences between the Ciherang that we sequenced and the Ciherang that was used as the parent of Ciherang-Sub1, which could be due to seed admixture and/or pollen contamination during seed multiplication. In the absence of genotyping quality control methods, such issues are common across multiple species regardless of the mating system, which varied from 3% to 28% [41].

2.4. Dissection of qSub8.1 Region

The qSub8.1 physical interval in the Nipponbare genome varied from 12.4 to 24.1 Mb on chromosome 8 [13]. As shown in Figure 3, most parts of the upper portion of the qSub8.1 region in the Ciherang-Sub1 originated mainly from the Ciherang background, but the lower portion was inherited from both Ciherang and IR64-Sub1 plus other unknown sources, as described above. The structures profiled with a 50 kb window size and a 100 kb window size were slightly different, which could be due to the noise of the smaller window size. The ideal window size of adjacent markers balances noise reduction with signal identification and maximizing power, which depends on the extent of the linkage disequilibrium (LD), genome size, marker density, and recombination frequency. For example, in common bean, LD levels were found to be higher within the Mesoamerican gene pool and decay more rapidly within the Andean gene pool. The recombination rate across the genome was 2.13 cM/Mb, but it was highly suppressed around the centromeres [34]. The extent of LD varies in rice, and the average LD is about 75 kb in the indica subgroup and longer in the japonica subgroup [42]. Hence, we selected the two windows of 50 and 100 kb for this study. Based on our analysis, the favorable allele for qSub8.1 likely originated from IR64-Sub1, but it could also be originated either from a susceptible Ciherang parent or an unknown source due to seed admixture, pollen contamination, or other causes described above. Susceptible parents have previously been reported as the origin of favorable alleles, which includes a major-effect QTL (qtl12.1) associated with grain yield under severe drought stress in rice [43].

The SNPs identified in the present study will enable us to develop suitable DNA markers and identify the candidate gene(s) underlying qSub8.1 for further functional characterization. Additionally, transcriptomic studies have been performed to help elucidate the candidate genes underlying the QTL (Bailey-Serres, unpublished data). As described in the introduction section, qSub8.1 was discovered by using a RIL population derived from the cross between Ciherang-Sub1 that harbored the SUB1 gene and the submergence and stagnant flooding-tolerant IR10F365 [20]. However, because of complications in importing seeds of IR10F365 (a breeding line), we did not include this parent in the present study, which restricted our ability to identify polymorphic SNPs between IR10F365 and Ciherang-Sub1 around qSub8.1 that would be useful to narrow the large confidence interval and design breeder-friendly markers that can be used for MAS in the future. Hence, further studies are needed to characterize qSub8.1 in detail, including fine mapping to narrow its physical interval, validate its effect across different genetic backgrounds, determine/verify the exact parental origin of the favorable allele, and identify polymorphic SNPs within the qSub8.1 physical interval.
smaller window size. The ideal window size of adjacent markers balances noise reduction. A 100 kb window size were slightly different, which could be due to the noise of the known sources, as described above. The structures profiled with a 50 kb window size and but the lower portion was inherited from both Ciherang and IR64-Sub1 plus other un-known sources. As shown in Figure 3, most parts of the upper portion of the chromosome 8 [13]. As shown in Figure 3, most parts of the upper portion of the chromosome 8 [13].

2.4. Dissection of qSub8.1 Region

The qSub8.1 physical interval in Nipponbare genome varied from 12.4 to 24.1 Mb. The qSub8.1 region in the Ciherang-Sub1 originated mainly from the Ciherang background, whereas that in IR64-Sub1 was discovered by using a RIL population derived from the cross between Ciherang-Sub1 that harbored the SUB1 gene, and the submergence-susceptible Ciherang as a recurrent parent and the submergence-tolerant IR64-Sub1 as a donor parent [8]. To characterize qSub8.1, we used Ciherang-Sub1, Ciherang, and IR64-Sub1 for the whole genome sequence and assembly. The seeds of Ciherang-Sub1, Ciherang, and IR64-Sub1 were imported from the International Rice Research Institute (IRRI), Philippines, for the experiment and were planted in a quarantined greenhouse in the Texas A&M AgriLife Research Center at Beaumont, Texas, in 2017 for seed multiplication to conduct the research experiment.

3. Materials and Methods

3.1. Plant Materials

Ciherang-Sub1, which carries the novel submergence-tolerant QTL qSub8.1 [20], was derived through marker-assisted backcrossing from a cross between the submergence-susceptible Ciherang as a recurrent parent and the submergence-tolerant IR64-Sub1 as a donor parent [8]. To characterize qSub8.1, we used Ciherang-Sub1, Ciherang, and IR64-Sub1 for the whole genome sequence and assembly. The seeds of Ciherang-Sub1, Ciherang, and IR64-Sub1 were imported from the International Rice Research Institute (IRRI), Philippines, for the experiment and were planted in a quarantined greenhouse in the Texas A&M AgriLife Research Center at Beaumont, Texas, in 2017 for seed multiplication to conduct the research experiment.

3.2. Whole Genome Sequencing and Assembly

DNA was collected from healthy leaves of the three cultivars and stored in a −80 °C freezer before extraction. Leaves were ground by mortar and pastel with liquid nitrogen. A QIAGEN DNeasy Plant Mini Kit was used to extract genomic DNA. The quality of the DNA was checked using an Agilent 2100 Bioanalyzer, and the libraries were prepared following the Illumina protocol at the Texas A&M AgriLife Genomics and Bioinformatics Service (TxGen). Whole-genome shotgun sequencing of the three rice genomes was performed using the Illumina HiSeq4000 platform to provide at least 80–100 million reads per sample, with 150 bp pair-ends. The quality of raw reads was checked using Trimmomatic version 0.36 [44] with the PE-Phred 33 command as follows: (1) raw sequencing reads were trimmed to remove adaptors; (2) low-quality bases with a quality score less than 20 on the ends and tails of reads were removed; (3) reads were scanned with a 5 bp sliding window, and those with an average quality of sequences per bp below 20 were removed; (4) reads of less than 25 bases long were dropped; and (5) reads without correspondence read pairs were dropped. We used SOAPdenovo2 version r240 [45] to perform the de novo genome assembly with both -63mer and -127mer commands. The first command is suitable for

![Figure 3. Zoom-in version of the qSub8.1 region using (a) a 50 kb window and (b) a 100 kb window size. The blue, red, and green colors represent the Ciherang-like genotype, the IR64-Sub1-like genotype, and neither of them. The white space indicates that the SNP information is missing in the 50 or 100 kb window. The physical map position (Mb) is shown on the left. This QTL region spans between LOC_Os08g20660 and LOC_Os08g38020.](image-url)
assembly with k-mer values less than 63 bp, while the latter one works for k-mer values less than 127 bp. MUMmer version 3.23 [46] was used for aligning de novo scaffolds and contigs to the *O. sativa* L. spp. *japonica* reference genome (IRGSP-1.0) and the *O. sativa* L. spp. *indica* reference genome. The *indica* rice cultivar MH63 version 2 (MH63RS2) reference genome was downloaded from the Rice Information GateWay [47]. Following this, three genomes were constructed by re-orienting and connecting all the scaffolds and contigs according to their order.

3.3. Variants Calling

The *O. sativa* L. spp. *Japonica* reference genome (IRGSP-1.0, https://rapdb.dna.affrc.go.jp/download/irgsp1.html; accessed on 15 December 2019) was used as a reference genome in the variants calling. Reads were sorted by individual sample and aligned to the reference genome using Bowtie2 v2.2.9 [48] with the default parameters for end-to-end mode. The likelihood of each genotype was computed using the `samtools mpileup` function in SAMtools v0.1.19 [49], and the actual variant calling was performed using the `bcftools -bvcg` function. SNPs were identified when there were polymorphisms between the three cultivars, and SNPs were filtered with a minimum quality score of 30. To generate the genome structure graph, SNPs of Ciherang-Sub1 were compiled into two different window sizes, (blocks) of 50 and 100 kb, and the dominant SNP types (Ciherang-like genotype or IR64-Sub1-like genotype) within the block were used to represent each block (Figure S1).

4. Conclusions

This study demonstrates the potential of whole genomic sequence and genomic information in crop improvement. The results showed that the Ciherang-Sub1 genome is composed of 59–63% Ciherang, 22–24% of IR64-Sub1, and 15–17% of unknown sources. The genome profile derived from SNP information provided a more detailed picture of the previous Ciherang-Sub1 marker-assisted breeding. High-resolution SNP markers allow us to better understand *qSub8.1* chromosome 8. The SNPs identified in the current study can be used to assist in analyzing the candidate genes underlying *qSub8.1* and to develop DNA markers for future breeding efforts to further improve the level of submergence tolerance in rice. However, further studies are needed to fine-map the QTL, design breeder-friendly SNPs for MAS in the future, validate its effect across different genetic backgrounds, determine/verify the exact parental origin of the favorable allele, and conduct gene expression analysis on the QTL region.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants10122740/s1, Figure S1: The illustration of examined SNP blocks using a 50 or 100 kb window size, Table S1: Raw sequence outputs of three rice genomes, Table S2: De novo assembly of three rice genomes using different assembly functions.

Author Contributions: E.M.S., J.B.-S. and Y.L. conceived the project; Y.L. and E.M.S. designed the experiments; Y.L., C.L.H., N.K.S. and R.E.T. performed the experiments; C.D.J.’s lab performed the whole genome sequencing; Y.L. and S.W. performed the bioinformatic analysis; E.M.S. supervised the project; E.M.S., J.B.-S. and Y.L. performed critical data interpretation; Y.L. wrote the first draft of the manuscript; E.M.S. and J.B.-S. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This project is funded by USDA NIFA # 2017-67013-26194 to E.M.S. and J.B.-S.

Data Availability Statement: Data are available as Supplementary Materials.

Acknowledgments: We thank Fu-Jin Wei, Backki Kim, Yi-Pin Lai, and Patrick Carre for technical assistance, Texas A&M High Performance and Research Computing (HPRC) for providing a server, and Kevin Childs for providing a server for part of the sequence analysis.

Conflicts of Interest: The authors declare no conflict of interest.
References

1. Rice Is Life: Scientific Perspectives for the 21st Century; Toriyama, K.; Heong, K.L.; Hardy, B., Eds.; International Rice Research Institute: Los Baños, Philippines; Tsukuba, Japan, 2005.

2. Fukao, T.; Bailey-Serres, J. Plant responses to hypoxia—Is survival a balancing act? Trends Plant Sci. 2004, 9, 449–456. [CrossRef]

3. Fannin, B. Texas Agricultural Losses from Hurricane Harvey Estimated at more than $200 Million. AgriLife TODAY 2017. Available online: https://agrilifetoday.tamu.edu/2017/10/27/texas-agricultural-losses-hurricane-harvey-estimated-200-million/ (accessed on 8 December 2021).

4. Xu, K.; Xu, X.; Fukao, T.; Canlas, P.; Maghirang-Rodriguez, R.; Heuer, S.; Ismail, A.M.; Bailey-Serres, J.; Ronald, P.C.; Mackill, D.J. Sub1A is an ethylene-response-factor-like gene that confers submergence tolerance to rice. Nature 2006, 442, 705–708. [CrossRef]

5. Neeraja, C.N.; Maghirang-Rodriguez, R.; Pamplona, A.; Heuer, S.; Collard, B.C.Y.; Septiningsih, E.M.; Vergara, G.; Sanchez, D.; Xu, K.; Ismail, A.M.; et al. A marker-assisted backcross approach for developing submergence-tolerant rice cultivars. Theor. Appl. Genet. 2007, 115, 767–776. [CrossRef] [PubMed]

6. Septiningsih, E.M.; Pamplona, A.M.; Sanchez, D.L.; Neeraja, C.N.; Vergara, G.V.; Heuer, S.; Ismail, A.M.; Mackill, D.J. Development of submergence-tolerant rice cultivars: The Sub1 locus and beyond. Ann. Bot. 2009, 103, 151–160. [CrossRef]

7. Septiningsih, E.M.; Mackill, D.J. Genetics and breeding of flooding tolerance in rice. In Rice Genomics, Genetics and Breeding; Springer: Berlin/Heidelberg, Germany, 2018; pp. 275–295.

8. Septiningsih, E.M.; Hidayatun, N.; Sanchez, D.L.; Nugraha, Y.; Carandang, J.; Pamplona, A.M.; Collard, B.C.; Ismail, A.M.; Mackill, D.J. Accelerating the development of new submergence tolerant rice varieties: The case of Chihgang-Sub1 and PSB Rc18-Sub1. Euphytica 2015, 202, 259–268. [CrossRef]

9. Iftekharuddaula, K.M.; Ahmed, H.U.; Ghosal, S.; Amin, A.; Moni, Z.R.; Ray, B.P.; Barman, H.N.; Siddique, M.A.; Collard, B.C.; Septiningsih, E.M. Development of early maturing submergence-tolerant rice varieties for Bangladesh. Field Crops Res. 2016, 190, 44–53. [CrossRef]

10. Iftekharuddaula, K.M.; Newaz, M.A.; Salam, M.A.; Ahmed, H.U.; Mahbub, M.a.A.; Septiningsih, E.M.; Collard, B.C.Y.; Sanchez, D.L.; Pamplona, A.M.; Mackill, D.J. Rapid and high-precision marker assisted backcrossing to introgress the SUB1 QTL into BR11, the rained lowland rice mega variety of Bangladesh. Euphytica 2010, 178, 83–97. [CrossRef]

11. Singh, R.; Singh, Y.; Xalaso, S.; Verulkar, S.; Yadav, N.; Singh, S.; Singh, N.; Prasad, K.; Kondayya, K.; Rao, P.R. From QTL to variety-harnessing the benefits of QTLs for drought, flood and salt tolerance in mega rice varieties of India through a multi-institutional network. Plant Sci. 2016, 242, 278–287. [CrossRef]

12. Septiningsih, E.M.; Collard, B.C.; Heuer, S.; Bailey-Serres, J.; Ismail, A.M.; Mackill, D.J. Applying genomics tools for breeding submergence tolerance in rice. Transl. Genom. Crop Breed. 2013, 2, 9–30.

13. Fukao, T.; Xu, K.; Ronald, P.C.; Bailey-Serres, J. A variable cluster of ethylene response factor–like genes regulates metabolic and developmental acclimation responses to submergence in domesticated rice. Ann. Bot. 2009, 103, 143–150. [CrossRef]

14. Fukao, T.; Harris, T.; Bailey-Serres, J. Evolutionary analysis of the Sub1 gene cluster that confers submergence tolerance to rice. Plant Cell 2006, 18, 2021–2034. [CrossRef]

15. Nioroula, R.K.; Pucciariello, C.; Ho, V.T.; Novi, G.; Fukao, T.; Perata, P. SUB1A-dependent and -independent mechanisms are involved in the flooding tolerance of wild rice species. Plant J. 2012, 72, 282–293. [CrossRef]

16. Singh, N.; Dang, T.T.; Pandey, D.M.; Sanchez, D.; Neeraja, C.; Septiningsih, E.M.; Mendioro, M.; Tecson-Mendoza, E.M.; Ismail, A.M. Molecular marker survey and expression analyses of the rice submergence-tolerance gene SUB1A. Theor. Appl. Genet. 2010, 121, 1441–1453. [CrossRef] [PubMed]

17. Iftekharuddaula, K.M.; Ghosal, S.; Gonzaga, Z.J.; Amin, A.; Barman, H.N.; Yaseen, R.; Haque, M.M.; Carandang, J.; Collard, B.C.; Septiningsih, E.M. Allelic diversity of newly characterized submergence-tolerant rice (Oryza sativa L.) germplasm from Bangladesh. Genet. Resour. Crop Evol. 2016, 63, 859–867. [CrossRef]

18. Li, Z.-X.; Septiningsih, E.; Quillloy-Mercado, S.; McNally, K.; Mackill, D. Identification of SUB1A alleles from wild rice Oryza rufipogon Griff. Genet. Resour. Crop Evol. 2011, 58, 1237–1242. [CrossRef]

19. Gonzaga, Z.J.C.; Carandang, J.; Sanchez, D.L.; Mackill, D.J.; Septiningsih, E.M. Mapping additional QTLs from FR13A to increase submergence tolerance in rice beyond SUB1. Euphytica 2016, 209, 627–636. [CrossRef]

20. Gonzaga, Z.J.C.; Carandang, J.; Singh, A.; Collard, B.C.Y.; Thomson, M.J.; Septiningsih, E.M. Mapping QTLs for submergence tolerance in rice using a population fixed for SUB1A tolerant allele. Mol. Breeding. 2017, 37, 47. [CrossRef]

21. Septiningsih, E.M.; Sanchez, D.L.; Singh, N.; Sendon, P.M.; Pamplona, A.M.; Heuer, S.; Mackill, D.J. Identifying novel QTLs for submergence tolerance in rice cultivars IR72 and Madabaru. Appl. Genet 2012, 124, 867–874. [CrossRef] [PubMed]

22. Singh, A.; Singh, Y.; Mahato, A.K.; Jayaswal, P.K.; Singh, S.; Singh, R.; Yadav, N.; Singh, A.; Singh, P.; Singh, R. Allelic sequence variation in the Sub1A, Sub1B and Sub1C genes among diverse rice cultivars and its association with submergence tolerance. Sci. Rep. 2020, 10, 1–18. [CrossRef] [PubMed]

23. Fukao, T.; Yeung, E.; Bailey-Serres, J. The submergence tolerance regulator SUB1A mediates crosstalk between submergence and drought tolerance in rice. Plant Cell 2011, 23, 412–427. [CrossRef]

24. Chaudhary, B.; Shrestha, S.M.; Singh, U.S.; Manandhar, H.K.; Zaidi, N.W.; Thapa, R.B. Submergence MEDIATES Leaf Blast Resistance in Sub1 and Non-Sub1 Rice Genotypes. Glob. J. Biol. Agric. Health Sci. 2015, 4, 231–237.
