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Research Paper

Mikan Genome Database (MiGD): integrated database of genome annotation, genomic diversity, and CAPS marker information for mandarin molecular breeding

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Citrus species are some of the most valuable and widely consumed fruits globally. The genome sequences of representative citrus (e.g., *Citrus clementina*, *C. sinensis*, *C. grandis*) species have been released but the research base for mandarin molecular breeding is still poor. We assembled the genomes of *Citrus unshiu* and *Poncirus trifoliata*, two important species for citrus industry in Japan, using hybrid *de novo* assembly of Illumina and PacBio sequence data, and developed the Mikan Genome Database (MiGD). The assembled genome sizes of *C. unshiu* and *P. trifoliata* are 346 and 292 Mb, respectively, similar to those of citrus species in public databases; they are predicted to possess 41,489 and 34,333 protein-coding genes in their draft genome sequences, with 9,642 and 8,377 specific genes when compared to *C. clementina*, respectively. MiGD is an integrated database of genome annotation, genetic diversity, and Cleaved Amplified Polymorphic Sequence (CAPS) marker information, with these contents being mutually linked by genes. MiGD facilitates access to genome sequences of interest from previously reported linkage maps through CAPS markers and obtains polymorphism information through the multiple genome browser TASUKE. The genomic resources in MiGD (https://mikan.dna.affrc.go.jp) could provide valuable information for mandarin molecular breeding in Japan.

Key Words: *Citrus unshiu*, *Poncirus trifoliata*, hybrid genome assembly, Illumina sequencing-by-synthesis technology, PacBio single-molecule sequencing technology, genome annotation database, CAPS marker.

Introduction

Citrus species are some of the most valuable fruits globally and are widely cultivated in all suitable subtropical and tropical climates, comprising various species such as sweet oranges, mandarins, grapefruits, limes, lemons, and so on. More than 25 species of citrus are cultivated in Japan and the satsuma mandarin (*Citrus unshiu* Marc.) has been predominantly cultivated for over 100 years in Japan (Hodgson 1967). Numerous promising cultivars such as the ‘Kiyomi’, ‘Shiranuhi’, ‘Harumi’, ‘Setoka’, ‘Kanpei’ and so on have been released through the conventional breeding programs of public research organizations. These new cultivars immensely benefit the citrus industry and their cultivation area has been growing; however, it cannot match that of the satsuma mandarin. Satsuma mandarin offers many superior characteristics such as seedless-ness, easy peeling ability, early maturing, disease resistance, and high and stable productivity, which facilitates its cultivation and consumption. Most citrus trees are grafted on trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstock in the orchard. The trifoliate orange is closely related to the genus *Citrus* although its flowering habit is deciduous against the evergreen habit of general citrus species. It is quite suitable for satsuma mandarin and other citrus trees, and the grafted citrus trees generally form a compact canopy with high productivity and high fruit quality (Kawase *et al.* 1987). In addition, trifoliate orange is a cold-hardy citrus and is resistant to phytophthora root and collar rot caused by *Phytophthora citrophthora*, citrus tristeza virus (CTV) and citrus nematode

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(*Tylenchulus semipenetrans*), which cause severe damage to citrus trees. The grafted citrus trees can tolerate these biotic and abiotic stresses. Thus, these two citrus and relative cultivars are important resources for sustainability and expansion in the citrus industry in Japan, as well as for elucidating the genetic composition and molecular mechanisms of agronomically important traits and the isolation of related genes. Recently, the Japanese citrus industry has faced various obstacles such as lack of labor due to high-aging of industrial carriers, declining domestic demand, and an increase in low-priced imported fruits due to the progress of global economization. The development of new citrus cultivars with a top level of fruit quality in the world have contributed to protect the Japanese citrus industry; therefore, sustainable development of a new cultivar is indispensable to maintain the present international advantage of the Japanese brand. Owing to the long period required for conventional cross breeding, introduction of genome information assisted molecular breeding technology is required to efficiently generate a new superior cultivar.

Recently, the second-generation sequencing technology (e.g., Illumina HiSeq system) has drastically accelerated the whole genome sequencing process of all living organisms. Furthermore, long read information generated from the third-generation sequencing technology (e.g., PacBio RS II and Oxford Nanopore sequencers) promises to significantly improve the quality of genome assembly. However, despite decreasing the turnaround time, the costs of third-generation sequencing are at least an order of magnitude more expensive than those of Illumina sequencing. Several hybrid *de novo* assembly methods using both short (Illumina) and long (PacBio/Nanopore) read information, such as PacBioToCA, SPAdes, and DBG2OLC, have been reported using the advantageous points of both second- and third-generation sequencing technologies (Antipov *et al.* 2016, Koren *et al.* 2012, Ye *et al.* 2016). The *de novo* assembly of highly heterozygous genomes is still a complex and challenging task. Recently, several genome assemblers and analysis pipelines, such as Platanus and Redundans, that are specifically designed for the assembly of highly heterozygous genomes have been developed (Kajitani *et al.* 2014, Pryszcz and Gabaldón 2016). The International Citrus Genome Consortium (ICGC, composed of researchers from Australia, Brazil, China, France, Israel, Italy, Japan, Spain, and USA) was established in 2003 to sequence the genomes of sweet orange (*C. sinensis* L.) and clementine mandarin (*C. clementina* Hort ex Tan). The genome sequences of sweet orange (diploid) and mandarin (haploid) have been determined (Wu *et al.* 2014), and their draft sequences are now available in Phytozome (https://phytozome.jgi.doe.gov). General citrus cultivars are diploids with nine pairs of chromosomes and genome size varies among citrus species; the genomes of mandarin (*C. reticulata* Blanco) and sweet orange are approximately estimated to be 360 Mb and 367 Mb, respectively (Arumuganathan and Earle 1991, Ollitrault *et al.* 1994). Therefore, the assembled sequences of clementine mandarin (301.4 Mb in JGI ver. 1.0) and sweet orange (319.2 Mb in JGI ver. 1.0) cover 83.7% and 87.0% of this estimated genome size, respectively. In addition, various citrus genomes, such as that of Ponkan mandarin (*C. reticulata* Blanco) and Chandler pummelo (*C. grandis* (L.) Osbeck), have been sequenced and compared to understand the complex citrus phylogeny and sequence-directed genetic improvement (Wu *et al.* 2014). In a recent study, a high-quality haploid pumelo genome was assembled using single-molecule sequences generated by the PacBio RS II platform, and the draft genomes of three heterozygous *Citrinae* species were assembled using Illumina reads (Wang *et al.* 2017). In addition, the draft genome of satsuma mandarin was assembled to understand the structural features of this major Japanese mandarin species (Shimizu *et al.* 2017). These advances in genome research have extended to molecular breeding and the isolation of agronomically important genes, resulting in many worldwide reviews and publications on citrus genomics, genetics, and breeding (Gmitter *et al.* 2007, 2012, Khan 2007, Talon and Gmitter 2008). On the contrary, information integration between the various genetic linkage maps reported previously and these assembled genome sequences is an upcoming task and is desired to access the genomic regions responsible for agronomically important traits through linkage and phylogenetic DNA markers.

Herein, to enforce the progress of mandarin molecular breeding in Japan, we developed an integrated genome database named as Mikan Genome Database (MiGD) (https://mikan.dna.affrc.go.jp), which comprises the genome annotation database of *C. unshiu* and *P. trifoliata*, a genome diversity database among nine citrus species, and a Cleaved Amplified Polymorphic Sequence (CAPS) marker database. MiGD could facilitate connection of interesting genetic loci on the genetic linkage map to the genome sequences of *C. clementina* and *C. unshiu* through the CAPS marker information. The newly assembled genome sequence of *P. trifoliata* and enrichment of the *C. unshiu* genome sequences by re-sequencing are valuable genetic resources to explore the genes responsible for agriculturally important traits underlying the two major cultivated species, satsuma mandarin and trifoliate orange, in Japan.

Materials and Methods

Plant material and genome sequencing of C. unshiu and P. trifoliata

“Miyagawa wase”, one of the major cultivated satsuma mandarin cultivars (NIAS Genebank registration number: 117351 (https://www.gene.affrc.go.jp/databases-plant_search_detail.php?jp=117351)) and trifoliate orange (NIAS Genebank registration number: 113401 (https://www.gene.affrc.go.jp/databases-plant_search_detail.php?jp=113401)), grown at Okitsu Citrus Research Station of NIFTS in Japan were used as the genetic sources for genome sequencing of

C. unshiu and *P. trifoliata*. Genomic DNA was extracted from fresh and fully expanded leaves of these cultivars, according to the method described by Dellaporta *et al.* (1983). For *C. unshiu*, two Illumina paired-end (PE) libraries with average insert sizes of 350 and 550 bp were constructed using the TruSeq DNA PCR-Free Library Preparation Kit. Each library was sequenced using a one-lane of a flow cell in the Illumina HiSeq 2000 system. The read lengths were 101 and 151 bp for each library, respectively. For PacBio RSII sequencing, the DNA sequencing library constructed by the standard protocol was sequenced using P6C4 chemistry and eight SMART cells on the PacBio RS II system. For *P. trifoliata*, one Illumina paired-end and three mate-pair (MP) libraries with average insert sizes of 3, 5, and 8 kb were constructed using the standard protocol. All the libraries were sequenced using the Illumina HiSeq 2000 system with a read length of 101 bp. PacBio RS II sequencing was performed in the same manner as for Satsuma mandarin. All the sequence data are available in DRA/ERA/SRA (DRA008432).

Hybrid de novo genome assembly

Low-quality bases and Illumina sequencing adapters were trimmed using Trimmomatic v.0.36 (ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10 LEADING:20 TRAILING:20 SLIDINGWINDOW:10:15 MINLEN:50) and NxTrim (--minlength 50) for Illumina PE and MP reads, respectively (Bolger *et al.* 2014, O'Connell *et al.* 2015). The Illumina PE reads were assembled using Platanus v1.2.4 with the following parameters: -k 61 -u 1.0 for satsuma mandarin and -k 41 -u 1.0 for trifoliate orange (Kajitani *et al.* 2014). High quality consensus sequences were constructed with the contigs assembled with Platanus and PacBio subreads using the hybrid *de novo* genome assembler DBG2OLC of November 1, 2016 (Ye *et al.* 2016). Alternative heterozygous contigs were removed using the Redundans pipeline (Pryszcz and Gabaldón 2016). The PacBio subreads were used for scaffolding with the SSPACE-LongRead package and two rounds of gap-closing with PBJelly implemented in PBSuite v15.8.24 (Boetzer and Pirovano 2014, English *et al.* 2012). For *P. trifoliata*, an additional scaffolding process with Illumina MP reads using the SSPACE-STANDARD package v.3.0 was performed before gap-closing (Boetzer *et al.* 2011). Finally, all Illumina PE reads were aligned to the scaffolds using BWA-MEM v.0.7.15, sequencing errors (single nucleotide polymorphisms (SNPs) and indels) were detected and filtered using the HaplotypeCaller in GATK v.3.7, and errors were corrected using in-house scripts (DePristo *et al.* 2011, Li and Durbin 2009). The genome sizes of the two species were estimated using MaSuRCA v3.2.2 (Zimin *et al.* 2013). Quality assessment and calculation of statistics for the genome assembly was performed using in-house scripts and QUAST v4.2 with the *C. clementina* genome and gene annotation (v1.0) downloaded from Phytozome (https://phytozome.jgi.doe.gov) as a reference (Gurevich *et al.* 2013). Sequence comparisons between the published *C. unshiu* draft genome and assembled scaffolds in this study were performed using MUMmer (v4.0.0) with the following parameters: the default parameters for nucmer, "-r -q -i 98.0 -l 1000" for delta-filter and "-c -r" for show-coords. Dot plot of the draft genome and assembled scaffolds were drawn using the R script "dotPlotly (https://github.com/tpoorten/dotPlotly)". LTR_retriever v2.5 was used to calculate LTR Assembly Index (LAI) (Ou *et al.* 2018).

Genome annotation

Genome annotation on the assembled *C. unshiu* and *P. trifoliata* genome sequences was conducted with a web-based annotation system MEGANTE (Numa and Itoh 2014). *C. sinensis* was selected as the parameter for query sequences since the amount of available EST data for *C. sinensis* is the largest among citrus species in MEGANTE. For annotation, full-length cDNAs (FLcDNAs) and ESTs of *C. sinensis* obtained from INSDC (updated on Apr 2016) and UniProtKB (plant division of Swiss-Prot and TrEMBL in release 2016_03) were used as references for the transcript and protein sequence alignment. InterPro v56.0 was used for the functional domain search. To simply compare the gene annotation among three citrus species (*C. clementina*, *C. unshiu* and *P. trifoliata*), we also carried out re-annotation of the *C. clementina* genome (only for scaffold 1-9 of JGI v1.0 genome) using MEGANTE.

Gene conservation and orthologous relationships among the three citrus species were examined by an all-against-all blastp (NCBI Blast v2.6.0+) search of protein sequences and clustering using OrthoFinder v1.1.4 with the default parameters (Camacho *et al.* 2009, Emms and Kelly 2015). GO and InterPro enrichment analyses were performed using Fisher's exact tests in combination with False discovery rate (FDR) correction.

Mapping of CAPS markers on the C. unshiu genome assembly

The molecular DNA marker information (e.g., primer sequences, STS, positions on *C. clementina* scaffolds, and AGI genetic linkage map) applicable for *C. unshiu* was collected from a previous study (Shimada *et al.* 2014). AGI genetic map was constructed as a standard genetic map to progress molecular breeding of mandarin in Japan. The positions of DNA markers on the *C. unshiu* draft genome were determined using MFEprimer v2.0 (Qu *et al.* 2012). The best marker positions were selected using the following criteria: 1) a primer pair coverage (PPC) score more than 30, 2) Tm values of both forward and reverse primers more than 35°C, and 3) expected product size in the range of 0.5–1.5-fold of the experimentally validated product size.

Genetic diversity among C. unshiu, P. trifoliata, and other citrus species

The aforementioned Illumina PE data of *C. unshiu* and

P. trifoliata, and the recently published genome resequencing data of the representative citrus species: *C. reticulata* (SRR3747540), *C. sinensis* (SRR4240447), *C. grandis* (SRR4294213, SRR4294216), *C. ichangensis* (SRR4007116, SRR4006763, SRR4006743, SRR4006657), *C. medica* (SRR4010249, SRR4009988), and *A. buxifolia* (SRR4254787, SRR4254698) were downloaded from DRA/ERA/SRA (Wang *et al.* 2017). After trimming the low-quality bases and sequencing adapters using Trimmomatic, the reads were aligned to the *C. clementina* reference genome (v1.0) using BWA-MEM v.0.7.15. The SNVs were detected using the GATK HaplotypeCaller command according to the best practice protocols for SNP and indel discovery in whole genome sequences from the GATK website (https://software.broadinstitute.org/gatk/best-practices/). For sliding window analysis of genome-wide nucleotide diversity between *C. clementina* and other citrus genomes, the rates of sequence variations were calculated for each 200 kb window with 100 kb sliding.

Development of CAPS markers

During construction of the mandarin genetic standard map with 708 gene-based markers (Shimada *et al.* 2014), more than 4,000 CAPS markers were designed with reference to the expression sequencing tags (ESTs) of various cDNA libraries. Their primers were designed using Oligo ver. 5.0 (National Bioscience, Inc. Plymouth, MN, USA). Most of them were eliminated in the screening due to no polymorphism between parent lines (A255 and G434) and for technical reasons such as no amplification, unstable PCR fragment pattern, and so on. These CAPS markers without technical problems are valuable to advance mandarin molecular breeding in Japan; however, their information has been unpublished so far. A total of 2,696 CAPS markers were thus selected and deposited in the CAPS marker database.

Construction of MiGD

Draft genome sequences and gene annotation of *C. unshiu* and *P. trifoliata* were obtained from JBrowse v1.12.1 (Buels *et al.* 2016). BLAST DB of nucleotide transcript and protein sequences were constructed using ncbi-blast-2.6.0+. The BLAST search function against the genome and transcriptome sequences of *C. unshiu* and *P. trifoliata* was implemented in SequenceServer v1.0.9 (https://www.sequenceserver.com). All genome sequences and the genomic feature information (e.g., genes, repeats, and DNA markers) are available on the download page (https://mikan.dna.affrc.go.jp/data_download/index.html). To demonstrate the genome-wide variations between *C. clementina* and other citrus species, the Multiple Genome Browser: TASUKE version 1.5.3 (Kumagai *et al.* 2013) was installed in the MiGD server. The MiGD website is implemented on a Linux server with a CentOS and Apache web server. All CAPS marker data are stored in the MariaDB database. The user-interface and functions of the CAPS marker database were developed using PHP and JavaScript.

Results

Genome sequencing and hybrid de novo assembly of C. unshiu and P. trifoliata

We sequenced the genomes of *C. unshiu* (satsuma mandarin) and *P. trifoliata* (trifoliate orange), which are agronomically important species in Japan. For *C. unshiu*, 97 Gb of Illumina paired-end reads (288x coverage) and 8 Gb of PacBio RS II subreads (23x coverage) were obtained (**Table 1**). For *P. trifoliata*, 34 Gb of Illumina paired-end reads (113x coverage) and 10 Gb of PacBio RS II subreads (34x coverage) were obtained. Furthermore, Illumina mate-pair reads were used for scaffolding. Using k-mer analysis with the Illumina PE reads, the genome sizes of two species were estimated to be 336 Mb and 296 Mb for satsuma mandarin and trifoliate orange, respectively. K-mer spectra also showed the high heterozygosity of two genomes (**Supplemental Fig. 1**). In our assembly strategy, Illumina PE reads were assembled using Platanus followed by the hybrid *de novo* genome assembly of Platanus contig sequences and PacBio subreads using DBG2OLC (**Fig. 1**) (Kajitani *et al.* 2014, Ye *et al.* 2016). To remove the alternative heterozygous contigs, we used the Redundans pipeline (Pryszcz and Gabaldón 2016). At this step, we obtained a 336.2 Mb *C. unshiu* genome assembly containing 5,269 contigs (N50 = 114,572) and a 289.4 Mb *P. trifoliata* genome assembly containing 3,328 contigs (N50 = 176,694) without any unambiguous bases (Ns) in the assemblies. Assembled contigs were scaffolded with PacBio subreads by SSPACE-LongRead. In addition, for *P. trifoliata*, Illumina mate-pair (MP) reads were also used for further scaffolding processes. The obtained scaffolds would still have sequencing errors derived from the high sequencing error rate in the PacBio subreads. We corrected the sequencing errors (both SNPs and indels) detected by the alignment of Illumina PE reads and the GATK pipeline. Finally, the total lengths of the assembled scaffolds of *C. unshiu* and *P. trifoliata* were 346.4 Mb and 291.9 Mb, which comprised 3,151 and 1,313 scaffolds for *C. unshiu* and *P. trifoliata*, respectively (**Table 2**). The scaffold N50 of the final assemblies were 206 kb and 424 kb for *C. unshiu* and *P. trifoliata*, respectively. The 2-fold higher sequence contiguity for the *P. trifoliata* assembly might largely be due to the additional Illumina Mate-pair sequences. The sequence contiguities of the two assemblies were lower than those of the recently published high-quality reference genome of the haploid pummelo *C. grandis* (N50 = 4.2 Mb), but were comparable to the draft genomes of primitive citrus *A. buxifolia* (N50 = 1,074 kb), *C. ichangensis* (N50 = 504 kb) and citron *C. medica* (N50 = 367 kb) (Wang *et al.* 2017). The sizes of the assembled genomes were very close to the length estimated by k-mer analysis. The assembled genome sizes of *C. clementina* (301 Mb), *C. sinensis* (321 Mb), *C. grandis*

Table 1. Sequence data used for hybrid *de novo* genome assembly

Species	Type	# of reads	# of bases	Coverage (x)[a]
C. unshiu	Illumina, PE101	532,000,280	54,540,028,280	162
	Illumina, PE151	279,626,232	42,223,561,032	126
	PacBio, P6C4	1,099,957	7,705,209,842	23
P. trifoliata	Illumina, PE101	332,587,118	33,591,298,918	113
	Illumina, MP3k	97,164,256	9,813,589,856	33
	Illumina, MP5k	96,069,378	9,703,007,178	33
	Illumina, MP8k	131,459,878	13,277,447,678	45
	PacBio, P6C4	900,552	10,097,873,768	34

[a] Estimated genome sizes of 336 Mb for *C. unshiu* and 296 Mb for *P. trifoliata* were used for the calculation.

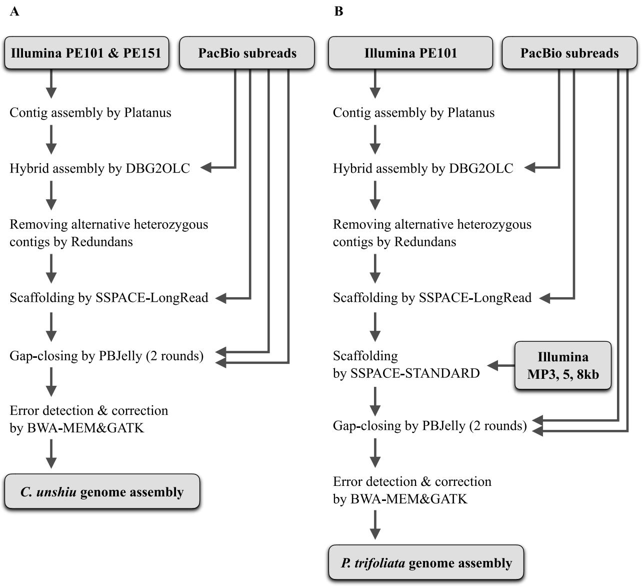

A
Illumina PE101 & PE151
PacBio subreads
Contig assembly by Platanus
Hybrid assembly by DBG2OLC
Removing alternative heterozygous contigs by Redundans
Scaffolding by SSPACE-LongRead
Gap-closing by PBJelly (2 rounds)
Error detection & correction by BWA-MEM&GATK
C. unshiu genome assembly
B
Illumina PE101
PacBio subreads
Contig assembly by Platanus
Hybrid assembly by DBG2OLC
Removing alternative heterozygous contigs by Redundans
Scaffolding by SSPACE-LongRead
Scaffolding by SSPACE-STANDARD
Illumina MP3, 5, 8kb
Gap-closing by PBJelly (2 rounds)
Error detection & correction by BWA-MEM&GATK
P. trifoliata genome assembly

Fig. 1. The workflow for hybrid *de novo* assembly. The *C. unshiu* (A) and *P. trifoliata* (B) genome was assembled using Illumina paired-end reads and PacBio subreads using the hybrid *de novo* genome assembly strategy. Furthermore, for *P. trifoliata*, Illumina mate-pair reads were used for scaffolding of the contigs.

(345 Mb), *C. ichangensis* (335 Mb), *C. medica* (368 Mb), and *C. unshiu* (346 Mb) were relatively larger than those of *A. buxifolia* (288 Mb) and *P. trifoliata* (292 Mb). The GC content values were similar among the citrus species and are 34.0 and 33.8 for *C. unshiu* and *P. trifoliata*, respectively (**Table 2**). The quality of the *C. unshiu* assembly was evaluated using the recently proposed measurement, the LTR assembly index (LAI), estimates the ratio of intact LTR retrotransposons in a genome assembly (Ou *et al.* 2018). The LAI of the *C. unshiu* assembly was estimated to be 11.93 (reference quality) and higher than 8.18 (draft quality) of the published *C. unshiu* draft genome (Shimizu *et al.* 2017).

The accuracy of the *C. unshiu* assembly was evaluated by comparing the positions of publicly available DNA markers on the *C. unshiu* assembly and the integrated genetic linkage map (AGI) (**Supplemental Table 1**). In all, 403 markers could be uniquely placed on 266 *C. unshiu* scaffolds and 75 of those scaffolds had two or more markers. For each scaffold, we examined whether the DNA markers on a certain *C. unshiu* scaffold were assigned to a single linkage group or a *C. clementina* chromosome. As a result, only 8 *C. unshiu* scaffolds had inconsistent orders of the markers with both the *C. clementina* genome and AGI map, suggesting that the *C. unshiu* scaffolds was almost properly assembled overall. However, the number of currently available DNA markers was not sufficient for

Table 2. Statistics of genome assemblies and annotations

	C. clementina (JGI v1.0)	*C. sinensis* (HZAU v2)	*C. unshiu* (Shimizu *et al.* 2017)	*C. unshiu* (MiGD)	*P. trifoliata* (MiGD)
Number of scaffolds	1,398	10	20,470[b]	3,151	1,313
Total scaffold length (bp)	301,386,998	327,944,670	359,692,661	346,435,163	291,927,159
Mean scaffold length (bp)	215,584	32,794,467	17,571.7	109,944.5	222,336
Largest scaffolds (bp)	51,050,279	88,947,451	27,672,184	2,674,519	2,044,385
N50	31,410,901	30,837,053	14,331,940	206,057	424,026
Number of Ns (%)	6,218,033 (2.1)	26,794,082 (8.2)	28,235,341 (7.9)	2,333,253 (0.7)	1,743,151 (0.6)
%GC	35.0	34.6	33.9	34.0	33.8
LTR assembly index (LAI)	18.61	1.99	8.18	11.93	11.65
Repeat bases, Mb (%)	134.5 (44.6)	128.0 (39.0)	145.3 (40.4)	149.7 (43.2)	116.8 (40.0)
- SINEs, Kb (%)	122.3 (0.04)	120.6 (0.04)	128.8 (0.04)	154.1 (0.04)	134.0 (0.05)
- LINEs, Kb (%)	5,278.1 (1.75)	5,661.3 (1.73)	6,015.3 (1.67)	6,481.0 (1.87)	4,946.5 (1.69)
- LTR elements, Kb (%)	61,011.0 (20.24)	57,240.4 (17.45)	66,211.2 (18.41)	68,770.9 (19.85)	52,632.3 (18.03)
- DNA elements, Kb (%)	14,867.3 (4.93)	15,470.9 (4.72)	17,115.4 (4.76)	18,097.9 (5.22)	13,179.5 (4.51)
- Unclassified repeats, Kb (%)	52,053.7 (17.27)	48,327.7 (14.74)	54,497.3 (15.15)	54,737.5 (15.80)	44,786.4 (15.34)
Number of genes	24,533 (36,163)[a]	29,655	29,024	41,489	34,333
Number of transcripts	33,929 (37,570)[a]	44,275	37,970	42,913	35,291

[a] Number of genes and transcripts in the parentheses are based on the re-annotation data using MEGANTE.
[b] Satsuma pseudomolecule sequences (9 chromosomes and 20,461 unanchored scaffolds) was used.

anchoring and sorting the scaffolds into pseudomolecules. Further development of marker information and additional long-read sequences are thus needed for refining our genome assemblies. By mapping of the scaffold sequences against the published *C. unshiu* draft genome (Shimizu *et al.* 2017), 62.0% (1,954/3,151) of the scaffolds can be anchored on the chromosome 1-9 (**Supplemental Fig. 2**, **Supplemental Table 2**).

Genome annotation of C. unshiu and P. trifoliata

Annotation of the assembled genome was carried out using the web-based annotation pipeline MEGANTE. The result indicted that 41,489 and 34,333 protein-coding genes with 42,913 and 35,291 transcripts were predicted for *C. unshiu* and *P. trifoliata*, respectively. The transcripts had an average length of 1,130.6 and 1,135.6, and the mean amino acid sequence sizes of 341.3 and 347.2 aa for *C. unshiu* and *P. trifoliata*, respectively. To compare these statistics of gene annotation with *C. clementina*, we re-annotated the *C. clementina* scaffold 1–9 which covered more than 96% sequence of total scaffolds using MEGANTE with the same parameters. As a result, 36,163 protein-coding genes with 37,570 transcripts were predicted in the *C. clementina* genome and the average length of transcript and amino acid sequences was 1,252.9 bp and 378.7 aa, respectively. These results suggest that the number and length distribution of genes was comparable among the three citrus species.

Evolutionary conservation of genes and gene families among the three species was examined by a similarity search and clustering analysis of protein sequences. All protein sequences of three species were clustered into 51,615 gene groups (**Fig. 2**). Of those groups, 7,499, 9,639 and 8,355 were specifically observed in the *C. clementina*, *C. unshiu*, and *P. trifoliata* genomes, respectively (species-specific gene groups) (**Supplemental Tables 3–5**). Furthermore, 4,706 gene groups were observed only in two mandarin species (mandarin-specific gene groups) (**Supplemental Table 6**). Among three species, large gene families were observed in *C. unshiu* and *P. trifoliata* including terpene synthase, cytochrome P450, protein kinase, leucine-rich repeat protein, UDP-glucosyltransferase, ribonuclease H-like domain protein, pectin esterase inhibitor domain protein, zinc finger protein, ankyrin repeat proteins, and so on. Enrichment analyses of Gene Ontology and InterPro domains revealed that viral movement proteins (IPR028919) were significantly (FDR = 0.01) enriched in *P. trifoliata* specific genes (**Supplemental Tables 7**, **8**). On the contrary, genes required for the maintenance of basic or "housekeeping" functions (e.g., regulation of transcription, protein phosphorylation) were depleted among *P. trifoliata* specific genes.

The gene set in this study was compared with those in the previously published draft genome (Shimizu *et al.* 2017) using the same similarity search and clustering analyses. As a result, 17,218 (41.5%) protein-coding genes were annotated specifically in our genome assembly and 1,289 (7.5%) of those specific genes were supported by EST or mRNA sequences.

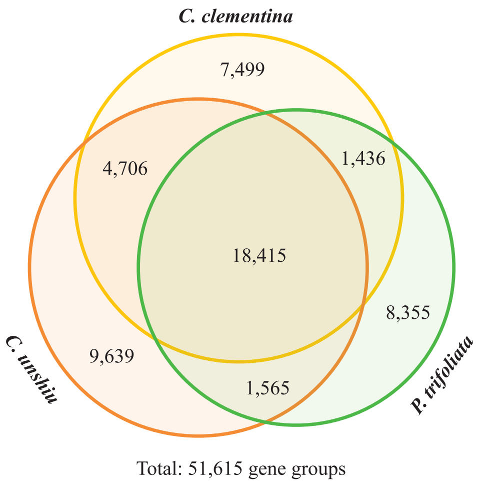
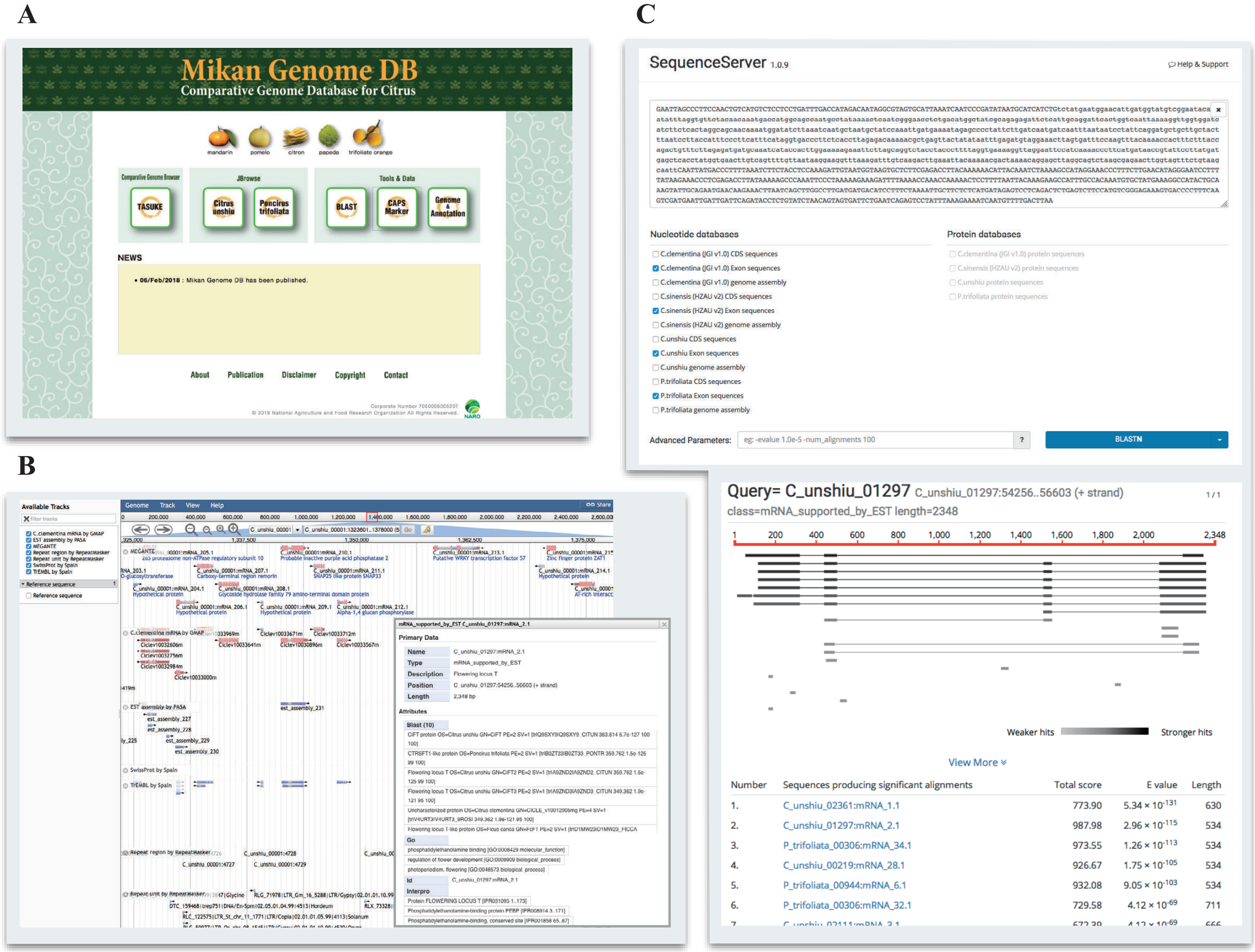


Fig. 2. Gene conservation among three citrus species. Orthologous gene groups were defined based on the all-against-all blastp search and clustering by OrthoFinder. The numbers of gene groups that were conserved in all, two, and a specific species are represented in the Venn diagram.

Database contents and functions of MiGD

The MiGD comprises three databases as follows: genome and annotation database of *C. unshiu* and *P. trifoliata*, genetic diversity database of nine citrus and relative species, and the CAPS marker database (**Fig. 3A**). The genome browser "JBrowse" provides the genome sequences and gene annotation data for *C. unshiu* and *P. trifoliata* (**Fig. 3B**). The SequenceServer system provides a BLAST search service against the genome, transcript, and protein sequences of *C. clementina* (JGI v1.0), *C. sinensis* (HZAU v2), *C. unshiu* (Shimizu *et al.* 2017), *C. unshiu* (MiGD), and *P. trifoliata* (MiGD) (**Fig. 3C**). All information regarding genome sequences in the FASTA format and gene annotations in GFF file are available on the download page. In JBrowse, users can search a gene with a transcript ID and obtain gene annotation data, such as description, GO terms, InterPro domains, and nucleotide sequences.

Genome-wide sequence polymorphisms (SNPs and indels) among nine citrus and relative species (*C. clementina*, *C. sinensis*, *C. reticulata*, *C. unshiu*, *C. grandis*, *C. medica*, *C. ichangensis*, *P. trifoliata* and *A. buxifolia*) are provided through the multiple genome browser "TASUKE" (Kumagai *et al.* 2013) (**Fig. 4**). The main panel indicates the sequence alignment information among them, and the distribution and frequencies of SNPs and indels can be feasibly displayed around any regions on the nine chromosomes against the referencing genome of *C. clementina* (JGI ver. 1.0). The top menu bar helps users to search functions to find identifiers or genomic positions. At the most precise level, individual nucleotides and translated amino acids can be displayed. By turning on the "snpEff" function


A
Mikan Genome DB
Comparative Genome Database for Citrus
mandarin
pomelo
citron
papeda
trifoliate orange
Comparative Genome Browser
TASUKE
JBrowse
Citrus unshiu
Poncirus trifoliata
Tools & Data
BLAST
CAPS Marker
Genome & Annotation
NEWS
• 06/Feb/2018 : Mikan Genome DB has been published.
About
Publication
Disclaimer
Copyright
Contact
B
Available Tracks
Genome
Track
View
Help
Primary Data
Name C_unshiu_01297:mRNA_2.1
Type mRNA_supported_by_EST
Description Flowering locus T
Position C_unshiu_01297:54256..56603 (+ strand)
Length 2,348 bp
Attributes
C
SequenceServer 1.0.9
Help & Support
Nucleotide databases
☐ C.clementina (JGI v1.0) CDS sequences
☑ C.clementina (JGI v1.0) Exon sequences
☐ C.clementina (JGI v1.0) genome assembly
☐ C.sinensis (HZAU v2) CDS sequences
☑ C.sinensis (HZAU v2) Exon sequences
☐ C.sinensis (HZAU v2) genome assembly
☐ C.unshiu CDS sequences
☑ C.unshiu Exon sequences
☐ C.unshiu genome assembly
☐ P.trifoliata CDS sequences
☑ P.trifoliata Exon sequences
☐ P.trifoliata genome assembly
Protein databases
☐ C.clementina (JGI v1.0) protein sequences
☐ C.sinensis (HZAU v2) protein sequences
☐ C.unshiu protein sequences
☐ P.trifoliata protein sequences
Advanced Parameters: eg: -evalue 1.0e-5 -num_alignments 100
BLASTN
Query= C_unshiu_01297 C_unshiu_01297:54256..56603 (+ strand)
class=mRNA_supported_by_EST length=2348
1/1
1
200
400
600
800
1,000
1,200
1,400
1,600
1,800
2,000
2,348
Weaker hits
Stronger hits
View More

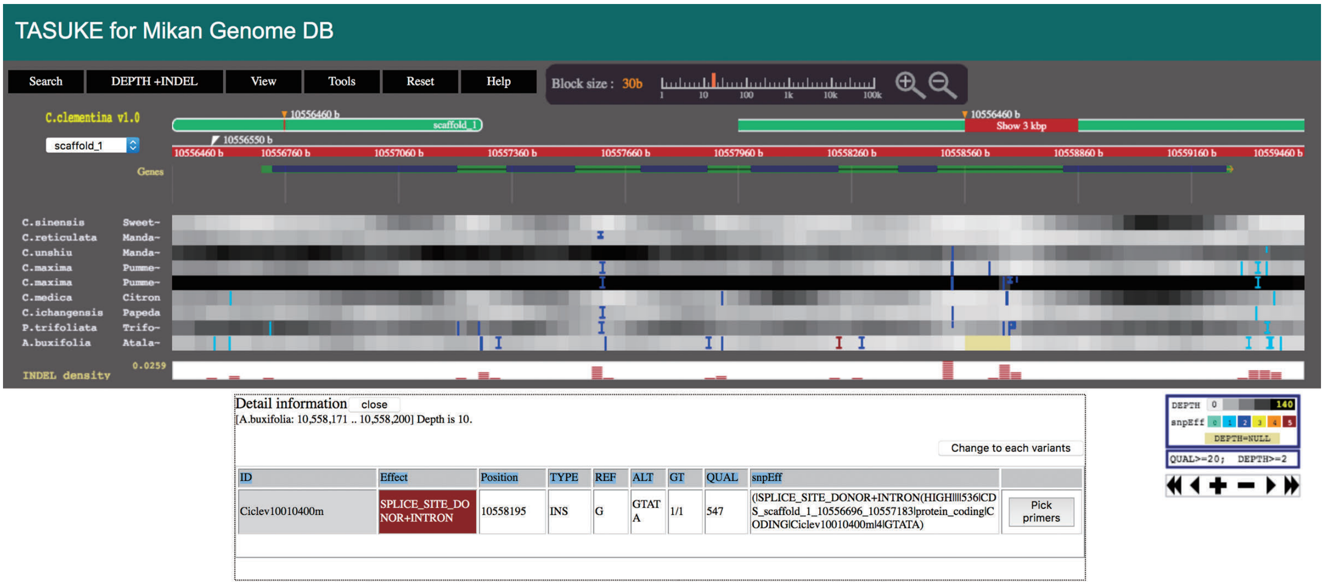
Number	Sequences producing significant alignments	Total score	E value	Length
1.	C_unshiu_02361:mRNA_1.1	773.90	5.34×10^{-131}	630
2.	C_unshiu_01297:mRNA_2.1	987.98	2.96×10^{-115}	534
3.	P_trifoliata_00306:mRNA_34.1	973.55	1.26×10^{-113}	534
4.	C_unshiu_00219:mRNA_28.1	926.67	1.75×10^{-105}	534
5.	P_trifoliata_00944:mRNA_6.1	932.08	9.05×10^{-103}	534
6.	P_trifoliata_00306:mRNA_32.1	729.58	4.12×10^{-69}	711
7.	C_unshiu_02111:mRNA_3.1	672.39	4.12×10^{-69}	666

Fig. 3. Genome annotation on JBrowse and the BLAST service in MiGD. (A) The top page of MiGD provides links to the genome browser JBrowse, BLAST, the multiple genome browser TASUKE, CAPS marker database, data download page, and so on. (B) Gene and genome annotation of *C. unshiu* and *P. trifoliata* are provided through JBrowse. (C) SequenceServer allows users to perform a sequence similarity search against the genome and gene sequences of *C. unshiu*, *P. trifoliata*, *C. clementina*, and *C. sinensis*.

for each polymorphism, users can obtain variant effect information on whether the sequence variation triggers silent substitution, evolutionary substitution, and frame shift changes (Cingolani *et al.* 2012). A total of 1,225,594 homozygous SNPs (4.6 SNPs/kb) and 168,907 indels (0.6 indels/kb) were detected in *C. unshiu* against the reference genome of *C. clementina*, whereas a total of 4,681,886 SNPs (21.6 SNPs/kb) and 720,315 indels (3.3 indels/kb) were detected in *P. trifoliata* against the reference. A total of heterozygous 3,079,344 SNPs (11.7 SNPs/kb) and 467,683 indels (1.8 InDels/kb) were detected in *C. unshiu* against the reference genome of *C. clementina*, while, a total of 2,450,018 SNPs (11.3 SNPs/kb) and 401,371 indels (1.9 indels/kb) were detected in *P. trifoliata* against the reference. The number of SNPs in *C. unshiu* was almost similar to that in *C. sinensis* (3.6 SNPs/kb) (Xu *et al.* 2013) whereas many SNPs in *P. trifoliata* suggested that *P. trifoliata* was diverged from the general citrus species.

The CAPS marker database possesses the following information on 2,696 CAPS markers: PCR product size, primer sequence, electrophoresis pattern of representative citrus cultivars, the genetic locus of the previously reported genetic maps (Omura *et al.* 2003, Shimada *et al.* 2014), track records of the 384 SNP array genotyping (Fujii *et al.* 2013), cultivar identification (Ninomiya *et al.* 2015, Nonaka *et al.* 2017), and transcript IDs of *C. clementina* and *C. unshiu* draft genomes, although some of this information is not available (**Fig. 5A**).

In citrus, various type of DNA markers, such as simple sequence repeat (SSR), CAPS, SNP and indel, have been developed and have been applied to phylogenetic studies, linkage analysis, GWAS and so on (Fang *et al.* 2018, Fujii *et al.* 2013, Luro *et al.* 2008, Minamikawa *et al.* 2017, Shimada *et al.* 2014, Shimizu *et al.* 2016). Out of these DNA markers, we selected CAPS markers for DNA marker database in MiGD because our developed CAPS markers were gene-based markers that generated from highly conservative ESTs among citrus and related species, moreover,

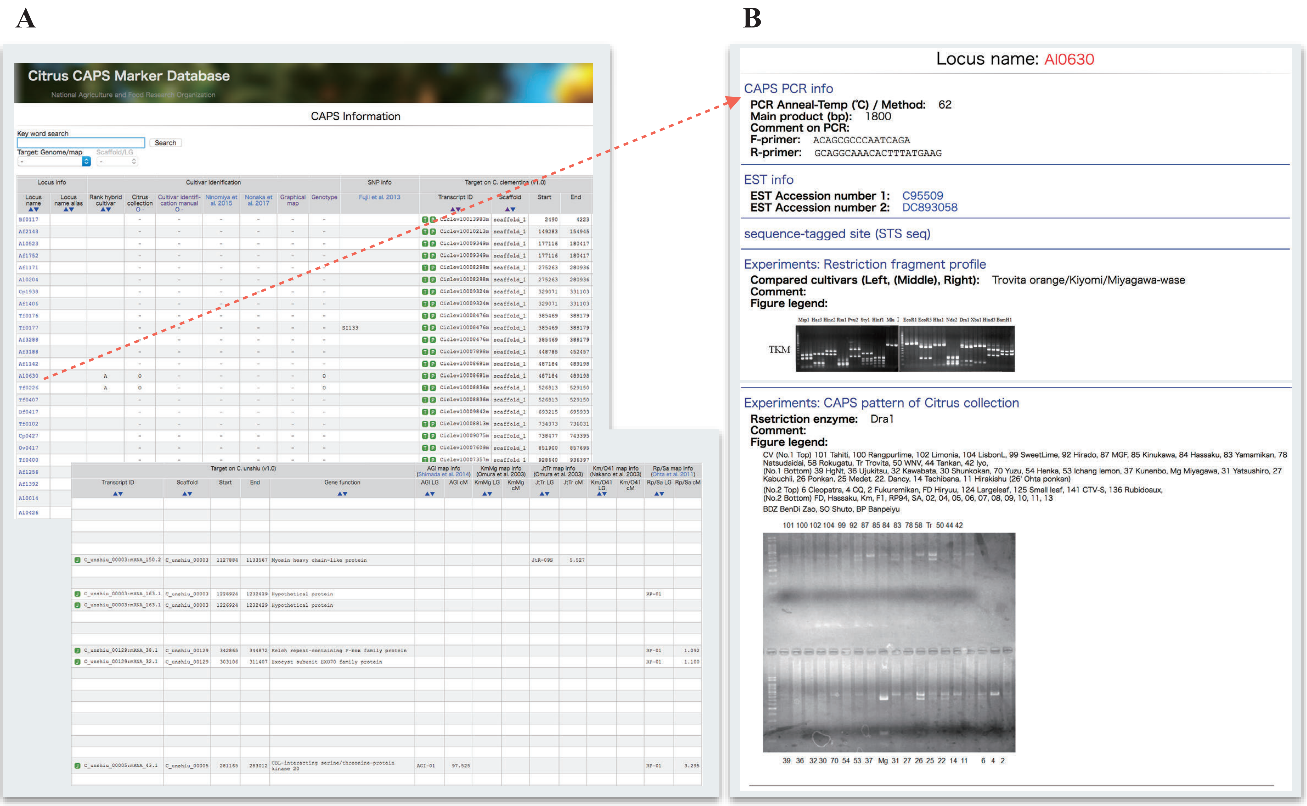


Detail information close
[A.buxifolia: 10,558,171 .. 10,558,200] Depth is 10.

Change to each variants

ID	Effect	Position	TYPE	REF	ALT	GT	QUAL	snpEff	
Ciclev10010400m	SPLICE_SITE_DONOR+INTRON	10558195	INS	G	GTATA	1/1	547	(SPLICE_SITE_DONOR+INTRON(HIGH||||536|CDS_scaffold_1_10556696_10557183|protein_coding|CODING|Ciclev10010400m|4|GTATA)	Pick primers

Fig. 4. Genomic variations between *C. clementina* and nine citrus and related species on TASUKE. Sequence variant information (frequencies of SNPs and indels against the *C. clementina* genome) within each 30 bp block are represented in the top panel. The detailed information of each variant is shown by clicking a block as the bottom panel.


A
B
Citrus CAPS Marker Database
National Agriculture and Food Research Organization
TKM
Msp1 Hae3 Hinc2 Rsa1 Pvu2 Sty1 Hinf1 Mlu I EcoR1 EcoR5 Hha1 Nde2 Dra1 Xba1 Hind3 BamH1
101 100 102 104 99 92 87 85 84 83 78 58 Tr 50 44 42
39 36 32 30 70 54 53 37 Mg 31 27 26 25 22 14 11 6 4 2

CAPS Information

Key word search
Search
Target: Genome/map - Scaffold/LG -

Locus info		Cultivar Idenification							SNP info	Target on C. clementina (v1.0)			
Locus name	Locus name alias	Rank hybrid cultivar	Citrus collection O -	Cultivar identification manual O -	Ninomiya et al. 2015	Nonaka et al. 2017	Graphical map	Genotype	Fujii et al. 2013	Transcript ID	Scaffold	Start	End
Bf0117			-	-	-	-	-	-		Ciclev10013983m	scaffold_1	2490	4223
Af2143			-	-	-	-	-	-		Ciclev10010213m	scaffold_1	149283	154945
Al0523			-	-	-	-	-	-		Ciclev10009349m	scaffold_1	177116	180417
Af1752			-	-	-	-	-	-		Ciclev10009349m	scaffold_1	177116	180417
Af1171			-	-	-	-	-	-		Ciclev10008298m	scaffold_1	275263	280936
Al0204			-	-	-	-	-	-		Ciclev10008298m	scaffold_1	275263	280936
Cp1938			-	-	-	-	-	-		Ciclev10009324m	scaffold_1	329071	331103
Af1406			-	-	-	-	-	-		Ciclev10009324m	scaffold_1	329071	331103
Tf0176			-	-	-	-	-	-		Ciclev10008476m	scaffold_1	385469	388179
Tf0177			-	-	-	-	-	-	SI133	Ciclev10008476m	scaffold_1	385469	388179
Af3288			-	-	-	-	-	-		Ciclev10008476m	scaffold_1	385469	388179
Af3188			-	-	-	-	-	-		Ciclev10007898m	scaffold_1	448785	452457
Af1142			-	-	-	-	-	-		Ciclev10008681m	scaffold_1	487184	489198
Al0630		A	O	-	-	-	-	O		Ciclev10008681m	scaffold_1	487184	489198
Tf0226		A	O	-	-	-	-	O		Ciclev10008836m	scaffold_1	526813	529150
Tf0407			-	-	-	-	-	-		Ciclev10008836m	scaffold_1	526813	529150
Bf0417			-	-	-	-	-	-		Ciclev10009842m	scaffold_1	693215	695933
Tf0102			-	-	-	-	-	-		Ciclev10008813m	scaffold_1	734373	736031
Cp0427			-	-	-	-	-	-		Ciclev10009075m	scaffold_1	738477	743395
Ov0417			-	-	-	-	-	-		Ciclev10007609m	scaffold_1	851900	857695
Tf0400			-	-	-	-	-	-		Ciclev10007357m	scaffold_1	928640	936397
Af1256													
Af1392													
Al0014													
Al0426													

Target on C. unshiu (v1.0)					AGI map info (Shimada et al. 2014)		KmMg map info (Omura et al. 2003)		JtTr map info (Omura et al. 2003)		Km/O41 map info (Nakano et al. 2003)		Rp/Sa map info (Ohta et al. 2011)	
Transcript ID	Scaffold	Start	End	Gene function	AGI LG	AGI cM	KmMg LG	KmMg cM	JtTr LG	JtTr cM	Km/O41 LG	Km/O41 cM	Rp/Sa LG	Rp/Sa cM
C_unshiu_00003:mRNA_150.2	C_unshiu_00003	1127884	1133567	Myosin heavy chain-like protein					JtR-09S	5.527				
C_unshiu_00003:mRNA_163.1	C_unshiu_00003	1226924	1232429	Hypothetical protein									RP-01	
C_unshiu_00003:mRNA_163.1	C_unshiu_00003	1226924	1232429	Hypothetical protein										
C_unshiu_00129:mRNA_38.1	C_unshiu_00129	342865	344872	Kelch repeat-containing F-box family protein									RP-01	1.092
C_unshiu_00129:mRNA_32.1	C_unshiu_00129	303106	311407	Exocyst subunit EXO70 family protein									RP-01	1.100
C_unshiu_00005:mRNA_43.1	C_unshiu_00005	281165	283012	CBL-interacting serine/threonine-protein kinase 20	AGI-01	97.525							RP-01	3.295

Locus name: Al0630

CAPS PCR info
PCR Anneal-Temp (℃) / Method: 62
Main product (bp): 1800
Comment on PCR:
F-primer: ACAGCGCCCAATCAGA
R-primer: GCAGGCAAACACTTTATGAAG

EST info
EST Accession number 1: C95509
EST Accession number 2: DC893058

sequence-tagged site (STS seq)

Experiments: Restriction fragment profile
Compared cultivars (Left, (Middle), Right): Trovita orange/Kiyomi/Miyagawa-wase
Comment:
Figure legend:

Experiments: CAPS pattern of Citrus collection
Rsetriction enzyme: Dra1
Comment:
Figure legend:
CV (No.1 Top) 101 Tahiti, 100 Rangpurlime, 102 Limonia, 104 LisbonL, 99 SweetLime, 92 Hirado, 87 MGF, 85 Kinukawa, 84 Hassaku, 83 Yamamikan, 78 Natsudaidai, 58 Rokugatu, Tr Trovita, 50 WNV, 44 Tankan, 42 Iyo,
(No.1 Bottom) 39 HgNt, 36 Ujukitsu, 32 Kawabata, 30 Shunkokan, 70 Yuzu, 54 Henka, 53 Ichang lemon, 37 Kunenbo, Mg Miyagawa, 31 Yatsushiro, 27 Kabuchii, 26 Ponkan, 25 Medet. 22. Dancy, 14 Tachibana, 11 Hirakishu (26' Ohta ponkan)
(No.2 Top) 6 Cleopatra, 4 CQ, 2 Fukuremikan, FD Hiryuu, 124 Largeleaf, 125 Small leaf, 141 CTV-S, 136 Rubidoaux,
(No.2 Bottom) FD, Hassaku, Km, F1, RP94, SA, 02, 04, 05, 06, 07, 08, 09, 10, 11, 13
BDZ BenDi Zao, SO Shuto, BP Banpeiyu

Fig. 5. CAPS marker information in MiGD. (A) All marker information is listed in the table. (B) The detailed information of each marker is shown by clicking the "Locus name" in the table.

it was confirmed that PCR fragments were stably amplified among major citrus and related species. Therefore, our developed CAPS markers could be linked feasibly to the other databases in MiGD through transcription ID and it is promised to apply them to a wide range of citrus and related species. By clicking the icons on the left side of each transcript ID, users directly jump on the locus on the *C. clementina* and *C. unshiu* draft genomes in JBrowse and TASUKE of MiGD as well as in Phytozome (https://phytozome.jgi.doe.gov). The order of CAPS markers could be sorted by each linkage group of the AGI map or each scaffold of *C. clementina* and *C. unshiu* genome sequences, providing feasible access to the genome sequence around the target genetic locus through CAPS markers. In addition, genotyping data of 37 representative citrus cultivars for 213 CAPS markers, the graphical genotyping map of 35 representative citrus cultivars using 158 CAPS markers, and the genotyping data of 48 citrus cultivar identification using 26 CAPS markers are also available in the database (**Fig. 5B**). The graphical genotyping map is easily interpretable to understand the features of homogenous and heterozygous loci among the examined citrus cultivars. For example, LG7, which corresponded to scaffold 4 of *C. clementina*, revealed frequent homogenous loci in the graphical map of *C. clementina* and *C. unshiu*, whereas LG2 (scaffold 7 of *C. clementina*) and LG5 (scaffold 3 of *C. clementina*) revealed frequent heterozygous loci. These genotyping data among major citrus cultivars are helpful to select the optimal polymorphic CAPS markers for the user's experimental purposes such as cultivar identification, construction of a genetic map, development of a linkage marker, and so on.

Discussion

MiGD is an integrated database of genome annotation, genetic diversity, and CAPS marker information and each database is mutually connected by the transcript ID, which could feasibly compare the locus of the DNA marker in a genetic linkage map with the corresponding locus in the assembled genome sequences of *C. clementina* and *C. unshiu*. This relational database is the first attempt among public citrus databases and MiGD could help users to link the information from the 5 previously generated genetic linkage maps of AGI (Shimada *et al.* 2014), KmMg (Omura *et al.* 2003), JtTr (Omura *et al.* 2003), KmO41 (Nakano *et al.* 2003), and RpSa (Ohta *et al.* 2011) with the draft genome sequences of *C. clementina* and *C. unshiu*. In the genetic diversity database, whole genome sequence data of nine citrus and related species were analyzed, and the detected nucleotide variations and depth of coverages were stored in the TASUKE browser. Citrus species are known to have highly heterozygous genomes with numerous SNPs and indels diverged among species and cultivars. Therefore, we considered that sequence comparison among nine citrus and related species would help determining the functional SNP and indel information around the target locus by removing the null sequence variation and to develop a new DNA marker aiming for mapped based cloning of agronomically important traits. The TASUKE browser has high scalability and we will add additional sequence information of various agronomically important cultivars in the future. Moreover, the optimal DNA marker can be easily selected for cultivar identification of a new cultivar and for the detection of chimera and hybrid embryos in citrus breeding based on the information of genotyping data on CAPS markers among major citrus cultivars. Thus, MiGD would be a useful database to extract the necessary information for the advancing mandarin molecular breeding and cultivar identification for protecting the breeder's rights in Japan, without browsing multiple public databases.

In this study, we reported the first draft genome sequence of *P. trifoliata* assembled by a hybrid *de novo* assembly strategy using Illumina and PacBio data. The total size of the assembled scaffold of *P. trifoliata* is 292 Mb and it almost covers the entire genome sequence as the estimated genome size of *P. trifoliata* is 296 Mb. Interestingly, the assembled genome size of *P. trifoliata* (292 Mb) is smaller than that of *C. clementina* (301 Mb), *C. sinensis* (321 Mb), *C. grandis* (345 Mb), *C. ichangensis* (335 Mb), *C. medica* (368 Mb), and *C. unshiu* (346 Mb), suggesting that the genomic contents of *P. trifoliata* might be similar to those of the wild citrus, *A. buxifolia* (288 Mb). It is clear that the genome structure of *P. trifoliata* is different from cultivated citrus species, particularly in the non-coding region. Through evaluation of genome assemblies by QUAST, the assembled scaffolds of *C. unshiu* and *P. trifoliata* can be aligned to 76.8% and 30.8% of the *C. clementina* reference genome and 93.1% (19,667 + 3,174 partial) and 63.1% (10,628 + 4,862 partial) of 24,533 *C. clementina* reference genes were covered. Enrichment analyses of Gene Ontology and InterPro domains could not fully capture the unique features of *P. trifoliata* specific genes. Therefore, high diversity in the non-coding region is likely to explain the unique responses to biotic and abiotic stresses in *P. trifoliata.* Elucidation of whole-genome sequences is a key milestone for research to explore agronomically and academically important traits specific to the trifoliate orange such as various disease resistances for *Phytophthora citrophthora*, CTV, *Tylenchulus semipenetrans*, cold-hardiness, the aptitude of root stock, and so on. Recently, various research efforts are underway to elucidate the molecular mechanisms and identify the causative gene for cold stress tolerance (Wang *et al.* 2015), Huanglongbing tolerance (Rawat *et al.* 2017), *Phytophthora* disease resistance (Dalio *et al.* 2018) and so on. The genomic resources of *P. trifoliata* in MiGD can contribute to find functional mutations on the genome structure and the specific genes responsible for them.

The first genome assembly of *C. unshiu* was published in 2017 (Shimizu *et al.* 2017). We conducted re-sequencing and re-assembly of the *C. unshiu* genome for enrichment of the draft sequence to develop highly accurate DNA markers. Compared with previously published *C. unshiu* scaffold sequences, the number of scaffolds and N-ratio were clearly reduced to approximately 1/10, whereas the ratio of repeat bases was slightly increased. Enrichment of the draft sequence would be achieved by improving the assembly method by which PacBio subreads were used for contig assembly, scaffolding, and gap-closing steps in the hybrid *de novo* assembly. The genomes of citrus species are known to be highly heterogenous and have an admixture structure. The graphical genotyping maps also showed that numerous heterozygous loci existed throughout the linkage groups among the examined cultivars. Therefore, in the process of hybrid genome assembling, it is predicted that important sequence information located on one side of the haplotype might have fallen off. For example, most polyembryonic citrus cultivars show a heterogenous genotype on the polyembryonic locus and have polyembryonic and monoembryonic alleles (Shimada *et al.* 2018). The genomic structures of the polyembryonic and monoembryonic alleles are very similar except for a miniature inverted-repeat transposable element (MITE) inserted in the promoter region responsible for the transcription of *CitRKD1*. MITE comprises 185 bps of a short sequence element and possibly happened to fall off in the hybrid genome assembly in the previously published draft genome sequences. Recently, the molecular mechanism of various agronomically important traits in fruit trees is becoming apparent owing to the advances in deciphering the genome. Anthocyanin accumulation in blood orange (Butelli *et al.* 2012), grape (Kobayashi *et al.* 2004), and apple (Zhang *et al.* 2019), columnar tree type in apple (Okada *et al.* 2016), and non-melting flesh in stony hard peaches (Tatsuki *et al.* 2018) is controlled by transcriptional regulation mediated by insertion or deletion of transposons. Therefore, the advancement of technologies in assembling hybrid genomes and long sequence reads is extremely important to assign transposons, retrotransposons, and repeat elements to accurate positions in the draft sequences.

The most economically important citrus cultivars originate from repeated natural interspecific hybridization among four ancestral taxa (*C. reticulata*, *C. grandis*, *C. medica* and *C. micrantha*) and possess complex interspecific mosaic genomes (García-Lor *et al.* 2012). Therefore, modern breeding utilizing these conventional cultivars is hampered by these complex heterozygous genomic structures and the typical phenotypes observed in conventional cultivars are frequently broken in their progenies by the admixture of genomes through sexual hybridization (Curk *et al.* 2014). This observation is also applicable to the Japanese breeding program, as the present citrus economic cultivars and breeding lines are originally derived from a limited genetic source of 14 ancestral citrus cultivars by pedigree analysis (Imai *et al.* 2017). Therefore, the most desirable phenotypic traits in economic cultivars do not come only from a specific gene of a specific cultivar but also arise from a desirable allelic combination of commonly possessed genes among ancestral cultivars. Considering this background of the complex citrus genomic structures, whole genome sequencing and structural comparison among various citrus cultivars could be a powerful approach to decipher the admixture genomic structure of current cultivars and would enable us to understand how to build superior traits among them to advance molecular breeding efficiently. The draft sequences of *C. unshiu* and *P. trifoliata* genomes in this study would be useful to explore the genes responsible for the agronomically important traits originated from these major cultivated citruses in Japan. MiGD could accelerate the molecular breeding in citrus to renew major cultivation of the satsuma mandarin.

Author Contribution Statement

Dr. Yoshihiro Kawahara assembled the genome sequence of satsuma mandarin and trifoliate orange to develop genome annotation database, and managed Mikan Genome Database. Dr. Tomoko Endo conducted trifoliate orange genome sequencing. Dr. Mitsuo Omura developed 2,696 CAPS marker and genotyped the major citrus cultivars by CAPS markers. Ms. Yumiko Teramoto developed Mikan Genome Database. Dr. Takeshi Itoh analyzed genome sequence data of satsuma mandarin and trifoliate orange. Dr. Hiroshi Fujii annotated the information of transcription ID, linkage map locus etc. for 2,696 CAPS markers. He is a corresponding author to organize Mikan Genome Database. Dr. Takehiko Shimada conducted satsuma mandarin genome sequencing and is a corresponding author to organize the manuscript.

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