

1 **Genome sequencing and analysis of two early-flowering cherry (*Cerasus* × *kanzakura*) varieties,**
2 **'Kawazu-zakura' and 'Atami-zakura'**

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16 **Running title:** Genomes of early-flowering cherry varieties

17

18 Abstract

19 To gain genetic insights into the early-flowering phenotype of ornamental cherry, also known as sakura, we
 20 determined the genome sequences of two early-flowering cherry (*Cerasus* × *kanzakura*) varieties,
 21 'Kawazu-zakura' and 'Atami-zakura'. Since the two varieties are interspecific hybrids, likely derived from
 22 crosses between *Cerasus campanulata* (early-flowering species) and *Cerasus speciosa*, we employed the
 23 haplotype-resolved sequence assembly strategy. Genome sequence reads obtained from each variety by
 24 single molecule real-time sequencing (SMRT) were split into two subsets, based on the genome sequence
 25 information of the two probable ancestors, and assembled to obtain haplotype-phased genome sequences.
 26 The resultant genome assembly of 'Kawazu-zakura' spanned 519.8 Mb with 1,544 contigs and an N50
 27 value of 1,220.5 kb, while that of 'Atami-zakura' totaled 509.6 Mb with 2,180 contigs and an N50 value of
 28 709.1 kb. A total of 72,702 and 72,528 potential protein-coding genes were predicted in the genome
 29 assemblies of 'Kawazu-zakura' and 'Atami-zakura', respectively. Gene clustering analysis identified 2,634
 30 clusters uniquely presented in the *C. campanulata* haplotype sequences, which might contribute to its
 31 early-flowering phenotype. Genome sequences determined in this study provide fundamental information
 32 for elucidating the molecular and genetic mechanisms underlying the early-flowering phenotype of
 33 ornamental cherry tree varieties and their relatives.

34

35 **Keywords:** early-flowering, genome assembly, haplotype-phased genome sequence, long-read sequencing,
 36 sakura

37

38 Introduction

39 Flowering cherry, called sakura in Japanese, is an ornamental plant popular worldwide. A major *Cerasus* ×
 40 *yedoensis* cultivar 'Somei-Yoshino', which is an interspecific hybrid of *Cerasus spachiana* and *Cerasus*
 41 *speciosa*¹, usually blooms from March to April in Japan. In addition, early-flowering sakura species, such
 42 as *Cerasus campanulata*, usually bloom 1–2 months earlier than 'Somei-Yoshino', and its interspecific
 43 hybrids such as *Cerasus* × *kanzakura* also exhibit early flowering. *C.* × *kanzakura* is considered a hybrid

44 between *C. campanulata* and *Cerasus speciosa* and/or *Cerasus jamasakura*², but its origin is still debated.
 45 Two *C. × kanzakura* cultivars, ‘Kawazu-zakura’ and ‘Atami-zakura’, also bloom early (January and
 46 February, respectively); however, the molecular mechanisms underlying their early-flowering phenotype
 47 remain unknown. Although the mechanisms of early flowering in Rosaceae family members, Japanese
 48 plum (*Prunus mume*) and peach (*Prunus persica*), which flower in February and March, respectively, are
 49 well known³, it remains unclear whether these mechanisms are common between *Cerasus* and *Prunus*.

50 Genome sequence analysis provides information on nucleotide polymorphisms and gene copy
 51 number variation, which can lead to phenotypic differences among individuals and cultivars⁴.
 52 Pan-genomics, which involves de novo genome sequencing of multiple lines within a species, is conducted
 53 to obtain information on variation in all genes within a species to understand the origin of the organism
 54 under study^{5,6}. In ‘Somei-Yoshino’, haplotype-phased genome sequences have been reported, and
 55 comprehensive changes in gene expression during floral bud development that contribute toward flowering
 56 have been revealed by time-course transcriptome analysis⁷. Therefore, comparative genomics of multiple
 57 lines of flowering cherry varieties, such as ‘Kawazu-zakura’, ‘Atami-zakura’, and ‘Somei-Yoshino’, could
 58 provide genetic insights into their early-flowering phenotypes.

59 A trio-binning strategy⁸, previously used in a bovine F1 hybrid to resolve two haplotype-phased
 60 genome sequences, was recently applied to ‘Somei-Yoshino’⁷. Genes associated with the early-flowering
 61 phenotype of ‘Kawazu-zakura’ and ‘Atami-zakura’ were assumed to be encoded by the *C. campanulata*
 62 haplotype sequences. Therefore, in this study, we used the trio-binning strategy to determine the
 63 haplotype-phased sequences of ‘Kawazu-zakura’ and ‘Atami-zakura’. Comparative analysis of three
 64 sakura genomes (‘Kawazu-zakura’, ‘Atami-zakura’, and ‘Somei-Yoshino’) facilitated the identification of
 65 genes unique to the *C. campanulata* haplotype sequences of ‘Kawazu-zakura’ and ‘Atami-zakura’ as
 66 candidates responsible for the early-flowering phenotype of these varieties.

67

68 **Materials and methods**

69 *Plant materials and DNA extraction*

Two early-flowering cherry (*Cerasus* × *kanzakura*) varieties, 'Kawazu-zakura' and 'Atami-zakura', were used in this study. Both varieties were planted at the orchard of Kyoto Prefectural University (Kyoto, Japan). Genome DNA was extracted from young leaves by a modified sodium dodecyl sulfate (SDS) method⁹.

Genome size estimation

Software tools used for data analyses are listed in Supplementary Table S1. Genome libraries for short-read sequencing were prepared with the TruSeq DNA PCR-Free Sample Prep Kit (Illumina, San Diego, CA, USA), and sequenced on the NextSeq 500 platform (Illumina, San Diego, CA, USA) in paired-end, 150 bp mode. The genome size was estimated with Jellyfish.

De novo genome sequence assembly and reference-guided contig ordering and orientation

Genomes of the two cherry varieties were sequenced using the single molecule real-time (SMRT) sequencing technology. Long-read DNA libraries were constructed using the SMRTbell Express Template Prep Kit 2.0 (PacBio, Menlo Park, CA, USA) and sequenced on SMRT cells (1M v3 LR) in a PacBio Sequel system (PacBio). Raw sequence reads of each variety were divided into two subsets with the trio-binning strategy⁸ using the short-read data of *C. campanulata* ('Kanhi-zakura') and *C. speciosa* ('Ohshima-zakura') (DDBJ sequence archive accession no.: DRA008096)⁷. The sequence read subsets were assembled separately with Falcon or Canu to build haplotype-phased diploid genome sequences. Sequence errors in the contigs were corrected twice using long reads with ARROW. Potential contaminating sequence reads from organelle genomes were identified by alignments with the chloroplast and mitochondrial genome sequences of *Prunus avium* (GenBank accession no.: MK622380 and MK816392) with Minimap2, and then removed from the final assemblies. Haplotype-phased sequences, based on binning with *C. campanulata* and *C. speciosa*, were aligned against the *C. spachiana* and *C. speciosa* haplotype sequences, respectively, of the 'Somei-Yoshino' genome using Ragoo to build pseudomolecule sequences. Genome sequences were compared with D-Genies.

96

97 *Gene prediction and repetitive sequence analysis*

98 Potential protein-coding genes were predicted with the MAKER pipeline, which was based on peptide
99 sequences predicted from the genome sequences of sweet cherry (PAV_r1.0)¹⁰, peach (v2.0.a1)¹¹, and
100 Japanese plum¹². Short genes (<300 bp) as well as genes predicted with an annotation edit distance (AED)
101 greater than 0.5, which is proposed as a threshold for good annotations in the MAKER protocol, were
102 removed to facilitate the selection of high-confidence (HC) genes. Functional annotation of the predicted
103 genes was performed with Hayai-Annotation Plants. Gene clustering was performed with OrthoFinder.

104 Repetitive sequences in the pseudomolecules were identified with RepeatMasker using repeat
105 sequences registered in Repbase and a *de novo* repeat library built with RepeatModeler. The identified
106 repetitive sequences were classified into nine types, in accordance with RepeatMasker: short interspersed
107 nuclear elements (SINEs), long interspersed nuclear elements (LINEs), long terminal repeat (LTR)
108 elements, DNA elements, small RNAs, satellites, simple repeats, low complexity repeats, and unclassified.

109

110 **Results and data description**

111 *De novo assembly of ‘Kawazu-zakura’ and ‘Atami-zakura’ genomes*

112 Short reads amounting to 64.0 and 127.7 Gb were obtained for ‘Kawazu-zakura’ and ‘Atami-zakura’,
113 respectively. The genome sizes of ‘Kawazu-zakura’ and ‘Atami-zakura’ were estimated at 672.7 and 675.2
114 Mb, respectively (Figure 1). Since ‘Kawazu-zakura’ and ‘Atami-zakura’ are interspecific hybrids, we used
115 a trio-binning strategy to establish haplotype-resolved genome assemblies representing each parental
116 genome sequence.

117 Long-read data (34.9 Gb) of ‘Kawazu-zakura’ obtained from two SMRT cells were divided into two
118 subsets (17.2 and 17.6 Gb), in accordance with the short-read data of potential parental species, *C.*
119 *campanulata* and *C. speciosa*⁷, respectively. Reads in each subset were independently assembled with
120 Falcon to construct contigs representing the two haplotype sequences. Potential errors in the haplotype
121 sequences were corrected with long reads, and sequences of organelle genomes were removed to obtain the

final assembly of the diploid genome of ‘Kawazu-zakura’. The resulting assemblies consisted of *C. campanulata* (262.2 Mb, N50 = 1.4 Mb) and *C. speciosa* (257.6 Mb, N50 = 1.1 Mb) haplotypes (Table 1), and were designated as KWZcam_r1.0 and KWZspe_r1.0, respectively. Although the total assembly size was shorter than the estimated size, the complete BUSCO scores of KWZcam_r1.0 and KWZspe_r1.0 were 93.1% and 96.7%, respectively, indicating that the assemblies were complete (Table 1). The two assemblies were merged to generate KWZ_r1.0, with a complete BUSCO score of 98.0%.

The ‘Atami-zakura’ genome was sequenced in parallel with the ‘Kawazu-zakura’ genome. Long-read data of ‘Atami-zakura’ (14.3 Gb) were obtained from two SMRT cells, and divided into two subsets (7.4 and 6.8 Gb) using the short-read data of *C. campanulata* and *C. speciosa*⁷, respectively. The reads were assembled with Falcon to generate two haplotype contig sequences. However, the sizes of the two assemblies (139.9 and 110.0 Mb) were much smaller than the estimated sizes. Therefore, we used Canu to obtain long assemblies. This was followed by potential sequence error correction and organelle genome sequence removal. The sizes of the resultant assemblies were improved to 267.4 Mb (N50 = 853.5 kb) and 242.2 Mb (N50 = 569.4 Mb) for the *C. campanulata* and *C. speciosa* haplotypes, respectively (Table 1), and the assemblies were designated as ATMcam_r1.0 and ATMspe_r1.0, respectively. The complete BUSCO scores were 93.4% and 93.5% for ATMcam_r1.0 and ATMspe_r1.0, respectively (Table 1), and 98.2% for the merged assembly (ATM_r1.0).

Reference-guided pseudomolecule sequence construction

Since the genome structures are well conserved across the *Cerasus* and *Prunus* species⁷, we used the two haplotype pseudomolecule sequences of the ‘Somei-Yoshino’ genome, CYEspachiana_r3.1 and CYEspeciosa_r3.1, as references to establish the pseudomolecule sequences of ‘Kawazu-zakura’ and ‘Atami-zakura’. A total of 777 and 746 contigs of KWZcam_r1.0 and KWZspe_r1.0, respectively, were aligned against CYEspachiana_r3.1 and CYEspeciosa_r3.1 sequences, respectively. The lengths of the resultant ‘Kawazu-zakura’ pseudomolecule sequences were 256.7 Mb (KWZcam_r1.0) and 246.5 Mb (KWZspe_r1.0) (Table 2). On the other hand, 1,110 ATMcam_r1.0 and 1,041 ATMspe_r1.0 contigs were

aligned with the CYEspachiana_r3.1 and CYEspeciosa_r3.1 sequences, respectively, and the lengths of the ‘Atami-zakura’ pseudomolecule sequences obtained were 261.5 Mb (ATMcam_r1.0) and 238.9 Mb (ATMspe_r1.0) (Table 2). The pseudomolecule sequences of ‘Kawazu-zakura’ and ‘Atami-zakura’ genomes covered the entire genome sequence of ‘Somei-Yoshino’ (Figure 2).

Gene and repetitive sequence predictions

A total of 36,264 and 36,264 HC protein-coding genes were predicted in KWZcam_r1.0 and KWZspe_r1.0 assemblies, respectively (Table 2). The complete BUSCO scores of genes in the KWZcam_r1.0 and KWZspe_r1.0 were 88.3% and 86.6%, respectively, while the BUSCO score of all 72,582 genes was 97.0%. Functional gene annotation revealed that 9,430, 17,907, and 12,603 sequences were assigned to Gene Ontology (GO) slim terms in the biological process, cellular component, and molecular function categories, respectively, and 2,264 genes had enzyme commission numbers.

On the other hand, 36,281 and 36,421 HC genes were predicted in ATMcam_r1.0 and ATMspe_r1.0 assemblies, respectively (Table 2). Complete BUSCOs of genes in ATMcam_r1.0 and ATMspe_r1.0 were 88.3% and 86.6%, respectively, while that of all 72,702 genes was 96.8%. According to the functional gene annotation, 9,836, 18,586, and 13,020 sequences were assigned to GO slim terms in the biological process, cellular component, and molecular function categories, respectively, and 2,301 genes had enzyme commission numbers.

Repeat sequences occupied varying proportions of the different genome assemblies: 48.0% (KWZcam_r1.0), 45.7% (KWZspe_r1.0), 47.7% (ATMcam_r1.0), and 43.2% (ATMspe_r1.0). LTR elements were the most abundant repetitive sequences (15.1–17.7%), followed by unclassified repeats (12.7–13.7%) and DNA transposons (11.1–13.2%) (Table 3).

Clustering analysis of flowering time related genes in cherry varieties

Four sets of genes predicted in the haplotype-phased genomes of ‘Kawazu-zakura’ and ‘Atami-zakura’ clustered with two sets of genes in the two haploid sequences of ‘Somei-Yoshino’. A total of 35,226

clusters were obtained, of which 10,702 were common across all six gene sets. The early-flowering phenotype of *C. × kanzakura* could be explained by genes uniquely present in the *C. campanulata* haplotype sequences. In the *C. campanulata* haplotype sequences of ‘Kawazu-zakura’ and ‘Atami-zakura’ genomes, a total of 2,634 clusters were found to include 3,113 and 3,123 genes, respectively, suggesting that these genes might be associated with the early-flowering phenotype of ‘Kawazu-zakura’ and ‘Atami-zakura’.

Conclusion and future perspectives

Here, we report haplotype-phased genome assemblies of two early-flowering cherry (*C. × kanzakura*) cultivars, ‘Kawazu-zakura’ and ‘Atami-zakura’, both of which are interspecific hybrids derived from *C. campanulata* and *C. speciosa*. Although the origin of *C. × kanzakura* remains unclear, *C. campanulata* and *C. speciosa* and/or *C. jamasakura* are considered as its potential parents². Another possibility is that ‘Atami-zakura’ originated from *C. jamasakura* and *C. campanulata*¹³. This is supported by the fact that our attempt to divide the long reads of ‘Atami-zakura’ into two subsets using short-read data of *C. serrulata* (closely related to *C. jamasakura*⁷) and *C. campanulata* failed (data not shown). Therefore, we used short reads of *C. campanulata* and *C. speciosa* for both ‘Kawazu-zakura’ and ‘Atami-zakura’. This result suggests that both ‘Kawazu-zakura’ and ‘Atami-zakura’ are closely related to *C. campanulata* and *C. speciosa*.

Clustering analysis of genes predicted in the genomes of ‘Kawazu-zakura’ and ‘Atami-zakura’ together with those of ‘Somei-Yoshino’ revealed that 2,634 gene clusters were uniquely present in the genome of *C. campanulata* but absent from the genomes of *C. spachiana* and *C. speciosa*. Such copy number variation (or presence/absence variation) of genes could explain the early-flowering phenotype of ‘Kawazu-zakura’ and ‘Atami-zakura’. Previously, we performed a time-course transcriptome analysis of the floral buds and flowers of ‘Somei-Yoshino’ to clarify gene expression patterns during flowering⁷. A similar time-course transcriptome analysis could be applied to ‘Kawazu-zakura’ and ‘Atami-zakura’. Comparative transcriptome analysis of three cultivars could identify the genes responsible for the

early-flowering phenotype of sakura. Furthermore, comparative transcriptome analysis of Japanese apricot and peach³ could reveal the genetic mechanisms controlling flowering time across all *Prunus* and *Cerasus* species.

Although several flowering cherry cultivars are known to bloom in late-spring, fall, and winter seasons⁷, genome sequences of only a few of these cultivars are publicly available^{7,14,15}. Comparative genomics and transcriptomics, also known as pan-genomics⁴⁻⁶, of sakura would provide insights into the origins of these cultivars and their flowering mechanisms, which could facilitate the development of new cultivars with attractive flower characteristics and provide us with the ability to forecast the date of sakura blooming.

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Data availability

Sequence reads are available from the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (DRA) database (accession no.: DRA012553). The DDBJ accession numbers of assembled sequences are BPUM01000001–BPUM01000783 (KWZcam_r1.0), BPUM01000784–BPUM01001544 (KWZspe_r1.0), BPUL01000001–BPUL01001124 (ATMcam_r1.0), and BPUL01001125–BPUL01002180 (ATMspe_r1.0). The genome sequence information generated in this study is available at Plant GARDEN (<https://plantgarden.jp>).

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Conflict of interest

226 None declared.

227

228 **Supporting information**

229 **Supplementary Table S1** Software tools used for genome assembly and gene prediction.

230

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271 **Table 1** Statistics of the contig sequences of two flowering cherry (*Cerasus × kanzakura*) cultivars, ‘Kawazu-zakura’ and ‘Atami-zakura’

	KWZ_r1.0	KWZcam_r1.0	KWZspe_r1.0	ATM_r1.0	ATMcam_r1.0	ATMspe_r1.0
Total contig size (bases)	519,843,677	262,196,010	257,647,667	509,633,549	267,393,285	242,240,264
Number of contigs	1,544	783	761	2,180	1,124	1,056
Contig N50 length (bases)	1,220,495	1,445,144	1,108,133	709,113	853,547	569,444
Longest contig size (bases)	8,019,066	5,955,677	8,019,066	5,799,312	5,799,312	3,381,444
Gap (bases)	0	0	0	0	0	0
Complete BUSCOs	98.2%	93.1%	96.7%	98.0%	93.4%	93.5%
Single-copy BUSCOs	7.5%	86.7%	89.0%	16.0%	86.5%	87.8%
Duplicated BUSCOs	90.7%	6.4%	7.7%	82.0%	6.9%	5.7%
Fragmented BUSCOs	0.3%	0.7%	0.4%	0.4%	0.7%	1.6%
Missing BUSCOs	1.5%	6.2%	2.9%	1.6%	5.9%	4.9%
#Genes	72,702	36,281	36,421	72,528	36,264	36,264

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Table 2 Statistics of the pseudomolecule sequences of flowering cherry (*C. × kanzakura*) cultivars, ‘Kawazu-zakura’ and ‘Atami-zakura’

		‘Kawazu-zakura’						‘Atami-zakura’					
Chrom.		Total length	%	Number of contigs	%	Number of genes	%	Total length	%	Number of contigs	%	Number of genes	%
<i>C. campanulata</i> haplotype	1	38,834,322	14.8	86	11.0	5,173	14.3	37,943,013	14.2	123	10.9	5,180	14.3
	2	45,216,402	17.2	216	27.6	6,881	19.0	49,279,926	18.4	289	25.7	6,710	18.5
	3	28,286,294	10.8	74	9.5	3,837	10.6	29,043,603	10.9	108	9.6	3,885	10.7
	4	31,150,796	11.9	110	14.0	3,747	10.3	33,258,136	12.4	157	14.0	4,442	12.2
	5	28,296,805	10.8	82	10.5	3,852	10.6	26,412,617	9.9	83	7.4	3,625	10.0
	6	33,630,106	12.8	83	10.6	4,908	13.5	34,947,855	13.1	132	11.7	4,871	13.4
	7	20,603,721	7.9	61	7.8	2,702	7.4	21,052,878	7.9	92	8.2	3,003	8.3
	8	30,708,646	11.7	65	8.3	4,559	12.6	29,538,870	11.0	126	11.2	3,745	10.3
	Unassigned	5,546,318	2.1	6	0.8	622	1.7	6,027,887	2.3	14	1.2	803	2.2
	Total	262,273,410	100.0	783	100.0	36,281	100.0	267,504,785	100.0	1,124	100.0	36,264	100.0
<i>C. speciosa</i> haplotype	1	42,661,824	16.6	82	10.8	5,912	16.2	38,473,692	15.9	136	12.9	5,644	17.0
	2	33,947,397	13.2	125	16.4	4,804	13.2	32,131,565	13.3	179	17.0	4,495	13.5
	3	29,126,079	11.3	81	10.6	4,367	12.0	30,781,260	12.7	111	10.5	4,269	12.8
	4	34,989,196	13.6	162	21.3	4,668	12.8	35,466,104	14.6	214	20.3	4,532	13.6
	5	25,691,272	10.0	99	13.0	3,729	10.2	23,383,776	9.6	100	9.5	3,062	9.2
	6	29,111,560	11.3	56	7.4	4,240	11.6	32,639,480	13.5	120	11.4	4,532	13.6
	7	16,872,426	6.5	20	2.6	2,328	6.4	16,033,168	6.6	40	3.8	2,174	6.5
	8	34,141,902	13.2	121	15.9	5,125	14.1	30,022,323	12.4	141	13.4	4,075	12.3
	Unassigned	11,181,211	4.3	15	2.0	1,248	3.4	3,413,596	1.4	15	1.4	481	1.4
	Total	257,722,867	100.0	761	100.0	36,421	100.0	242,344,964	100.0	1,056	100.0	33,264	100.0

Table 3 Repetitive sequences in two flowering cherry (*C. × kanzakura*) cultivars, ‘Kawazu-zakura’ and ‘Atami-zakura’

Repeat type	‘Kawazu-zakura’						‘Atami-zakura’					
	<i>C. campanulata</i> haplotype			<i>C. speciosa</i> haplotype			<i>C. campanulata</i> haplotype			<i>C. speciosa</i> haplotype		
	Number of elements	Length occupied (bp)	%	Number of elements	Length occupied (bp)	%	Number of elements	Length occupied (bp)	%	Number of elements	Length occupied (bp)	%
SINEs	5,278	495,207	0.2	7,013	665,451	0.3	8,832	896,223	0.3	6,537	608,541	0.3
LINEs	9,358	3,548,357	1.4	9,980	3,635,040	1.4	9,242	3,175,048	1.2	9,285	3,460,432	1.4
LTR elements	63,025	45,423,275	17.3	57,175	42,749,594	16.6	61,503	47,443,444	17.7	52,221	36,517,551	15.1
DNA transposons	85,647	33,936,563	12.9	84,151	30,984,015	12.0	88,999	35,176,601	13.2	77,636	26,829,236	11.1
Unclassified	131,199	36,041,455	13.7	116,201	32,825,941	12.7	130,209	34,407,194	12.9	112,663	31,370,494	12.9
Small RNA	5,384	657,326	0.3	7,211	1,536,541	0.6	6,949	828,201	0.3	2,598	503,911	0.2
Satellites	1,072	277,222	0.1	297	53,425	0.0	1,083	399,307	0.2	342	75,860	0.0
Simple repeats	75,567	3,104,558	1.2	77,082	3,144,046	1.2	77,750	3,266,196	1.2	74,414	3,048,442	1.3
Low complexity	14,265	706,271	0.3	14,754	717,629	0.3	14,352	695,481	0.3	14,137	693,339	0.3

276 **Figure legends**

277 **Figure 1** Estimation of the genome size of two flowering cherry (*Cerasus* × *kanzakura*) varieties,
278 ‘Kawazu-zakura’ and ‘Atami-zakura’, based on *k*-mer analysis ($k = 17$), with the given multiplicity
279 values.

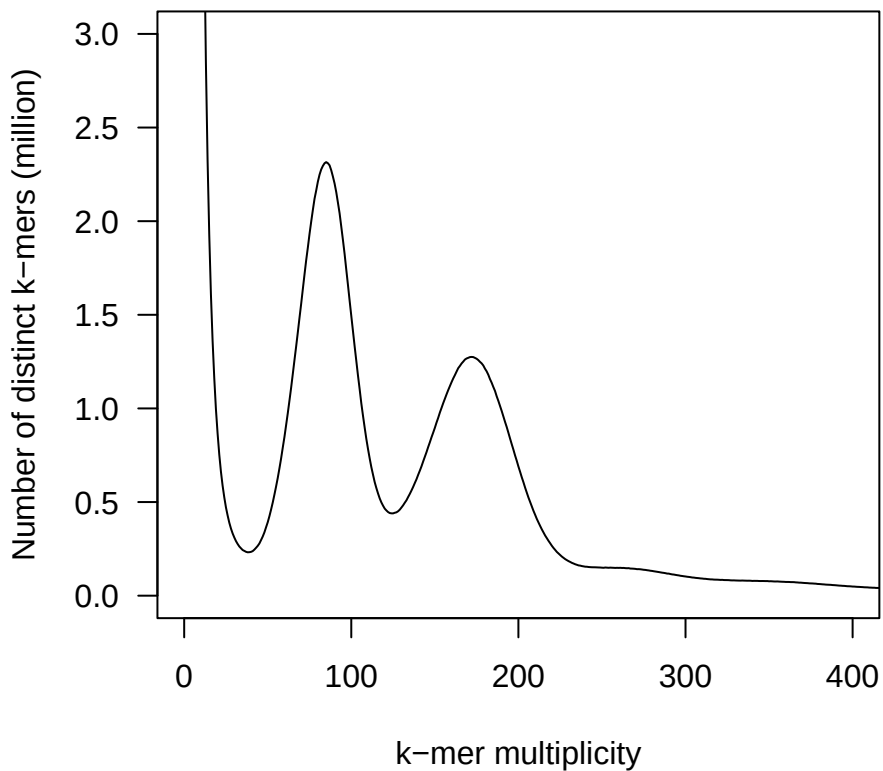
280

281 **Figure 2** Comparative analysis of the genome sequence and structure of flowering cherry varieties,
282 ‘Atami-zakura’, ‘Kawazu-zakura’, and ‘Somei-Yoshino’.

283 Chromosome numbers are indicated above the x-axis and on the right side of the y-axis. Genome
284 sizes (Mb) are below the x-axis and on the left side of the y-axis.

285

'Kawazu-zakura'



'Atami-zakura'

