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A Key Study on Pollen-Specific *SFB* Genotype and Identification of Novel *SFB* Alleles from 48 Accessions in Japanese Apricot (*Prunus mume* Sieb. et Zucc.)

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Abstract: Self-incompatibility (SI) is a common strategy to avoid inbreeding and, consequently, keep genetic diversity within a species. In its mechanism, pollen rejection happens in the style when the single multiallelic locus (*SFB* in *prunus* species) of the haploid pollen matches one of the S-alleles existing in the diploid pistil. The *SFB* gene for the pollen S gene has been identified in many *Prunus* species. However, Japanese apricot is a species with a typical gametophytic self-incompatibility (GSI), and its *SFB* alleles available are limited, although they are required for studying GSI. Therefore, we used an AS-PCR amplification method, sequencing, and the pair primers SFB-C1F and Pm-Vb designed based on the conserved region of the *Prunus SFB* gene to identify *SFB* genotypes of 48 Japanese apricot (*P. mume*) accessions. Eleven novel *SFB* alleles were isolated from these accessions and shared typical structural features with *SFB* alleles from other *Prunus* species. These novel *SFB* alleles were uniquely expressed in pollen. Hence, we concluded that these 11 *PmSFB* were pollen S determinants of *P. mume*. This current study offers the novel *SFB* genes of the *P. mume S* locus, which could be a useful potential resource for studies on pollen SI mechanisms.

Keywords: Japanese apricot; self-incompatibility; *Pm SFB*; *SFB* genotype; PCR

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1. Introduction

Self-incompatibility (SI) is the most widespread strategy that flowering plants use to avoid self-fertilization and promote outcrossing [1,2], which has been identified in almost half of blooming plants [3]. However, this system has only been studied in a limited number of families in which the fundamental molecular and genetic aspects are involved, and they have only been slightly characterized in detail in the gametophytic self-incompatibility (GSI) system, and in the sporophytic self-incompatibility (SSI) system [4]. In SSI, the genotype of the diploid parental plant (sporophyte), which acts as pollen donor, regulates the incompatibility type, while in GSI, the genotype of the haploid pollen itself (gametophyte) controls its incompatibility system [5,6].

However, GSI is the most prevalent in Plantaginaceae, Solanaceae, and Rosaceae, in which it is controlled via a single multiallelic locus (S locus) containing two related genes: the female part (pistil) pistil determinant S-ribonuclease gene (*S-RNase* gene) and the male

part (pollen) pollen S determinant (s) encoding an F-box protein [7–10]. The male part determinant is termed as *SLF* (S-locus F-box) in Plantaginaceae and in Solanaceae [11,12]; *SFBB* (S-locus F-box brothers) in the subtribe Malinae (Pyrus in Rosaceae) [13]; and *SFB* (S haplotype-specific F-box protein) in *Prunus* (Rosaceae) [14–16].

Subsequent to the first identification of *S-RNase* in the Solanaceae family [17], a key candidate gene for the male part was discovered in several *Prunus* species named *SFB*, including almond [18,19], sweet cherry [20–24], *P. armeniaca* [16,25–27], and *P. mume* [20,28,29]. However, *SFB* features including pollen-specific expression, a high level of allelic polymorphism, and a close physical proximity to *S-RNase*, are suitable for the pollen S gene [18,28,30]. The physical relationship of *SFB* gene with *S-RNase* has been established in other *Prunus* species including *P. mume* [28,31] and sweet and sour cherries [20,32]. Subsequently, the physical distance between *SFB* and *S-RNase* alleles in sweet cherry [24,33,34], Japanese plum [35,36], apricot [16,25,27], and Chinese cherry [37] has been established. One of the largest genus in the Rosaceae family is *Prunus* L., with over 200 species of evergreen and deciduous trees and shrubs that produce valuable fruits and nuts, [38], and most of them exhibit Gametophytic System (GSI).

Japanese apricot is an important stone fruit and an ornamental tree of this genus, that originates from China [39], which is sympathetic to typical GSI species [29]. *P. mume* blooms very early in the spring when pollination is severely restricted by several constraints (such as available insects, weather, and pollinizers). Thus, it is very important to determine the correct S haplotypes of cultivars/accessions. In *P. mume*, numerous *S-RNase* alleles have been reported [29,40–42] and Entani et al. [28] first discovered its pollen gene (S haplotype-specific F-box protein); then, other researchers reported the *SFB* alleles in a few cultivars [43]. To explore the GSI system in *P. mume*, the availability of *SFB* genes is sufficient, and its genotyping in several accessions are required. In this study, we identified the *SFB* genotype and novel *SFB* alleles of forty-eight *P. mume* accessions. These might contribute to the improvement of *SFB* alleles' basic data, which would be useful for the in-depth research aimed at understanding the self-(in)compatibility mechanism.

2. Materials and Methods

2.1. Materials

Forty-eight *P. mume* accessions from the National Field Gene-Bank for Japanese apricot in Nanjing, China were used in this research (Table 1). However, young fresh leaves of each accession were collected in spring, while styles, and pollen were collected from 'Sichuanbaimei' in winter. All of these plant materials were stored at −80 degrees Celsius until use.

2.2. Methods

2.2.1. DNA and RNA Extraction

The total genomic DNA from each of the 48 *P. mume* accessions was extracted from young fresh leaves using a modified CTAB method [44], treated with RNase (TaKaRa, Kyoto, Japan), and incubated at 37 °C for an hour. Subsequently, the extracted DNA concentration was determined with a BioPhotometer (Eppendorf, Hamburg, Germany), and its integrity was verified via electrophoresis.

Total RNA was extracted from young fresh leaves, styles, and pollen of 'Sichuanbaimei' according to Tao R. et al. [45], and then treated with gDNA Eraser (TaKaRa). The extracted RNA concentration was determined with a BioPhotometer, and its integrity was checked via electrophoresis.

2.2.2. PCR Amplification of SFB Alleles

An AS-PCR amplification method was used according to the protocols outlined in Junxia. X. et al. [46], to amplify *SFB* alleles using the primer pair SFB-C1F[RTTCGRTTTCTD TTTACRTG] [31] and Pm-Vb[ATCCAAGCAAGTTCTTGAAACA] [43]. However, PCR was carried out in a 25 µL reaction volume having 70 ng of genomic DNA, 2.0 µL of 10×

PCR buffer (TaKaRa), 1.5 mM $MgCl_2$, 0.15 mM dNTPs, 0.1 μM of each primer, and 1 U of Taq DNA polymerase (TaKaRa) in a PTC-100 thermal cycler (MJ Research, Cambridge, MA, USA). Then, a program of 35 cycles at 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min 30 s, with an initial denaturation of 94 °C for 3 min, and a final extension of 72 °C for 10 min, were used to run PCRs. PCR products were separated by 1.5% agarose gel electrophoresis in a 1× TAE buffer, and we observed the bands under ultraviolet light.

Table 1. *SFB* genotypes of 48 *P. mume* accessions.

Cultivars	*SFB* Genes	Novel Genes	Accession Numbers	Origin
Meilinhuang	SFB_2/SFB_{42}			Zhejiang
Changnong No. 17	SFB_{14}/SFB_{42}			Zhejiang
Changxing No. 1	SFB_{18}/SFB_{42}			Zhejiang
Changxing No. 2	SFB_{14}/SFB_{41}			Zhejiang
Changxing No. 3	SFB_7/SFB_{54}	SFB_{54}	MW186470	Zhejiang
Changxing No. 4	SFB_7/SFB_{42}			Zhejiang
Changxing No. 5	SFB_2/SFB_{42}			Zhejiang
Changxing No. 6	SFB_{12}/SFB_{41}			Zhejiang
Xianjulvmei	SFB_2/SFB_{41}			Zhejiang
Longyoubaimei	SFB_2/SFB_{14}			Zhejiang
Lizimei	SFB_2/SFB_{41}			Zhejiang
Hongding	SFB_{18}/SFB_{42}			Zhejiang
Xinbaimei	SFB_{24}/SFB_{31}			Guangdong
Puningqingzhumei	SFB_{24}/SFB_{41}			Guangdong
Guangdonghuangpi	SFB_{43}/SFB_{56}	SFB_{56}	MW186472	Guangdong
Daheqing	SFB_{24}/SFB_{43}			Guangdong
Huanghoumei	SFB_{24}/SFB_{31}			Guangdong
Dalizhong	SFB_{12}/SFB_{55}			Guangdong
Henghe	SFB_2/SFB_{42}			Guangdong
Yuanjiangroumei	SFB_2/SFB_{14}			Hunan
Yuanjiangdaqing	SFB_2/SFB_{47}			Hunan
Siyuemei	SFB_{14}/SFB_{18}			Hunan
Yunnanyanmei	SFB_{14}/SFB_{31}			Yunnan
Yunnanzhaoshuimei	SFB_{14}/SFB_{50}	SFB_{50}	MW186466	Yunnan
Zhaoanshuimei	SFB_2/SFB_{14}			Fujian
Fujianqingmei	SFB_{14}/SFB_{44}	SFB_{44}	MW186460	Fujian
Sichuanbaimei	SFB_{31}/SFB_{52}	SFB_{52}	MW186468	Sichuan
Sichuanhuangmei	SFB_{12}/SFB_{24}			Sichuan
Xinxiangxingmei	SFB_{14}/SFB_{24}			Henan
Kaidi	SFB_2/SFB_{45}	SFB_{45}	MW186461	Jiangsu
Xiaolve	SFB_1/SFB_{14}			Jiangsu
Yadanmei	SFB_2/SFB_{42}			Jiangsu
Liuliumei No. 1	SFB_2/SFB_{46}	SFB_{46}	MW186462	Jiangsu
Liuliumei No. 2	SFB_{24}/SFB_{56}	SFB_{56}	MW186472	Jiangsu
Nannongfengyan	SFB_{14}/SFB_{31}			Jiangsu
Nannongfengmao	SFB_{12}/SFB_{43}			Jiangsu
Nannonglongfeng	SFB_{31}/SFB_{43}			Jiangsu
Wanhong	SFB_2/SFB_{57}	SFB_{57}	MW786959	Jiangsu
Yunnankumei	SFB_{50}/SFB_{59}	SFB_{59}	MW786961	Yunnan
Hangzhoubaimei	SFB_{42}/SFB_{58}	SFB_{58}	MW786960	Zhejiang
Zhizhimei	SFB_{12}/SFB_{24}			Jiangsu
Fenbanguomei	SFB_2/SFB_7			Hubei
Dongshanlimei	SFB_{14}/SFB_{24}			Jiangsu
Dongqing	SFB_7/SFB_{42}			Zhejiang
Taihu No. 1	SFB_{24}/SFB_{46}			Jiangsu
Taihu No. 3	SFB_2/SFB_{24}			Jiangsu
Huangxiaoda	SFB_1/SFB_{43}			Zhejiang
Lvmei	SFB_{14}/SFB_{31}			Jiangsu

2.2.3. RT-PCR Amplification

The extracted RNA from 'Sichuanbaimei' tissues including leaves, pollen, and styles were reverse-transcribed into cDNAs according to the method of the reverse transcription kit (TaKaRa, Japan). However, RT-PCR was carried out using primers Pru-C2 and PCE-R, and cDNAs as templates following the manufacturer's protocol (TaKaRa, Japan). Specific reactions and procedures were carried out according to the provided descriptions in Junxia X et al. [47]. Primers for the PCR amplification were SFB-C1F and Pm-Vb as above. As in references, RT-PCR was also performed with an actin gene-specific primer pair, ActF1[ATGGTGAGGATATTCAACCC] [18], and ActRI[CTTCCTGTGGACAATGGATGG] [18]. RT-PCR products were separated by 1.5% agarose gel electrophoresis in a 1× TAE buffer, and we observed the bands under ultraviolet light.

2.2.4. PCR Products Sequencing

A DNA purification kit (TaKaRa, Japan) was used to extract/isolate all PCR products and cDNA fragments from 1.2% agarose gels. Refined target products were cloned into the 007 Simple Vector Kit according to the manufacturer's recommendations and transformed into an *Escherichia coli DH5α* cell. At least three positive samples of each target clones were sequenced by Kinco Biotechnology Co., Ltd. Company (Nanjing, China) to acquire accurate and correct sequences and avoid PCR amplification errors.

2.2.5. Sequence and Phylogenetic Analysis

Homology searches were performed using BLAST version +2.6.0 (Altschul, S.F., New York, NY, USA, http://www.ncbi.nlm.nih.gov/BLAST/, accessed on 27 August 2021) [48] program from the National Center for Biotechnology Information (NCBI), and we also determined whether a sequenced gene was a new *SFB* gene. *SFB* gene nucleotide sequence and amino acid sequence alignments were performed using DNAMAN Version V8 software (Lynnon Biosoft, Foster City, CA, USA, https://www.bioz.com/result/doap2%20proteins%20dnaman%20version%208%200%20software/product/Lynnon%20corporation, accessed on 27 August 2021) [49], MEGA X (Kumar, S., Philadelphia, PA, USA, www.megasoftware.net (accessed on 27 August 2021), and Jalview version 2.11.2.0 (Waterhouse, A, Dundee, UK, https://www.jalview.org/download, accessed on 27 August 2021). The online MEME tool: https://meme-suite.org/meme (accessed on 27 August 2021) [50], was used to analyze the proteins' conserved motifs distribution. Using MEGA X [51] software by the neighbor-joining approach [52], and Figtree (version V1.4.4) software (Rambaut, A, Edinburgh, UK, http://tree.bio.ed.ac.uk/, accessed on 27 August 2021), a phylogenetic tree was generated based on the putative amino acid sequences of the S-locus F-box genes in *Prunus*.

3. Results

3.1. SFB Alleles Identification from P. mume Accessions

The *Prunus SFB* primer pairs SFB-C1F and Pm-Vb were designed from conserved regions of *Prunus SFB* including the F-box motif and a downstream region of variable HVb, respectively. However, only one PCR amplification fragment of approximately 1000 bp was obtained from each *P. mume* accession (Figure 1). To identify, the *SFB* alleles from the forty-eight *P. mume* germplasm resources (accessions), a further analysis of the sequences was performed. According to the DNA homologous sequence analysis using DNAMAN (Version V6; Lynnon Biosoft) software [49], these sequences were categorized/classified into 25 types. Moreover, there were 11 novel *SFB* alleles sharing a high homology with the *SFB* alleles identified in other *Prunus* species, which were named pollen-specific *SFB* (*PmSFB*). The sequences of these 11 new *SFB* alleles were logged to the NCBI database by our research group, and their accession numbers were as follows: $PmSFB_{44}$ (MW186460), $PmSFB_{45}$ (MW186461), $PmSFB_{46}$ (MW186462), $PmSFB_{48}$ (MW186464), $PmSFB_{50}$ (MW186466), $PmSFB_{52}$ (MW186468), $PmSFB_{54}$ (MW186470), $PmSFB_{56}$ (MW186472), $PmSFB_{57}$ (MW786959),

PmSFB$_{58}$ (MW786960), and *PmSFB*$_{59}$ (MW786961). The other *PmSFB* alleles had previously been reported. The detailed information is accessible in Table 1.

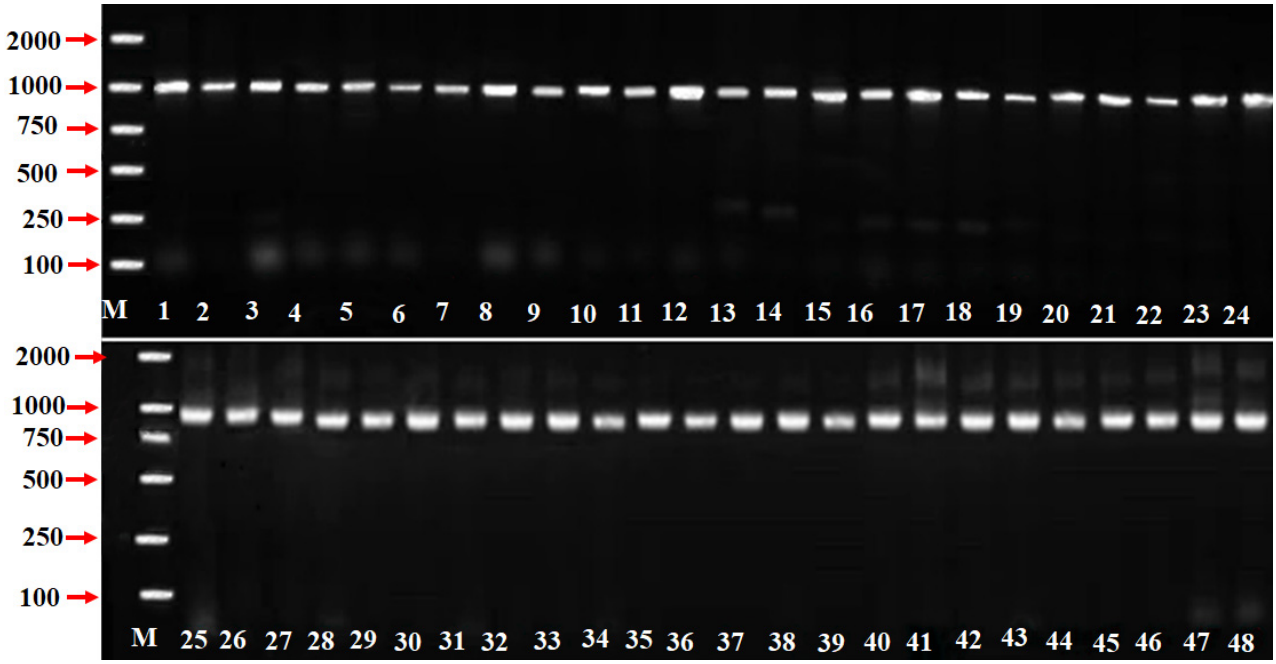

Figure 1. *SFB* alleles AS-PCR amplification in 48 *P. mume* accessions. Lane M = 2000 bp ladder marker, lane 1 to lane 48 represent the accessions in Table 1. Schemes follow the same formatting.

3.2. Specific Expression Analysis of SFB Alleles

To authenticate the Pm SFB isolated/identified in this study, an RT-PCR was conducted with the cDNAs of pollen, leaves, and styles of 'Sichuanbaimei' using SFB-C1F and Pm-Vb to explore the expression patterns of the PmSFB genes. The RT-PCR analysis of the actin gene was carried out using ActF1 and ActRI as positive controls (Figure 2B). Figure 2A (PmSFB) shows that the RT-PCR of pollen cDNA produced a DNA fragment of the same size as that of genomic DNA, but no fragment was amplified from leaf cDNA and style cDNA. The actin-gene-amplified fragments were obtained from genomic DNA, leaf cDNA, style cDNA, and pollen cDNA (Figure 2B). The fragments from leaf cDNA, style cDNA, and pollen cDNA had the same size, and they were shorter compared to the fragment from genomic DNA (the size of the fragment from genomic DNA was different compared to that of the fragments from pollen, style, and leaf) (Figure 2B), implying that the RNA preparations were free from genomic DNA. Consequently, these findings prove that the extracted RNA used in this study was exempted from genomic DNA contamination. As in the instance of other Prunus SFB alleles [53], the PmSFB alleles were exclusively expressed in pollen. The sequencing findings and a comparative analysis of the sequences showed that the amplification fragment of the SFB gene from pollen was identified as two SFB genes.

3.3. Comparative Analysis of SFB Alleles Identified in P. mume and Other Prunus Species

Ikeda et al. [21] were the first to characterize the structural features of the *PmSFB* gene based on a comparative analysis of the amino acid sequences. In the current study, the new 11 *SFB* alleles were similar to those of others *Prunus* species. However, the structure/feature is described as follows: two hypervariable areas (HVa and HVb) situated in the C-terminal zone and variable domains V2 and V1 situated upstream of the nonconserved HVa and downstream of the F-box motif, respectively (Figure 3). The putative amino acid identities of *Pm SFB* sequences ranged from 60.06 to 96.93% (Table 2), while their identities with other *Prunus* species ranged from 61.03 to 98.14% (the similarity between the *PmSFB* alleles and

other *Prunus* species was greater than the identities of the *PmSFB* alleles with each other for the Japanese apricot accessions) (Table 3).

3.4. Conserved Motifs Analysis

To acquire the motif structural composition, arrangement/order of the conserved motifs of *PmSFB* alleles, fourteen protein sequences of the *SFB* genes in Japanese apricot, including the eleven newly identified in this work, were examined for a conserved motif distribution analysis using MEME. The results revealed that the studied gene sequences shared exactly the same direction/order/organization for at least three most common motif structures (3, 2, 1) (Figure 4). Moreover, summary sequence LOGO and regular expression of each motif shared by *SFB* proteins displayed in Figure 5. The distance from motifs 3 and 2 in each amino acid sequence was approximately the same, as between motifs 2 and 1. The above results suggested that these proteins in *P. mume* shared a similar structure and function.

3.5. Phylogenetic Analysis of Pollen S genes (SLFL, SFB) in Prunus Species

To further explore the relationship between S-locus F-box genes in *Prunus* species, we constructed a phylogenetic tree by the neighbor-joining method using the amino acid sequences of 47 S pollen genes, including the *SLFL* and *SFB* genes, from different *Prunus* species (Figure 6). The results indicated that the *SFB* alleles clustered together, whereas all *SLFL* alleles clustered together (*SFB* alleles from different *Prunus* species grouped together, and the same for the *SLFL* genes). Hence, the S-locus F-box genes in *Prunus* species shared a high similarity. In addition, the newly identified 11 *PmSFB* alleles were placed within the *SFB* group and displayed a significant similarity with other *Prunus SFB* alleles. These findings indicated that the novel *PmSFB* alleles were orthologs of the *SFB* alleles in diverse *Prunus* species.

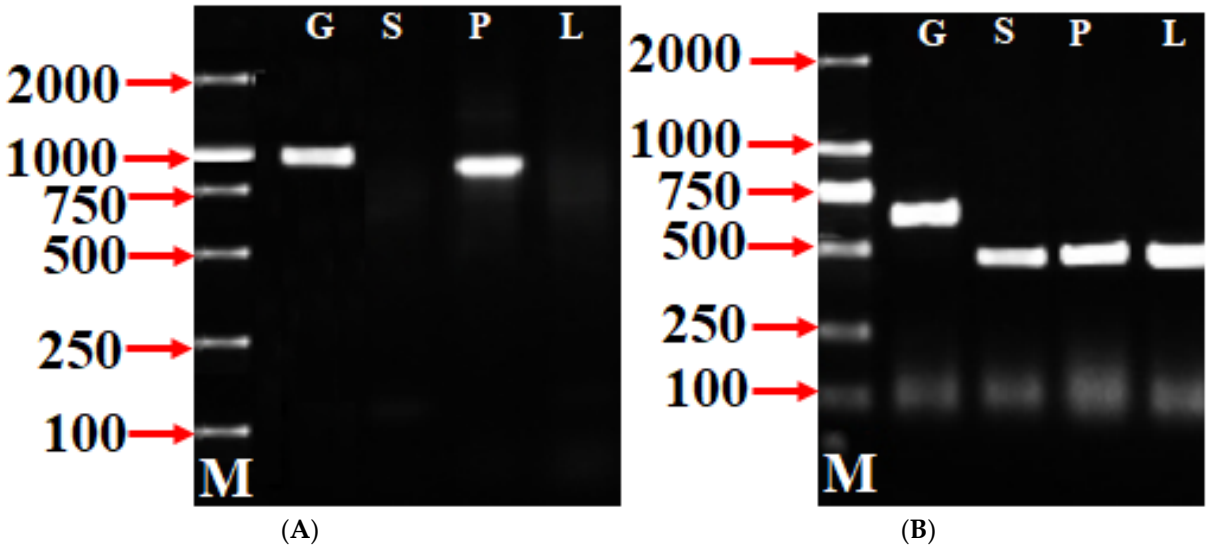

Figure 2. *PmSFB* (**A**) and actin gene (**B**) expression analysis in pollen (P), styles (S), and leaves (L), respectively, of 'Sichuanbaimei'. Lane G = genomic DNA; lane M = 2 kb DNA marker. The actin gene was used as positive control.

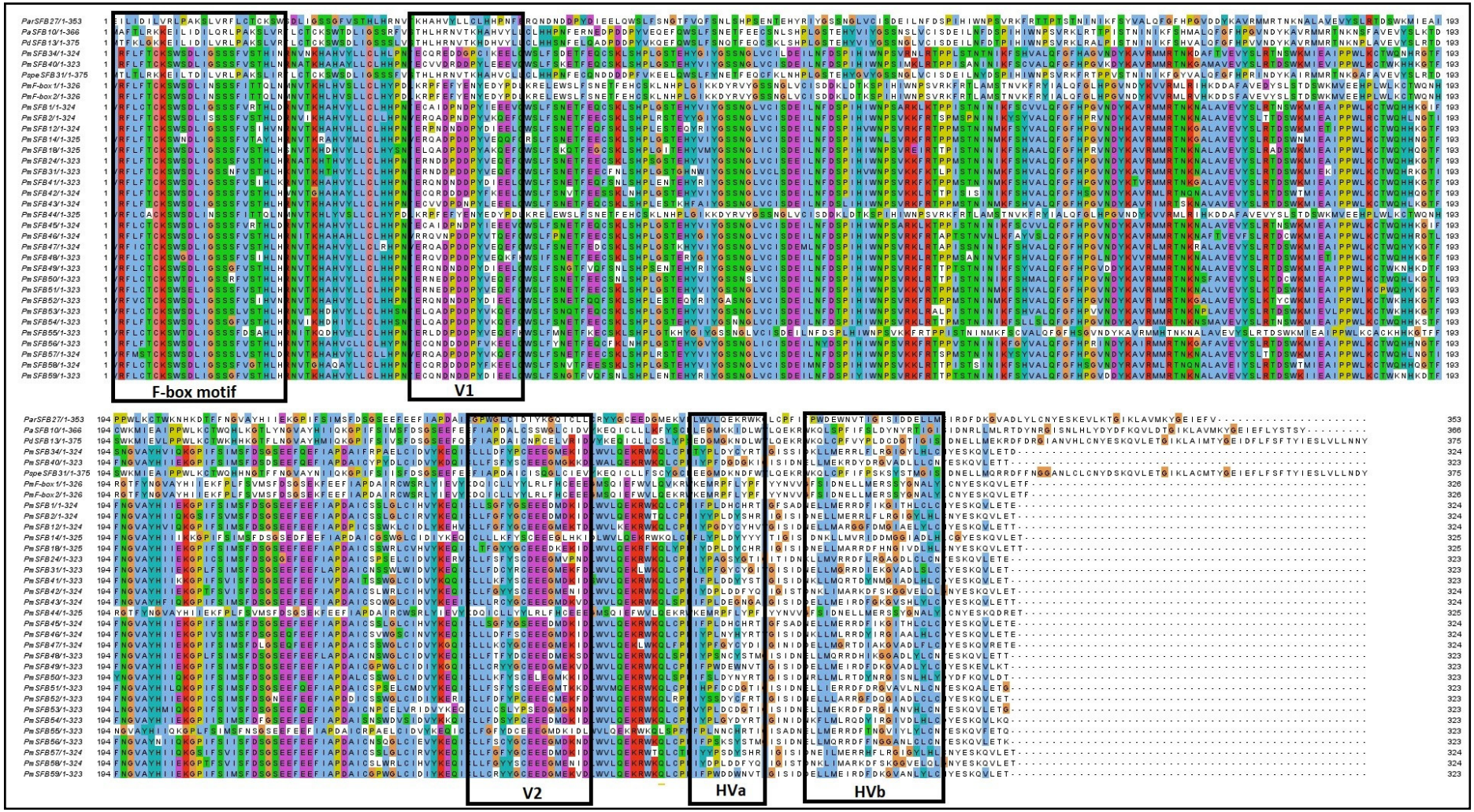

Figure 3. The amino acid sequences of *Prunus armeniaca* (*Par*), *Prunus avium* (*Pa*), *Prunus dulcis* (*Pd*), *Prunus speciosa* (*Pspe*), and *Prunus mume* (*Pm*) *SFB* genes source comparison. The dark areas show conserved residues and dots indicate gaps. The F-box domain, variable regions (V1 regions, V2 regions), and hypervariable regions (HVa regions and HVb regions) are, respectively, circled with boxes and marked.

Table 2. Homologies (%) of deduced amino acid sequences of *PmSFB* alleles.

SFB	***PmSFB***																								
Pm	***SFB$_1$***	***SFB$_2$***	***SFB$_{12}$***	***SFB$_{14}$***	***SFB$_{18}$***	***SFB$_{24}$***	***SFB$_{31}$***	***SFB$_{41}$***	***SFB$_{42}$***	***SFB$_{43}$***	***SFB$_{44}$***	***SFB$_{45}$***	***SFB$_{46}$***	***SFB$_{47}$***	***SFB$_{48}$***	***SFB$_{49}$***	***SFB$_{50}$***	***SFB$_{51}$***	***SFB$_{52}$***	***SFB$_{53}$***	***SFB$_{54}$***	***SFB$_{55}$***	***SFB$_{56}$***	***SFB$_{57}$***	***SFB$_{58}$***	***SFB$_{59}$***
SFB$_1$	/	82.41	80.19	80.00	82.10	81.73	78.95	83.25	79.94	83.28	60.06	96.93	79.26	81.73	82.35	80.80	81.11	82.10	80.50	80.31	79.88	81.42	79.94	82.41	80.25	81.73
SFB$_2$	82.41	/	79.32	79.08	81.17	81.17	77.78	79.32	78.09	79.63	62.96	82.72	80.25	79.63	80.25	76.23	79.01	80.31	77.78	76.99	79.01	78.09	79.38	95.06	76.54	76.54
SFB$_{12}$	80.19	79.32	/	79.69	77.78	80.80	78.95	84.83	77.78	82.04	64.71	80.50	78.02	78.64	81.12	81.73	76.47	79.94	89.69	76.37	74.54	76.47	79.63	79.01	76.54	82.04
SFB$_{14}$	80.00	79.08	79.69	/	78.46	82.46	79.69	84.31	76.62	79.38	63.69	79.69	81.54	82.46	81.58	77.85	81.54	80.98	79.08	77.68	79.38	78.46	77.30	79.69	76.31	77.85
SFB$_{18}$	82.10	81.17	77.78	78.46	/	79.63	78.40	78.09	81.48	78.09	63.27	81.79	77.16	80.86	79.01	78.09	78.70	78.77	77.47	80.06	80.86	77.47	78.15	80.86	80.56	78.40
SFB$_{24}$	81.73	81.17	80.80	82.46	79.63	/	83.28	81.11	79.94	81.73	63.04	82.04	81.42	81.73	82.61	79.57	80.80	86.38	79.88	80.86	81.42	81.11	80.19	81.17	78.70	78.95
SFB$_{31}$	78.95	77.78	78.95	79.69	78.40	83.28	/	78.95	76.85	79.57	64.09	78.64	78.02	83.59	82.66	77.71	78.95	82.10	76.78	77.54	78.02	79.26	76.85	78.09	75.93	77.71
SFB$_{41}$	83.25	79.32	84.83	84.31	78.09	81.11	78.95	/	79.63	82.35	65.33	82.04	80.05	80.80	82.04	83.59	82.35	80.25	83.90	76.92	79.26	77.40	80.86	79.94	79.01	83.59
SFB$_{42}$	79.94	78.09	77.78	76.62	81.48	79.94	76.85	79.63	/	78.09	62.96	80.25	76.85	78.40	77.47	77.16	76.85	77.23	75.00	75.46	77.16	75.31	77.54	78.09	96.91	78.40
SFB$_{43}$	83.28	79.63	82.04	79.38	78.09	81.73	79.57	82.35	78.09	/	64.71	83.59	78.33	82.04	80.50	83.59	80.19	82.10	79.88	78.15	76.47	79.88	80.25	79.01	76.85	84.21
SFB$_{44}$	60.06	62.96	64.71	63.69	63.27	63.04	64.09	65.33	62.96	64.71	/	62.85	65.63	65.02	66.46	63.78	61.92	62.85	62.45	61.11	60.68	62.85	65.63	63.27	62.35	63.78
SFB$_{45}$	96.93	82.72	80.50	79.69	81.79	82.04	78.64	82.04	80.25	83.59	62.85	/	79.57	81.42	82.04	80.50	80.80	81.79	80.19	80.00	80.19	81.11	80.25	82.10	79.94	81.42
SFB$_{46}$	79.26	80.25	78.02	81.54	77.16	81.42	78.02	80.05	76.85	78.33	65.63	79.57	/	79.88	79.88	76.16	81.73	78.70	76.47	75.83	81.73	77.09	78.40	79.32	75.62	76.47
SFB$_{47}$	81.73	79.63	78.64	82.46	80.86	81.73	83.59	80.80	78.40	82.04	65.02	81.42	79.88	/	82.97	80.50	81.73	81.79	78.95	78.46	79.88	78.33	77.16	80.56	78.09	79.88
SFB$_{48}$	82.35	80.25	81.12	81.58	79.01	82.61	82.66	82.04	77.47	80.50	66.46	82.04	79.88	82.97	/	79.26	80.19	82.35	82.04	78.07	79.26	81.73	80.08	80.25	77.16	79.26
SFB$_{49}$	80.80	76.23	81.73	77.85	78.09	79.57	77.71	83.59	77.16	83.59	63.78	80.50	76.16	80.50	79.26	/	77.71	79.63	82.35	79.08	76.47	77.71	78.09	76.85	77.16	96.56
SFB$_{50}$	81.11	79.01	76.47	81.54	78.70	80.80	78.95	82.35	76.85	80.19	61.92	80.80	81.73	81.73	80.19	77.71	/	82.10	77.71	78.77	81.11	78.33	75.93	79.63	76.85	78.02
SFB$_{51}$	82.10	80.31	79.94	80.98	78.77	86.38	82.10	80.25	77.23	82.10	62.85	81.79	78.70	81.79	82.35	79.63	82.10	/	79.94	83.02	77.47	82.10	78.38	81.23	77.23	79.94
SFB$_{52}$	80.50	77.78	89.69	79.08	77.47	79.88	76.78	83.90	75.00	79.88	62.45	80.19	76.47	78.95	82.04	82.35	77.71	79.94	/	76.00	75.54	76.16	77.47	79.01	75.00	82.35
SFB$_{53}$	80.31	76.99	76.37	77.68	80.06	80.86	77.54	76.92	75.46	78.15	61.11	80.00	75.83	78.46	78.07	79.08	78.77	83.02	76.00	/	79.08	77.85	77.47	78.22	75.46	80.00
SFB$_{54}$	79.88	79.01	74.54	79.38	80.86	81.42	78.02	79.26	77.16	76.47	60.68	80.19	81.73	79.88	79.26	76.47	81.11	77.47	75.54	79.08	/	74.92	75.62	78.40	75.93	75.85
SFB$_{55}$	81.42	78.09	76.47	78.46	77.47	81.11	79.26	77.40	75.31	79.88	62.85	81.11	77.09	78.33	81.73	77.71	78.33	82.10	76.16	77.85	74.92	/	75.93	78.09	75.31	78.02
SFB$_{56}$	79.94	79.38	79.63	77.30	78.15	80.19	76.85	80.86	77.54	80.25	65.63	80.25	78.40	77.16	80.08	78.09	75.93	78.38	77.47	77.47	75.62	75.93	/	78.77	75.69	79.32
SFB$_{57}$	82.41	95.06	79.01	79.69	80.86	81.17	78.09	79.94	78.09	79.01	63.27	82.10	79.32	80.56	80.25	76.85	79.63	81.23	79.01	78.22	78.40	78.09	78.77	/	77.78	76.85
SFB$_{58}$	80.25	76.54	76.54	76.31	80.56	78.70	75.93	79.01	96.91	76.85	62.35	79.94	75.62	78.09	77.16	77.16	76.85	77.23	75.00	75.46	75.93	75.31	75.69	77.78	/	78.40
SFB$_{59}$	81.73	76.54	82.04	77.85	78.40	78.95	77.71	83.59	78.40	84.21	63.78	81.42	76.47	79.88	79.26	96.56	78.02	79.94	82.35	80.00	75.85	78.02	79.32	76.85	78.40	/

Table 3. Homologies (%) of deduced amino acid sequences of *PmSFB* alleles with other *Prunus* species.

SFB		***Par***			***Pa***			***Pd***			***Ps***			***Pspe***	
Pm	***SFB$_2$***	***SFB$_{24}$***	***SFB$_{60}$***	***SFB$_3$***	***SFB$_6$***	***SFB$_{13}$***	***SFBc***	***SFBd***	***SFBe***	***SFB$_7$***	***SFB$_{10}$***	***SFBh***	***SFB$_{22}$***	***SFB$_{31}$***	***SFB$_{51}$***
F1-box	63.94	65.15	65.76	61.33	62.12	61.03	61.93	64.24	61.33	62.54	63.25	61.03	61.52	64.55	63.55
F2-box	64.55	65.15	65.76	61.93	62.12	61.33	61.33	63.94	61.14	62.65	63.86	61.33	61.21	65.15	64.15
SFB$_1$	81.73	84.52	83.90	81.73	80.50	80.19	76.78	82.35	78.95	77.23	85.49	79.23	65.53	80.80	70.80
SFB$_2$	78.70	79.94	79.94	76.85	79.01	79.01	74.38	80.25	75.62	75.00	81.23	75.00	69.14	79.63	69.63
SFB$_{12}$	81.42	80.19	79.88	82.04	78.64	75.54	75.23	78.64	84.21	79.26	78.95	79.32	73.46	79.88	78.88
SFB$_{14}$	80.00	79.38	79.08	79.08	81.54	80.92	77.54	82.15	79.08	76.31	78.46	78.77	69.33	77.54	73.54
SFB$_{18}$	77.16	79.94	79.63	76.23	77.78	82.72	73.15	78.70	76.23	75.31	79.94	75.00	69.75	77.16	72.16
SFB$_{24}$	80.12	83.59	83.28	80.50	97.20	79.57	76.47	82.04	79.26	77.09	82.35	75.31	71.83	80.43	77.43
SFB$_{31}$	76.16	83.59	83.28	77.40	81.73	77.09	74.92	78.95	79.57	75.23	78.95	73.46	72.22	76.78	74.78
SFB$_{41}$	82.97	80.50	80.19	83.59	79.26	81.42	79.88	81.73	83.28	82.04	79.26	82.10	69.66	81.11	71.11
SFB$_{42}$	75.62	80.25	79.63	75.93	77.47	77.47	76.54	76.85	76.23	75.31	78.09	73.77	67.59	77.78	70.78
SFB$_{43}$	79.26	82.35	81.73	81.73	79.88	78.02	75.85	80.19	79.88	81.42	81.11	76.54	72.53	80.50	78.64
SFB$_{44}$	63.94	64.85	65.45	61.03	62.12	60.73	61.93	64.24	60.54	62.24	63.14	60.73	61.28	64.24	61.03
SFB$_{45}$	81.42	84.21	83.59	82.04	80.19	79.88	76.47	82.66	78.64	77.40	86.07	79.01	72.22	81.11	82.04
SFB$_{46}$	77.40	80.19	79.57	77.71	78.95	79.57	77.40	82.04	74.92	74.30	79.26	77.47	68.50	78.64	81.11
SFB$_{47}$	79.57	84.21	83.59	77.40	81.42	80.50	74.92	80.19	82.04	77.71	78.02	75.62	73.15	78.02	80.19
SFB$_{48}$	81.37	83.90	83.28	78.95	81.68	78.02	74.61	81.73	81.42	77.40	81.42	75.62	73.99	81.73	80.50
SFB$_{49}$	79.57	81.11	80.50	83.28	78.95	76.47	77.71	78.02	82.35	86.69	78.95	80.25	69.66	78.95	78.33
SFB$_{50}$	78.64	79.88	79.26	76.47	80.50	90.40	74.92	81.11	75.23	76.23	79.57	76.85	70.06	77.40	78.95
SFB$_{51}$	80.19	83.90	83.99	80.25	86.07	80.25	75.93	81.79	79.01	77.78	83.02	74.85	72.53	79.26	78.40
SFB$_{52}$	81.42	79.02	78.64	82.97	79.88	75.85	75.23	78.64	87.00	79.88	77.71	77.47	71.91	78.33	77.71
SFB$_{53}$	76.85	80.31	80.00	77.23	79.94	81.23	76.31	79.69	76.62	75.69	79.69	75.15	69.23	78.09	79.08
SFB$_{54}$	76.47	79.88	79.57	74.92	78.95	81.73	75.23	81.42	70.23	74.30	78.33	76.23	69.44	75.54	82.66
SFB$_{55}$	75.85	82.04	81.73	79.57	81.68	77.09	72.76	79.26	77.71	75.54	84.21	74.38	69.44	76.78	78.33
SFB$_{56}$	78.33	79.63	79.32	77.47	77.71	76.85	76.23	79.63	76.85	77.16	79.01	75.38	71.30	98.14	78.40
SFB$_{57}$	74.32	79.56	84.33	79.45	80.27	81.36	74.36	78.54	77.86	78.36	78.25	79.36	80.22	85.31	78.12
SFB$_{58}$	84.32	75.23	77.56	85.32	74.32	76.23	77.23	75.32	78.86	78.64	79.62	83.25	84.32	86.32	78.23
SFB$_{59}$	83.23	75.23	78.63	76.51	77.53	78.54	78.24	79.53	86.23	75.23	77.12	76.24	75.23	80.23	81.32

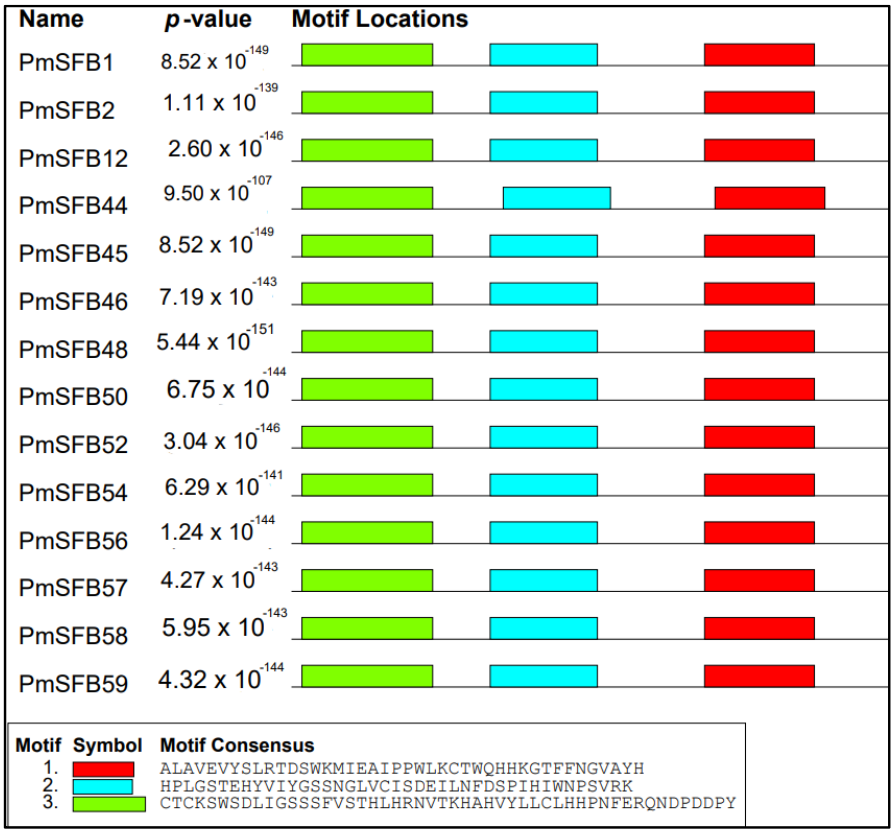

Figure 4. Motif distribution of *SFB* proteins in Japanese apricot. Each motif is denoted by a number in the colored box.

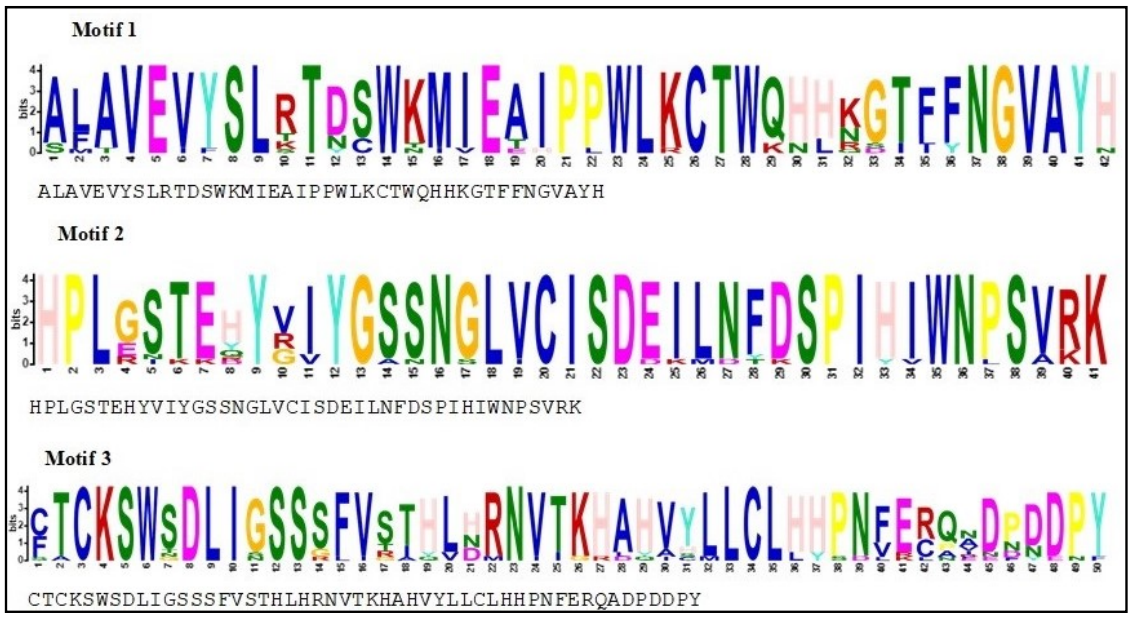

Figure 5. Summary sequence LOGO and regular expression of each motif shared by *SFB* proteins in Japanese apricot. At each location in the motif, the sequence logo features stacks of letters. The 'information content' of that place in the motif in bits is the whole height of the stack. The likelihood of the letter at that location multiplied by the overall information content of the stack determines the height of individual letters in a stack. The motif is described by the black letter below the sequence logo, which is a regular expression (RE). The RE includes all letters with observed frequencies greater than 0.2; less-frequent letters are not included.

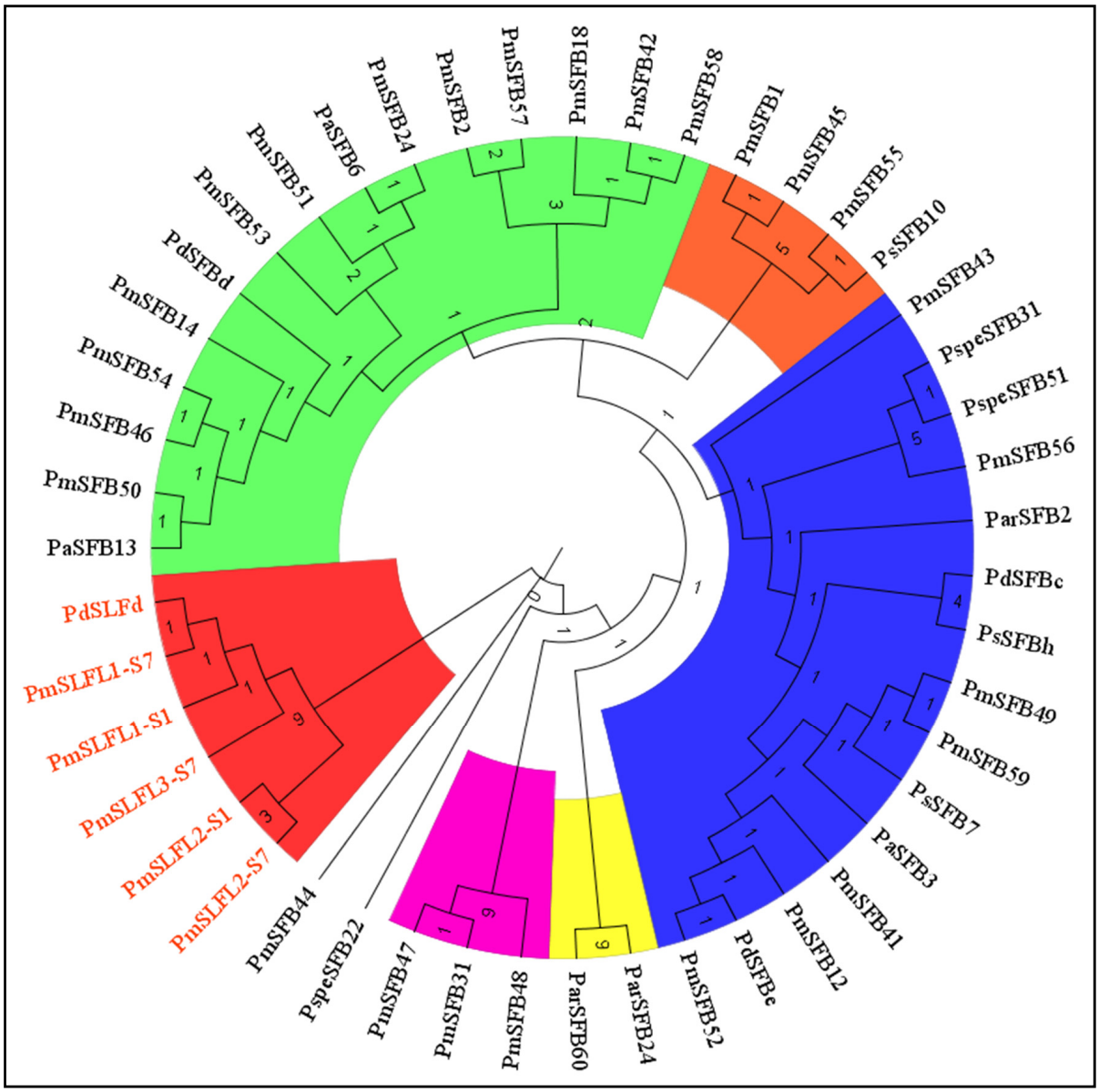

Figure 6. The phylogenetic tree was constructed based on the amino acid sequence using the neighbor-joining method of 47 S-locus F-box genes (*SFB* and *SLFL*) from, *P. armeniaca* (Par), *P. avium* (Pa), *P.* dulcis (Pd), *P. salicina* (Ps), *P. speciosa* (Pspe), and *P. mume* (Pm). GenBank accession numbers: *ParSFB1* (AY587563), *ParSFB*2 (AY587562), *PaSFB*1 (AY805048), *PdSFBa* (AB092966), *PdSFBd* (AB081648), *PdSFBc* (AB079776), *PmSLFL1-S1* (AB092623), *PmSLFL1-S7* (AB092624), *PmSLFL2-S1* (AB092625), *PmSLFL2-S7* (AB092626), *PmSLFL3-S7* (AB092627), *PdSLFd* (AB101660), *PaSLF1-S1* (AB360339), *PaSLF1-S2* (AB360340), *PsSFBb* (AB252412), *PsSFBc* (AB280792), *PspeSFB*1 (HM347508), and *PspeSFB*22 (HM347509); *PmSFB*1 (AB101440), *PmSFB*12 (JQ356586), *PmSFB*42 (JQ356581), *PmSFB*43 (JQ356578), *PmSFB*41 (JQ356593), *PmSFB*40 (JQ356585), and *PmSFB* accession numbers are detailed in the text.

4. Discussion

In previous studies, it has been reported that *P. salicina* [54], *P. armeniaca*, *P. mume*, and their cultivars/varieties are diploid [55]. Theoretically, these *P. mume* accessions should result in two amplified bands of *SFB* alleles. However, all the 48 accessions possessed two distinct sequences of *SFB* alleles (Table 2) using the *Prunus SFB* primer pair (SFB-C1F and Pm-Vb), which confirmed the exactness/correctness or accuracy of this pair of primers.

In Antirrhinum, it was first discovered that F-box genes (*AhSLF*) were physically connected to *S-RNase* and expressed only in pollen [30]. Yamane et al. [31] established a method for the molecular typing of *P. mume SFB* genes to determine the S haplotype utilizing a *PmSFB* probe and genomic DNA blots, while Zhang et al. [35] determined the *SFB* alleles of *P. salicina* (Japanese plum) cultivars using specific primers designed based on the hypervariable regions of *PsSFB*. Based on the PCR-amplified region, Vaughan et al. [23] established a rapid method to determine the S genotypes of sweet cherry cultivars. However, we successfully determined/identified the *SFB* alleles of 48 Japanese apricot accessions via the method based on AS-PCR amplification, which was less demanding in terms of instrumentation, more advantageous, and convenient.

The putative amino acid sequences of the eleven novel *PmSFB* alleles contained two hypervariable regions (HVa and HVb), two variable regions (V1 and V2), the F-box motif, and the same size as other *Prunus SFB* [43,56–58]. Furthermore, the motifs' structure was conserved among *SFB* alleles proteins, which could be important for the function and structure of *SFB* genes. In previous studies, *SFB* was reported to be a gene that recognized and protected the self *S-RNase* through some types of modification in peach (SC) [53]; others proposed that in *S-RNase*-based GSI, the ubiquitin/26S proteasome proteolytic pathway played a major role in nonself/self-pollen discrimination [18,28,30]. However, the four variable regions including HVa, HVb, V1, and V2 of *SFB* may play a crucial role in the haplotype-specific interaction mechanism with *S-RNase* for the discrimination of nonself/self-*S-RNase.*

Because the F-box motif is required for the formation of a complex termed SCF for the degradation of proteins, it is essential for the discrimination of self/nonself-pollen [56]. In *P. mume,* and *P. avium*, the self-compatible phenotypes have been reported to be associated to the insertion/deletions (indels) in *SFB* variable regions, which produce a frameshift in translation, resulting in a nonfunctional truncated amino acid/protein [56,59]. Likewise, in some cultivars of apricot (*P. armeniaca*), a 358 bp insertion was found in the *SFBc* gene located upstream from the HVa hypervariable region, resulting in the expression of a truncated protein; this gene alteration is associated with self-incompatibility breakdown [26]. On the other hand, Orlando Marchesano B.M. et al. (2022) [16] reported that in apricots, self-compatibility was related to a transposable element insertion within the coding sequence of *SFB* [16]. Furthermore, peach (*P. persica*) is a common self-compatible species [57], and its *SFB* mutation version was found in self-incompatible *Prunus* species, conferring SC to some of their cultivars [60,61]. However, these suggest that *SFB* alleles are essential in GSI study in general, specially its four variable regions.

For the *Pm SFB* alleles, the intraspecific amino acid identities were lower than interspecific identities when comparing with other *Prunus SFBs*. However, this could indicate the evolution of intraspecific identities in *Prunus.* The phylogenetic tree suggested that the newly identified *SFB* alleles in *P. mume* were orthologs rather than paralogs of *SFB* alleles from other *Prunus* species.

5. Conclusions

In this study, we identified the *SFB* genotypes of 48 Japanese apricot accessions, including 11 novel *SFB* alleles. Each accession possessed two distinct *SFB* alleles, the same as most of *Prunus.* The eleven new *SFB* alleles had the same typical features as *SFB* alleles from other *Prunus* species. The findings of this current study will enhance the accessible data/information on *SFB* alleles of *Prunus* and *P. mume*, which are required for the study of the self-(in)compatibility mechanism.

Author Contributions: Z.G. conceived and designed the study. G.H., D.C., C.M. and X.H. performed the experiment. G.H. and D.C. analyzed the data. D.C. and G.H. wrote the whole manuscript. C.M., X.H., Z.N. and K.O.O. assisted with the sample collection. K.O.O., S.I. and F.H. corrected the English language. S.I., F.H., T.S., B.K. and K.O.O. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data analyzed or generated during this study were comprised in this manuscript. Eleven new *Pm* SFB from this study were logged/submitted to the NCBI database (GenBank) under accession numbers: MW186460(*PmSFB*$_{44}$), MW186461(*PmSFB*$_{45}$), MW186462(*PmSFB*$_{46}$), MW186464(*PmSFB*$_{48}$), MW186466(*PmSFB*$_{50}$), MW186468(*PmSFB*$_{52}$), MW186470(*PmSFB*$_{54}$), MW186472 (*PmSFB*$_{56}$), MW786959(*PmSFB*$_{57}$), MW786960(*PmSFB*$_{58}$), MW786961(*PmSFB*$_{59}$).

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

Abbreviations

The following abbreviations are used in this manuscript:

PmSFB	*SFB* allele of *Prunus mume*
bp	Base pairs
SC	Self-compatibility
SI	Self-incompatibility
PCR	Polymerase chain reaction
AS-PCR	Allele-specific polymerase chain reaction
RT-PCR	Reverse-transcription polymerase chain reaction
CTAB	Cetyltrimethylammonium bromide

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