Spatio-genetic characterization of *S* receptor kinase (*SRK*) alleles in naturalized populations of *Raphanus sativus* L. var. *raphanistroides* on Yakushima island

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In various coastal areas of Japan, naturalized radish populations are observed. Radish is a cruciferous plant and exhibits self-incompatibility, involving a system controlled by a single locus with multiple *S* alleles. Although the *S* allele diversity of radish cultivars and wild radishes has been characterized, the *S* allele distribution in naturalized populations has not yet been analyzed in relation to the positions of the plants *in situ*. Here, we show the *S* allele distribution in naturalized radish populations of Yakushima, a small island in the East China Sea, with positions of the plants. Radish plants were sampled in coastal areas in Yakushima, and their *S* alleles were detected and characterized. Most of the *S* alleles had been previously identified in radish cultivars. However, four novel *S* alleles, which may be unique to Yakushima, were also found. Moreover, seeds in siliques from plants growing in the study areas were sampled, and *S* allele determination in DNA extracted from these seeds suggested that the plants had exchanged their pollen among their close neighbors. There was also a problem in that the PCR amplification of some *SRK* alleles was difficult because of their sequence diversity in the naturalized populations, as occurs in cultivars. Our results suggest that the exchange of *S* alleles between cultivars and naturalized populations occurs and that *S* alleles in naturalized populations are highly diverse. The methodology established in our study should be applicable to other self-incompatible species to dissect the diversity of *S* allele distribution in naturalized populations.

Key words: Naturalized population, *Raphanus sativus* L. var. *raphanistroides*, *S* allele, self-incompatibility, *SRK* gene

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INTRODUCTION

Many cultivars of radish, which is one of the most important vegetables, have been established by several seed companies in Japan, and are cultivated all over the world. The shape of the root is highly diverse among these cultivars (Mitsui et al., 2015). The root contains various secondary metabolites, glucosinolates (which are precursors of chemical defense compounds), biopesticides and flavor compounds (Halkier and Gershenzon, 2006). In addition to the agricultural importance of

radish, naturalized radish populations are observed in coastal areas in Japan, and their genetic diversity in allozymes and cytoplasmic male sterility-related genes has been partially characterized (Huh and Ohnishi, 2001; Yamagishi and Terachi, 2001). Wild populations of radish are also found in the USA, Canada and Australia, and the diversity in their *S* alleles, which regulate self-incompatibility traits, as described below, has been characterized (Sampson, 1967; Karron et al., 1990).

Self-incompatibility is an important trait to maintain genetic diversity within species (Watanabe et al., 2012). The self-incompatibility trait in cruciferous plants, including radish, cabbage and turnip, is controlled by a single locus with multiple *S* alleles (Bateman, 1955). To date, genes encoding the male and female *S* determinants have been identified and characterized as encoding a small cysteine-rich protein (SP11/SCR) and a receptor-like protein kinase (SRK), respectively (Stein et al., 1991; Watanabe et al., 1994; Schopfer et al., 1999; Suzuki et al., 1999; Takasaki et al., 2000; Takayama et al., 2000, 2001; Shiba et al., 2001). At the *S* locus, in addition to the two genes encoding SP11 and SRK, at least two additional genes, *SLG* and *Smi1/2*, are also located. *SLG* is required for full manifestation of self-incompatibility reactions (Takasaki et al., 2000), and *Smi1/2* encodes a small RNA that regulates the dominance relationship between *S* alleles on the pollen side (Hatakeyama et al., 1998b; Kakizaki et al., 2003; Tarutani et al., 2010; Yasuda et al., 2016). The *S* alleles *SP11*, *SRK* and *SLG* are divided into two classes (class-I and class-II; Nasrallah and Nasrallah, 1993), and the sequence identity at the nucleotide level within each class is higher than that between the classes (Hatakeyama et al., 1998a; Watanabe et al., 2012). Because of the complex nature of the *S* locus, as described above, the *S* alleles are referred to as the *S* haplotypes (Nasrallah and Nasrallah, 1993). In addition to the *S* locus, the *Brassica* genome contains several *SRK*-like genes, which are termed the *S* multigene family (Suzuki et al., 1995, 1997). *BcRK6*, one of the *SRK*-like genes, encodes a female determinant of unilateral incompatibility in *B. rapa* (Kai et al., 2001; Takada et al., 2017).

In tandem with the molecular dissection of the *S* locus, analysis of genetic diversity and distribution of *S* alleles in several cruciferous plants (*B. rapa*, *B. oleracea*, *Sinapis arvensis*, etc.) was performed by diallel crosses and test crosses (Bateman, 1955; Thompson, 1957; Sampson, 1967; Ockendon, 1974, 1980, 1982; Mackay, 1977; Wallace, 1979; Visser et al., 1982; Stevens and Kay, 1989; Nou et al., 1991, 1993). In radishes from both wild populations and cultivars, genetic diversity and distribution of *S* alleles have also been characterized (Sampson, 1957, 1964, 1967; Karron et al., 1990; Niikura and Matsuura, 1998). After identification and characterization of *S* locus genes, the *S* genotype was determined from the nucleotide

sequence polymorphism of *SLG* or *SRK* in *Brassica* and *Raphanus* species (Brace et al., 1994; Sakamoto et al., 1998; Park et al., 2002; Kim and Kim, 2019). Recently, two research groups independently defined the *S* alleles (*S* haplotypes) in radish according to the *S* locus genes *SLG*, *SRK* and *SP11* (Haseyama et al., 2018; Kim and Kim, 2019). Based on their data, at least 31 different *S* alleles have now been identified and characterized in radish cultivars. Although the distribution and frequency of *S* alleles have been surveyed in many cultivars and naturalized populations of radish using pollination tests or PCR methods, there are no published data on the distribution and frequency of *S* alleles in relation to the *in situ* positions of the plants in naturalized populations.

In this study, we dissected the distribution of *S* alleles of naturalized radish (*R. sativus* L. var. *raphanistroides*) populations in Yakushima, a small island in the East China Sea at latitude 30° 28' N and longitude 130° 30' E. In October 2019 and March 2020, seeds and/or leaves were sampled from the naturalized populations and the sampled positions were recorded. Their *S* alleles were determined by comparison with the *SRK* partial sequences amplified by PCR. We discuss the characteristics of the distribution of *S* alleles combined with the positions of the plants in the naturalized populations of Yakushima.

MATERIALS AND METHODS

Plant materials, sampling and conditions The tester lines for *S* alleles used in this study included three lines (Pekinmizu ($S^{204} \times$ unidentified *S* allele), No. 5 ($S^{205} \times S^{209}$) and Eisai ($(S^{202} \times S^{206}) \times (S^{201} \times S^{203})$)) from Tohoku Seed Co. (Niikura and Matsuura, 1998), and four lines (Kanpaku (S^1, S^4), Shinhasshu (S^1 , unidentified *S* allele), YR Kurama (S^2, S^3, S^4) and Oshin (S^{10}, S^{15} , unidentified *S* alleles)) from Takii & Co. (Sakamoto et al., 1998) (Supplementary Table S3). These plants were grown in an artificial growth chamber under 16-h light and 8-h dark conditions at Tohoku University, Sendai. Leaves were sampled and used for genomic DNA extraction. Radish seeds and plants growing in Yakushima were sampled in 2019 and 2020. In October 2019, siliques of radish plants were sampled in the coastal areas. The sampled seeds were germinated on petri dishes in December 2019. The germinated seeds were transferred to plastic pots and were grown in an artificial growth chamber under 16-h light and 8-h dark conditions until flowering; 80 (23 from Haruta and 57 from Tashiro) of these plants were used in the experiments. In March 2020, leaves were sampled from 54 flowering plants (12 in Tsukazaki, 12 in Kurio, six in Nakama, five in Haruta and 19 in Tashiro), and a plant with green siliques was sampled as a representative from each area.

Observation of pollen tube behavior To observe pollen tube behavior, the aniline blue staining method described by Wang et al. (1994) was used. Briefly, after pollination, flowers were mounted on a 1% agar plate. Softened pistils incubated in 1 M NaOH were stained with basic aniline blue solution. The pistils were mounted on a glass slide and observed by fluorescence microscopy (Axio Imager A2, Zeiss, Germany).

Detection of *S* alleles by *SRK* partial sequencing Genomic DNA from leaves or seeds was extracted according to Watanabe et al. (1992) and Takada et al. (2005) with slight modification. Extracted DNA was used as a template for PCR amplification. PCR amplifications of *SRK* partial sequences were performed using four different sets of *SRK*-specific primers, designed based on exon 4 and exon 7 sequences (Supplementary Table S2). The sequences of two sets of primers were from Kim and Kim (2019), and the other sets were designed based on a multiple sequence alignment of several *SRK* nucleotide sequences of *B. rapa* (Supplementary Table S2). PCR amplifications were performed with *Ex Taq* DNA polymerase (TaKaRa, Japan) with pre-denaturation for 4 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 57 °C and extension for 60 s at 72 °C, with a final extension at 72 °C for 10 min, using a TP650 DNA thermal cycler (TaKaRa) according to Kim and Kim (2019) with slight modification. The nucleotide sequences of the PCR products were determined by direct sequencing using a DNA sequencer (Genetic Analyzer 3500; ABI Hitachi, Japan). Products containing mixed sequences amplified from multiple *S* alleles were cloned into plasmid vectors (pTAKN2, BioDynamics Laboratory, Japan). The obtained data were analyzed by BLAST (<https://www.ncbi.nlm.nih.gov/>; Altschul et al., 1997; Johnson et al., 2008). In cases where the highest sequence identity against a known *SRK* sequence of *R. sativus* exceeded 99%, the determined *SRK* sequence was assigned to a known *SRK* allele based on Kim and Kim (2019) (Supplementary Table S1). On the other hand, in cases where the highest identity against any known *SRK* sequence was below 98%, we classified the *SRK* sequence as a novel allele that has not been identified previously (Supplementary Table S1). The partial nucleotide sequences determined in this study for *RsSRK*³⁴, *RsSRK*³⁵, *RsSRK*³⁶ and *RsSRK*³⁷ will be available in the DDBJ/EMBL/GenBank databases with the accession numbers LC591983, LC591984, LC591985 and LC591986, respectively.

Construction of a phylogenetic tree Multiple sequence alignment of *SRK* sequences of *Brassica* and *Raphanus* species was performed using MAFFT (Katoh et al., 2019). Suspicious regions of aligned sequences were removed using trimAl (Capella-Gutiérrez et al., 2009). A

phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). To evaluate clustering patterns, a bootstrap probability was calculated with 1,000 resamplings. The phylogenetic tree was visualized using iTOL (Letunic and Bork, 2019).

RESULTS AND DISCUSSION

Confirmation of the method of PCR amplification of *SRK* partial sequences in seven radish cultivars Before analyzing the *S* alleles of radish plants sampled in Yakushima, the utility of the PCR primer sets described in Materials and Methods was confirmed. We selected *SRK* for genotyping of *S* alleles because the nomenclature of *S* alleles and sequences of the kinase domain of *SRK* in radish are well established (Kim and Kim, 2019) (Supplementary Table S1). We did not attempt genotyping of *SP11* because the nucleotide sequences are highly diverse among the alleles, making the design of useful primer pairs difficult (Suzuki et al., 1999; Takayama et al., 2000; Watanabe et al., 2000). Genomic DNA extracted from seven radish cultivars established by two Japanese seed companies, Tohoku Seed Co. and Takii & Co., was used as a template for PCR amplifications of *SRK* partial sequences. Nomenclature of *S* alleles in radish cultivars was established by Niikura and Matsuura (1998) and Sakamoto et al. (1998). For PCR amplification of *SRK* partial sequences, two primer sets, PK and KK (Supplementary Table S2), were used. The nucleotide sequences of the *SRK* alleles amplified from each cultivar using either the PK or KK primer set (Supplementary Table S3) were identical, indicating that in addition to the KK primer set (Kim and Kim, 2019), the PK primer set is applicable for analysis of radish *SRK* sequences. Thus, in this study, we analyzed *S* alleles in radish from Yakushima using both PK and KK primer sets.

Interestingly, in five cultivars (Eisai, Kanpaku, Shinhas-shu, YR Kurama and Oshin) an *SRK*-like sequence (tentatively named *SRK*-#38), registered as an *SRK* allele of *Raphanus raphanistrum* in the DDBJ/EMBL/GenBank databases (accession no. KP117077), was amplified with the class-II primer sets (data not shown). PCR amplification of an *SRK*-like sequence was also reported by Kim and Kim (2018) and Kim and Kim (2019). Kim and Kim (2019) reported that *RsSRK-like8*, which was identified from a radish draft genome as an *SRK* homolog by Kim et al. (2016), was amplified by the class-II primer set as a non-*S* locus gene. Moreover, it was reported that *RsKD1*, which was identified from another radish draft genome as a gene showing high sequence identity in the kinase domain of the *SRK*, might complicate *S* genotyping because *RsKD1* is a non-*S* locus gene and could be amplified with class-II *SRK* primers (Kim and Kim, 2018). Our *SRK*-like sequence (*SRK*-#38) displayed over

99% identity with *RsSRK-like8* and *RsKD1* in BLAST analysis using the nucleotide sequences. Therefore, *SRK*-#38 is not an *SRK* allele and was eliminated as an *SRK* allele in this study. *SRK*-#38 may belong to the *S* multigene family (Suzuki et al., 1995, 1997), with an unknown function in radish.

In two of the cultivars, Pekinmizu and No. 5, two *SRK* alleles were amplified (Supplementary Table S3). The combination of *S* alleles was class-I and class-II *S* heterozygote in both these cultivars. The *SRK* alleles of Kanpaku and Shinhasshu were identical (Supplementary Table S3). The cultivar Eisai was established by a double-way crossing system, meaning that two different *S* heterozygotes, ($S^{202} \times S^{206}$) $\times$ ($S^{201} \times S^{203}$), were crossed in hybrid seed production. However, only two different *S* alleles, *RsS*² and *RsS*¹⁰, were detected in Eisai (Supplementary Table S3). This may have been due to the findings in previous studies that some *SRK* alleles in radish are difficult to detect because of nucleotide sequence diversity in the *S* locus genes (Okamoto et al., 2004; Kim et al., 2016; Haseyama et al., 2018).

Thus, the method of PCR amplification of *SRK* partial sequences could be applied to our radish samples based on the analysis and assignment of *S* alleles using the *SRK* partial sequences classified by Kim and Kim (2019), although there were several *SRK* alleles that could not be amplified and characterized, as also described below.

Characterization of two naturalized radish populations in October 2019 in Yakushima

In October 2019,

to sample siliques of radish, we surveyed seven areas in Yakushima (Tsukazaki (Tk), Kurio (Kr), Nakama (Nk), Hara (Hr), Haruta (Hh), Tashiro (Ts) and Kusakawa (Ks); Fig. 1). In only two areas, Haruta and Tashiro, could fallen siliques be sampled in the coastal areas (Fig. 1).

From the seeds sampled in Haruta and Tashiro, 23 and 57 plants, respectively, were grown in pots for use in experiments. To observe pollen tube behavior in self- and cross-pollination, aniline blue staining was performed. Nine plants were selected randomly and used for self-pollination tests. All plants showed the incompatible phenotype, i.e., inhibition of pollen tube penetration on papilla cells, as exemplified by plant #20-12-2 (Fig. 2A), suggesting that both the male and female *S* determinants were functional. Moreover, most of the plants of the Haruta and Tashiro populations are expected to be self-incompatible, because if these populations contained any self-compatible plants, the self-compatibility trait would spread rapidly within the populations (Igic et al., 2008). In the case of the cross #10-2-1 (female) and #5-1-1 (male), the compatible phenotype, i.e., pollen tube penetration and elongation, was observed (Fig. 2B). To confirm this compatible result, we determined the *S* genotype of the two plants: #10-2-1 had *RsS*¹⁴/*RsS*¹⁷ and #5-1-1 had *RsS*⁸/*RsS*²⁵. *RsS*¹⁴ and *RsS*²⁵ are classified as class-I, and *RsS*⁸ and *RsS*¹⁷ are classified as class-II (*RsS*⁸, *RsS*¹⁴ and *RsS*²⁵ were found in Tashiro in 2020, but *RsS*¹⁷ was not, as described below). Because class-I alleles are dominant over class-II alleles on the pollen side, the pollen phenotype would be *RsS*²⁵ and was thus different

Fig. 1. Locations of naturalized radish populations in Yakushima. (A) The location of Yakushima in East Asia. (B) The areas where the naturalized radish populations were found in Yakushima. Red circles indicate areas where plants with flowers and siliques were found in March 2020.

Fig. 2. Pollen tube behavior on stigmas stained by aniline blue. (A) Self-pollination of plant #20-12-2 ($R_sS^{12}/N.D.$). (B) Cross-pollination between plants #10-2-1 (R_sS^{14}/R_sS^{17}) as the female partner and #5-1-1 (R_sS^6/R_sS^{25}) as the pollen donor. N.D.: not determined. Scale bars = 100 μ m.

from the stigma phenotype, R_sS^{14}/R_sS^{17} . Therefore, this pollination would be compatible, as observed in the pollination test (Fig. 2B).

In the analysis of the plants sampled in October 2019, our aim was to determine the potential for amplification with the class-I and class-II primers in the populations. In the PCR amplification of *SRK*, three patterns were possible: amplification with only the class-I primer set, with both class-I and class-II primer sets (class-I/class-II), and with only the class-II primer set. Each plant in the samples from Haruta and Tashiro was classified according to the PCR amplification patterns, and the number of plants in each pattern was recorded (Fig. 3). The distribution of the PCR amplification patterns was different between Haruta and Tashiro. In Haruta, two patterns, class-I/class-II and class-II only, were present equally. In Tashiro, however, the class-I/class-II pattern was present at a higher frequency. To determine the independence of the distribution pattern between the two populations, Pearson's chi-squared test was performed. The test value (chi-squared = 9.79, $P < 0.05$, $df = 2$) confirmed that the distribution pattern was different, suggesting that each population had a specific allelic distribution.

Distribution of *S* alleles in five naturalized radish populations in March 2020 in Yakushima We observed plants with flowers and developing siliques in seven areas (Tsukazaki, Kurio, Nakama, Hara, Haruta, Tashiro and Kujukawa) in March 2020 (Supplementary Fig. S1A and S1B). To dissect the *S* allele variation in depth, we sampled plants in five of these areas (Tsukazaki, Kurio, Nakama, Haruta and Tashiro); these areas were

Fig. 3. Frequency of the three PCR amplification patterns (class-I only, class-I and class-II, and class-II only) in plants from Tashiro (Ts, $N = 57$) and Haruta (Hh, $N = 23$) in samples collected in October 2019 (chi-squared = 9.79, $P < 0.05$, $df = 2$).

selected because Haruta and Tashiro are located on the east side of Yakushima, whereas Tsukazaki, Kurio and Nakama are on the west side (Fig. 1). The number and positions of plants in randomly placed 1 m $\times$ 1 m quadrats were recorded. The *S* alleles of each plant sampled in the five areas were determined according to *SRK* partial sequences, as described in Materials and Methods (Fig. 4 and 5A, Supplementary Fig. S2, Supplementary Table S4).

Combining data from the five areas, six additional *SRK* alleles not described by Kim and Kim (2019) were detected; these *S* alleles were named R_sSRK^{32} to R_sSRK^{37} (to follow in sequence after the previously identified R_sS^{31} ; Supplementary Table S1). Among the six alleles, R_sSRK^{32} is registered as an *SRK* of *Raphanus sativus* in the databases and R_sSRK^{33} was reported by Haseyama et

	Tk	Kr	Nk	Hh	Ts
<i>RsS</i> ²					
<i>RsS</i> ⁷					
<i>RsS</i> ⁸					
<i>RsS</i> ¹¹					
<i>RsS</i> ¹²					
<i>RsS</i> ¹³					
<i>RsS</i> ¹⁴					
<i>RsS</i> ¹⁷					
<i>RsS</i> ²²					
<i>RsS</i> ²³					
<i>RsS</i> ²⁵					
<i>RsS</i> ²⁷					
<i>RsS</i> ²⁸					
<i>RsS</i> ³⁰					
<i>RsS</i> ³²					
<i>RsS</i> ³³					
<i>RsS</i> ³⁴					
<i>RsS</i> ³⁵					
<i>RsS</i> ³⁶					
<i>RsS</i> ³⁷					

Fig. 4. Identification of *S* alleles in the five areas from samples collected in March 2020. Identified *S* alleles are indicated by gray boxes. Tk, Tsukazaki; Kr, Kurio; Nk, Nakama; Hh, Haruta; Ts, Tashiro.

al. (2018) (Supplementary Table S1). Interestingly, four *S* alleles (*RsS*³⁴ to *RsS*³⁷) were novel, and may therefore be unique to Yakushima. Alignment and comparison with deduced amino acid sequences of *SRK* deposited in the databases suggested that *RsSRK*³⁴ to *RsSRK*³⁷ are functional genes (Supplementary Fig. S3).

On the west side of Yakushima, Kurio is located between Tsukazaki and Nakama (Fig. 1). In Kurio, seven *S* alleles were found and five of them (71%; *RsS*⁷, *RsS*¹², *RsS*¹⁴, *RsS*²², *RsS*³³) were shared with Tsukazaki and/or Nakama, suggesting that the three areas are genetically related (Fig. 4, Table 1). On the east side of Yakushima, Haruta is located next to Tashiro (Fig. 1). In Haruta, four *S* alleles were found and two of them (50%; *RsS*¹³,

*RsS*²³) were shared with Tashiro, suggesting that the two areas on the east side of the island are genetically related, as was found on the west side (Fig. 4, Table 1). Eleven *S* alleles were found in the west side whereas 12 *S* alleles were found in the east side (Fig. 4, Table 1). From a comparison of *S* alleles between the west and east sides, three *S* alleles (27% in the west side, 25% in the east side; *RsS*⁸, *RsS*¹⁴, *RsS*²⁸) were found to be shared. These data indicate that the number of shared *S* alleles was correlated with the distance between populations. During the research in Yakushima, it was observed that western honeybees (*Apis mellifera*), a potential pollinator, foraged flowers of these plants (data not shown). Their average foraging distance has been estimated to be about 1.5 km (Steffan-Dewenter and Kuhn, 2003), which is considered to be a potential pollination range. Also, our data suggest that the diversity of *S* alleles is maintained locally within areas in Yakushima.

Most of the *S* alleles detected in the five areas were classified as class-I (Supplementary Table S4). However, the number of identified *S* alleles varied in the five areas (Table 1). If the number of plants in a population decreases, the number of *S* alleles that are lost in the population would be expected to increase (Byers and Meagher, 1992). The number of *S* alleles identified in the higher population density areas, Tsukazaki, Kurio and Tashiro, was indeed larger than the number in the lower population density areas, Haruta and Nakama (Fig. 5A, Table 1, Supplementary Fig. S2). Therefore, our data support the previous study by Byers and Meagher (1992).

When we compared the identified *S* alleles in the cultivars and the naturalized plants, only five, *RsS*², *RsS*⁷, *RsS*⁸, *RsS*¹² and *RsS*¹⁷, were common to both (Fig. 4, Supplementary Tables S3 and S4). However, *RsS*¹ to *RsS*³¹ have previously been identified in cultivars, as described by Kim and Kim (2019) (Supplementary Table S1). Thus, most of the *S* alleles were common to cultivars and the naturalized populations. Interestingly, plants that are intermediate between radish cultivars and radish plants in naturalized populations were also observed in Yakushima and other locations (data not shown). These observations suggest that exchange of *S* alleles between cultivars and naturalized plants has occurred.

The *SRK*-like sequence *SRK*-#38, which was the same as in the cultivars, was amplified from all plants sampled in March 2020 (data not shown). Moreover, in only 12 plants (Tk9, Kr1, Kr2, Kr3, Kr6, Kr7, Kr8, Kr12, Nk1, Nk2, Ts2 and Ts4), two *SRK* alleles were amplified in addition to *SRK*-#38 (Supplementary Table S4). These results confirmed that *SRK*-#38 is not an allele of the *S* locus, as discussed above. The low number of plants in which two *SRK* alleles were amplified (12 of 54 plants) indicates that there were undetectable *SRK* alleles in most of the plants of the naturalized populations.

To explore how pollen was transferred among the popu-

Fig. 5. Relationship between *S* allele distribution and the position of each radish plant. (A) The positions of each radish plant (Ts1 to Ts19) in a 1 m × 1 m quadrat in Tashiro. Red circles indicate plants with flowers (sampled). Blue triangles indicate small plants without flowers (not sampled). (B) *S* alleles detected in the seeds of the siliques on branch D of plant Ts6 (*RsS*²⁷/N.D.). Solid lines indicate the branches with siliques. Dotted lines indicate clusters of flower buds. Numbers adjacent to the branch indicate internode length (cm). Boxes on the branches indicate individual seeds and rows of boxes are seeds within a silique. The *S* alleles identified in DNA extracted from each seed are shown in the boxes. (C) Structure of the whole plant Ts6 (see also Supplementary Fig. S1C). N.D.: not determined, N.A.: not available.

Table 1. Number of *S* alleles identified in samples from five different areas in March 2020

Area	Number of identified <i>S</i> alleles
Tsukazaki	7
Kurio	7
Nakama	4
Haruta	4
Tashiro	10
Total	20

lation, Tashiro was selected as a research area because the number of plants sampled there was the largest of the five areas. To analyze the *S* alleles of seeds, plant Ts6 was selected as representative. On plant Ts6 we recorded the internode lengths between siliques and the relative positions of seeds in the silique (Fig. 5B and 5C, Supplementary Fig. S1C). In the selected plant, one representative middle branch (branch D) was analyzed (Fig. 5B and 5C). The *S* alleles of the seeds on branch D were determined using DNA extracted from them (Fig. 5B). Interestingly, allele *RsS*²³, which was detected in seed D2, was also in other plants within the 1 m × 1 m quadrat at Tashiro. In contrast, allele *RsS*¹², which was detected in seeds D3, D4, D5, D6, D7, D10 and D11, was not present in other plants within the 1 m × 1 m quadrat at Tashiro. Therefore, the pollen with *RsS*¹² appears to have come from a plant outside the 1 m ×

1 m quadrat, and the pollen with *RsS*²³ appears to have come from inside this range. The seeds D3, D4, D8 and D9 did not have allele *RsS*²⁷, indicating that the other *S* allele, which was not determined in the plant Ts6, had been inherited. Another notable result was that three alleles, *RsS*⁸, *RsS*¹² and *RsS*²⁷, were detected in seed D1. *RsS*⁸ and *RsS*¹² must have been inherited from the pollen because *RsS*²⁷ was detected in plant Ts6. In wild-type *Arabidopsis thaliana*, another species in Brassicaceae, the frequency of spontaneous twin embryos is 0.02% (Vernon and Meinke, 1994). Therefore, the seed D1 may have been a twin produced by multiple independent fertilization, as occurs in *Arabidopsis*.

Phylogenetic analysis of *SRK* alleles in *Brassica* and *Raphanus* species To examine the evolutionary relationships among the *SRK* alleles characterized in Yakushima and other *SRK* alleles of *R. sativus*, *B. rapa*, *B. oleracea* and *B. napus*, a phylogenetic tree was constructed using the neighbor-joining method with the sequences determined in this study and those already deposited in the databases (Fig. 6). Two clusters (class-I and class-II) were clearly identified. Furthermore, the class-I cluster was divided into two subclusters. Recently, from crystal structure analysis, although the SP11–SRK complex structure was found to be similar in *BrS*⁸ and *BrS*⁹, in *B. rapa*, the interactive regions between SP11 and SRK were different between *BrS*⁸ and *BrS*⁹ (Murase et al., 2020). The class-I *SRK* alleles of *R.*

Fig. 6. Phylogenetic tree constructed from SRK sequences of *Brassica rapa*, *B. oleracea*, *B. napus* and *Raphanus sativus* using the neighbor-joining method. Bootstrap probabilities (%) are shown on the nodes. Asterisks indicate the SRKs of *R. sativus* found in Yakushima.

sativus were also classified into two different subclades (Fig. 6). In the class-I *BrS*⁹ subclade, the *SRK* alleles of each species were scattered within the cluster. However, in the class-I *BrS*⁸ subcluster and the class-II cluster, the *SRK* alleles were separated into a different clade in *Brassica* and *Raphanus*, except for a few alleles. Each cluster would have undergone different evolutionary processes. Interestingly, the *SRK* alleles identified in plants from Yakushima were found in all three of the clusters (class-I *BrS*⁸, class-I *BrS*⁹ and class-II), suggesting that the *S* alleles of the naturalized radish in Yakushima are highly diverse.

Conclusion Our results from Yakushima show the *S* allele distribution in relation to positions of the plants *in situ* in naturalized radish populations for the first time and provide evidence that pollination occurs between neighboring plants within a population. Interestingly, novel *SRK* alleles were identified in the naturalized populations. There were differences in *S* allele distribution between the naturalized populations in the different sampling areas, suggesting that *S* allele diversity in the naturalized populations is maintained. Our methodology, combining *S* allele distribution and positions of the plants *in situ*, should be applicable to other self-incompatible species to dissect the diversity of the *S* allele distribution and provide insight into the ecological diversity of their genetics.

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REFERENCES

- Altschul, S. F., Madden, T. L., Schffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.
- Bateman, A. J. (1955) Self-incompatibility systems in angiosperms. III. Cruciferae. *Heredity* **9**, 53–68.
- Brace, J., King, G. J., and Ockendon, D. J. (1994) A molecular approach to the identification of *S*-alleles in *Brassica oleracea*. *Sex. Plant Reprod.* **7**, 203–208.
- Byers, D. L., and Meagher, T. R. (1992) Mate availability in small populations of plant species with homomorphic sporophytic self-incompatibility. *Heredity* **68**, 353–359.
- Capella-Gutirrez, S., Silla-Martnez, J. M., and Gabaldn, T. (2009) trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* **25**, 1972–1973.
- Halkier, B. A., and Gershenzon, J. (2006) Biology and biochemistry of glucosinolates. *Annu. Rev. Plant Biol.* **57**, 303–333.
- Haseyama, Y., Kitashiba, H., Okamoto, S., Tonouchi, E., Sakamoto, K., and Nishio, T. (2018) Nucleotide sequence analysis of *S*-locus genes to unify *S* haplotype nomenclature in radish. *Mol. Breeding* **38**, 116.
- Hatakeyama, K., Takasaki, T., Watanabe, M., and Hinata, K. (1998a) Molecular characterization of *S* locus genes, *SLG* and *SRK*, in a pollen-recessive self-incompatibility haplotype of *Brassica rapa* L. *Genetics* **149**, 1587–1597.
- Hatakeyama, K., Watanabe, M., Takasaki, T., Ojima, K., and Hinata, K. (1998b) Dominance relationships between *S* alleles in self-incompatible *Brassica campestris* L. *Heredity* **80**, 241–247.
- Huh, M. K., and Ohnishi, O. (2001) Allozyme diversity and population structure of Japanese and Korean populations of wild radish, *Raphanus sativus* var. *hortensis* f. *raphanistroides* (Brassicaceae). *Genes Genet. Syst.* **76**, 15–23.
- Igic, B., Lande, R., and Kohn, J. R. (2008) Loss of self-incompatibility and its evolutionary consequences. *Int. J. Plant Sci.* **169**, 93–104.
- Johnson, M., Zaretskaya, I., Raytselis, Y., Merezuk, Y., McGinnis, S., and Madden, T. L. (2008) NCBI BLAST: a better web interface. *Nucleic Acids Res.* **36**, W5–W9.
- Kai, N., Suzuki, G., Watanabe, M., Isogai, A., and Hinata, K. (2001) Sequence comparisons among dispersed members of the *Brassica S* multigene family in an *S*⁹ genome. *Mol. Genet. Genomics* **265**, 526–534.
- Kakizaki, T., Takada, Y., Ito, A., Suzuki, G., Shiba, H., Takayama, S., Isogai, A., and Watanabe, M. (2003) Linear dominance relationship among four class-II *S* haplotypes in pollen is determined by the expression of *SP11* in *Brassica* self-incompatibility. *Plant Cell Physiol.* **44**, 70–75.
- Karron, J. D., Marshall, D. L., and Oliveras, D. M. (1990) Number of sporophytic self-incompatibility alleles in populations of wild radish. *Theor. Appl. Genet.* **79**, 457–460.
- Katoh, K., Rozewicki, J., and Yamada, K. D. (2019) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief. Bioinform.* **20**, 1160–1166.
- Kim, D., Jung, J., Choi, Y.-O., and Kim, S. (2016) Development of a system for *S* locus haplotyping based on the polymorphic *SLL2* gene tightly linked to the locus determining self-incompatibility in radish (*Raphanus sativus* L.). *Euphytica* **209**, 525–535.
- Kim, D. S., and Kim, S. (2018) Identification of the *S* locus core sequences determining self-incompatibility and *S* multigene family from draft genome sequences of radish (*Raphanus sativus* L.). *Euphytica* **214**, 16.
- Kim, D. S., and Kim, S. (2019) Development of a new *S* locus haplotyping system based on three tightly linked genes in the *S* locus controlling self-incompatibility in radish (*Raphanus sativus* L.). *Sci. Hort.* **243**, 70–77.
- Letunic, I., and Bork, P. (2019) Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res.* **47**, 256–259.
- Mackay, G. R. (1977) A diallel cross method for the recognition of *S* allele homozygotes in turnip, *Brassica campestris* L. ssp. *rapifera*. *Heredity* **38**, 201–208.
- Mitsui, Y., Shimomura, M., Komatsu, K., Namiki, N., Shibata-Hatta, M., Imai, M., Katayose, Y., Mukai, Y., Kanamori, H., Kurita, K., et al. (2015) The radish genome and comprehen-

- sive gene expression profile of tuberous root formation and development. *Sci. Rep.* **5**, 10835.
- Murase, K., Moriaki, Y., Mori, T., Liu, X., Masaka, C., Takada, Y., Maesaki, R., Mishima, M., Fujii, S., Hirano, Y., et al. (2020) Mechanism of self/nonself-discrimination in *Brassica* self-incompatibility. *Nat. Commun.* **11**, 4916.
- Nasrallah, J. B., and Nasrallah, M. E. (1993) Pollen-stigma signaling in the sporophytic self-incompatibility response. *Plant Cell* **5**, 1325–1335.
- Niikura, S., and Matsuura, S. (1998) Identification of self-incompatibility alleles (*S*) by PCR-RFLP in radish (*Raphanus sativus* L.). *Euphytica* **102**, 379–384.
- Nou, I. S., Watanabe, M., Isogai, A., and Hinata, K. (1993) Comparison of *S*-alleles and *S*-glycoproteins between two wild populations of *Brassica campestris* in Turkey and Japan. *Sex. Plant Reprod.* **6**, 79–86.
- Nou, I. S., Watanabe, M., Isogai, A., Shiozawa, H., Suzuki, A., and Hinata, K. (1991) Variation of *S*-alleles and *S*-glycoproteins in a naturalized population of self-incompatible *Brassica campestris* L. *Jpn. J. Genet.* **66**, 227–239.
- Ockendon, D. J. (1974) Distribution of self-incompatibility alleles and breeding structure of open-pollinated cultivars of Brussels sprouts. *Heredity* **33**, 159–171.
- Ockendon, D. J. (1980) Distribution of *S*-alleles and breeding structure of cape broccoli (*Brassica oleracea* var. 'italica'). *Theor. Appl. Genet.* **58**, 11–15.
- Ockendon, D. J. (1982) An *S*-allele survey of cabbage (*Brassica oleracea* var. *capitata*). *Euphytica* **31**, 325–331.
- Okamoto, S., Sato, Y., Sakamoto, K., and Nishio, T. (2004) Distribution of similar self-incompatibility (*S*) haplotypes in different genera, *Raphanus* and *Brassica*. *Sex. Plant Reprod.* **17**, 33–39.
- Park, J. I., Lee, S. S., Watanabe, M., Takahata, Y., and Nou, I. S. (2002) Identification of *S*-alleles using polymerase chain reaction-cleaved amplified polymorphic sequence of the *S*-locus receptor kinase in breeding lines of *Brassica oleracea*. *Plant Breed.* **121**, 192–197.
- Saitou, N., and Nei, M. (1987) The neighbor-joining methods: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.
- Sakamoto, K., Kusaba, M., and Nishio, T. (1998) Polymorphism of the *S*-locus glycoprotein gene (*SLG*) and the *S*-locus related gene (*SLR*) in *Raphanus sativus* L. and self-incompatible ornamental plants in the Brassicaceae. *Mol. Gen. Genet.* **258**, 397–403.
- Sampson, D. R. (1957) The genetics of self-incompatibility in the radish. *J. Hered.* **48**, 26–29.
- Sampson, D. R. (1964) A one-locus self-incompatibility system in *Raphanus raphanistrum*. *Can. J. Genet. Cytol.* **6**, 435–445.
- Sampson, D. R. (1967) Frequency and distribution of self-incompatibility alleles in *Raphanus raphanistrum*. *Genetics* **56**, 241–251.
- Schopfer, C. R., Nasrallah, M. E., and Nasrallah, J. B. (1999) The male determinant of self-incompatibility in *Brassica*. *Science* **286**, 1697–1700.
- Shiba, H., Takayama, S., Iwano, M., Shimosato, H., Funato, M., Nakagawa, T., Che, F.-S., Suzuki, G., Watanabe, M., Hinata, K., et al. (2001) A pollen coat protein, SP11/SCR, determines the pollen *S*-specificity in the self-incompatibility of *Brassica* species. *Plant Physiol.* **125**, 2095–2103.
- Steffan-Dewenter, I., and Kuhn, A. (2003) Honeybee foraging in differentially structured landscapes. *Proc. Biol. Sci.* **270**, 569–575.
- Stein, C. R., Howlett, B., Boyes, D. C., Nasrallah, M. E., and Nasrallah, J. B. (1991) Molecular cloning of putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*. *Proc. Natl. Acad. Sci. USA* **88**, 8816–8820.
- Stevens, J. P., and Kay, Q. O. N. (1989) The number, dominance relationships and frequencies of self-incompatibility alleles in a natural population of *Sinapis arvensis* L. in South Wales. *Heredity* **62**, 199–205.
- Suzuki, G., Kai, N., Hirose, T., Fukui, K., Nishio, T., Takayama, S., Isogai, A., Watanabe, M., and Hinata, K. (1999) Genomic organization of the *S* locus: identification and characterization of genes in *SLG/SRK* region of *S*⁹ haplotype of *Brassica campestris* (syn. *rapa*). *Genetics* **153**, 391–400.
- Suzuki, G., Watanabe, M., Kai, N., Matsuda, N., Toriyama, K., Takayama, S., Isogai, A., and Hinata, K. (1997) Three members of the *S* multigene family are linked to the *S* locus of *Brassica*. *Mol. Gen. Genet.* **256**, 257–264.
- Suzuki, G., Watanabe, M., Toriyama, K., Isogai, A., and Hinata, K. (1995) Molecular cloning of members of the *S*-multigene family in self-incompatible *Brassica campestris*. *Plant Cell Physiol.* **36**, 1273–1280.
- Takada, Y., Murase, K., Shimosato-Asano, Y., Sato, T., Nakanishi, H., Suwabe, K., Shimizu, K. K., Lim, Y. P., Takayama, S., Suzuki, G., et al. (2017) Duplicated pollen-pistil recognition loci control intraspecific unilateral incompatibility in *Brassica rapa*. *Nat. Plants* **3**, 17096.
- Takada, Y., Nakanowatari, T., Sato, J., Hatakeyama, K., Kakizaki, T., Ito, A., Suzuki, G., Shiba, H., Takayama, S., Isogai, A., et al. (2005) Genetic analysis of intra-species unilateral incompatibility between cultivar Osome and Turkish line in *Brassica rapa* (syn. *campestris*) L. *Sex. Plant Reprod.* **17**, 211–217.
- Takasaka, T., Hatakeyama, K., Suzuki, G., Watanabe, M., Isogai, A., and Hinata, K. (2000) The *S* receptor kinase determines self-incompatibility in *Brassica* stigma. *Nature* **403**, 913–916.
- Takayama, S., Shiba, H., Iwano, M., Shimosato, H., Che, F.-S., Kai, N., Watanabe, M., Suzuki, G., Hinata, K., and Isogai, A. (2000) The pollen determinant of self-incompatibility in *Brassica campestris*. *Proc. Natl. Acad. Sci. USA* **97**, 1920–1925.
- Takayama, S., Shimosato, H., Shiba, H., Funato, M., Che, F.-S., Watanabe, M., Iwano, M., and Isogai, A. (2001) Direct ligand-receptor complex interaction controls *Brassica* self-incompatibility. *Nature* **413**, 534–538.
- Tarutani, Y., Shiba, H., Iwano, M., Kakizaki, T., Suzuki, G., Watanabe, M., Isogai, A., and Takayama, S. (2010) Trans-acting small RNA determines dominance relationships in *Brassica* self-incompatibility. *Nature* **466**, 983–986.
- Thompson, K. F. (1957) Self-incompatibility in marrow-stem kale, *Brassica oleracea* var. *acephala*. I. Demonstration of a sporophytic system. *J. Genet.* **55**, 45–60.
- Vernon, D. M., and Meinke, D. W. (1994) Embryogenic transformation of the suspensor in *twinn*, a polyembryonic mutant of *Arabidopsis*. *Dev. Biol.* **165**, 566–573.
- Visser, D. L., Van Hal, J. G., and Verhoeven, W. H. (1982) Classification of *S*-alleles by their activity in *S*-heterozygotes of Brussels sprouts (*Brassica oleracea* var. *gemmifera* (DC.) Schultz). *Euphytica* **31**, 603–611.
- Wallace, D. H. (1979) Interactions of *S* alleles in sporophytically controlled self-incompatibility of *Brassica*. *Theor. Appl. Genet.* **54**, 193–201.
- Wang, K. C., Watanabe, M., and Hinata, K. (1994) Fluorescence and scanning electron microscopic study on self-incompatibility in distylous *Averrhoa carambola* L. *Sex. Plant Reprod.* **7**, 116–121.

- Watanabe, M., Ito, A., Takada, Y., Ninomiya, C., Kakizaki, T., Takahata, Y., Hatakeyama, K., Hinata, K., Suzuki, G., Takasaki, T., et al. (2000) Highly divergent sequences of the pollen self-incompatibility (*S*) gene in class-I *S* haplotypes of *Brassica campestris* (syn. *rapa*) L. *FEBS Lett.* **473**, 139–144.
- Watanabe, M., Nou, I. S., Takayama, S., Yamakawa, S., Isogai, A., Suzuki, A., Takeuchi, T., and Hinata, K. (1992) Variations in and inheritance of NS-glycoprotein in self-incompatible *Brassica campestris* L. *Plant Cell Physiol.* **33**, 343–351.
- Watanabe, M., Suwabe, K., and Suzuki, G. (2012) Molecular genetics, physiology and biology of self-incompatibility in Brassicaceae. *Proc. Jpn. Acad. Ser. B* **88**, 519–535.
- Watanabe, M., Takasaki, T., Toriyama, K., Yamakawa, S., Isogai, A., Suzuki, A., and Hinata, K. (1994) A high degree of homology exists between the protein encoded by *SLG* and the *S* receptor domain encoded by *SRK* in self-incompatible *Brassica campestris* L. *Plant Cell Physiol.* **35**, 1221–1229.
- Yamagishi, H., and Terachi, T. (2001) Intra- and Inter-specific variations in the mitochondrial gene *orf138* of Ogura-type male-sterile cytoplasm from *Raphanus sativus* and *Raphanus raphanistrum*. *Theor. Appl. Genet.* **103**, 725–732.
- Yasuda, S., Wada, Y., Kakizaki, T., Tarutani, Y., Miura-Uno, E., Murase, K., Fujii, S., Hioki, T., Shimoda, T., Takada, Y., et al. (2016) Complex dominance hierarchy is controlled by polymorphism of small RNAs and their targets. *Nat. Plants* **3**, 16206.

Supplementary Table S1. Nomenclature of S alleles in radish

S allele number	Class	Accession number of SRK	Reference
<i>RsS</i> ¹	I	AY052579	Kim and Kim (2019)
<i>RsS</i> ²	I	KX961696	Kim and Kim (2019)
<i>RsS</i> ³	II	Not determined	Kim and Kim (2019)
<i>RsS</i> ⁴	II	KX961697	Kim and Kim (2019)
<i>RsS</i> ⁵	I	KX961698	Kim and Kim (2019)
<i>RsS</i> ⁶	I	KX961699	Kim and Kim (2019)
<i>RsS</i> ⁷	I	KX961694	Kim and Kim (2019)
<i>RsS</i> ⁸	II	KX961700	Kim and Kim (2019)
<i>RsS</i> ⁹	I	KX961701	Kim and Kim (2019)
<i>RsS</i> ¹⁰	I	KX961702	Kim and Kim (2019)
<i>RsS</i> ¹¹	II	KX961703	Kim and Kim (2019)
<i>RsS</i> ¹²	I	AY534534	Kim and Kim (2019)
<i>RsS</i> ¹³	I	AY534535	Kim and Kim (2019)
<i>RsS</i> ¹⁴	I	AY052585	Kim and Kim (2019)
<i>RsS</i> ¹⁵	I	AY534536	Kim and Kim (2019)
<i>RsS</i> ¹⁶	I	KX961704	Kim and Kim (2019)
<i>RsS</i> ¹⁷	II	KX961695	Kim and Kim (2019)
<i>RsS</i> ¹⁸	I	KX961705	Kim and Kim (2019)
<i>RsS</i> ¹⁹	I	AY534539	Kim and Kim (2019)
<i>RsS</i> ²⁰	II	KX961706	Kim and Kim (2019)
<i>RsS</i> ²¹	I	KX961707	Kim and Kim (2019)
<i>RsS</i> ²²	II	KX961708	Kim and Kim (2019)
<i>RsS</i> ²³	I	KX961709	Kim and Kim (2019)
<i>RsS</i> ²⁴	I	AY534540	Kim and Kim (2019)
<i>RsS</i> ²⁵	I	KX961710	Kim and Kim (2019)
<i>RsS</i> ²⁶	I	Not determined	Kim and Kim (2019)
<i>RsS</i> ²⁷	I	KX961711	Kim and Kim (2019)
<i>RsS</i> ²⁸	I	KX961712	Kim and Kim (2019)
<i>RsS</i> ²⁹	I	AY534543	Kim and Kim (2019)
<i>RsS</i> ³⁰	I	KX961713	Kim and Kim (2019)
<i>RsS</i> ³¹	I	AY052581	Kim and Kim (2019)
<i>RsS</i> ³²	I	AY534542	Deposited in database
<i>RsS</i> ³³	I	LC341225	Haseyama et al. (2018)
<i>RsS</i> ³⁴	II	LC591983	This Study (Ts3)
<i>RsS</i> ³⁵	I	LC591984	This Study (Ts18)
<i>RsS</i> ³⁶	I	LC591985	This Study (Nk6)
<i>RsS</i> ³⁷	I	LC591986	This Study (Hh2)

Supplementary Table S2. Oligonucleotide primers used for PCR amplification of *SRK* partial sequences

Primer set	Target <i>SRK</i> gene	Primer	Primer sequence	Reference
PK	class-I	class-I SRK-PK-F	5'-TATGAATGAGGTGASATTRATCGCRAG-3'	designed in this study
		class-I SRK-PK-R	5'-TGAGCAGGTGTACTKGTTACACCGT-3'	
	class-II	class-II SRK-PK-F	5'-CATGAACGAAGTCAGGCTAATTGCAAA-3'	
		class-II SRK-PK-R	5'-CAAAGACTATCATATTACCGAGCGTCA-3'	
KK	class-I	class-I SRK-KK-F	5'-GATGARTTTATGAATGAGGTGASA-3'	Kim and Kim (2019)
		class-I SRK-KK-R	5'-GGCTGAGGAATCTCTGTTGC-3'	
	class-II	class-II SRK-KK-F	5'-TCCGACTTCTTGGCTGTTGT-3'	
		class-II SRK-KK-R	5'-AGAACTTYSGCTGACGCAAT-3'	

Supplementary Table S3. Assignment of amplified nucleotide sequences by BLAST search of established cultivars in a public database

Seed company	Cultivar	Reference	Identified S allele	Class
Tohoku Seed Co.	Pekinmizu (S^{204} x unidentified S allele)	Niikura and Matsuura (1998)	R_sS^8	II
			R_sS^{12}	I
	No. 5 (S^{205} x S^{209})	Niikura and Matsuura (1998)	R_sS^1	I
			R_sS^{17}	II
	Eisai ((S^{202} x S^{206}) x (S^{201} x S^{203}))	Niikura and Matsuura (1998)	R_sS^2	I
			R_sS^{10}	I
Takii & Co.	Kanpaku (S^1 , S^4)	Sakamoto et al. (1998)	R_sS^2	I
		Haseyama et al. (2018)	R_sS^{31}	I
	Shinhasshu (S^1 , unidentified S allele)	Sakamoto et al. (1998)	R_sS^2	I
		Haseyama et al. (2018)	R_sS^{31}	I
	YR Kurama (S^2 , S^3 , S^4)	Sakamoto et al. (1998)	R_sS^7	I
		Haseyama et al. (2018)	R_sS^{10}	I
	Oshin (S^{10} , S^{15} , unidentified S alleles)	Sakamoto et al. (1998)	R_sS^1	I
		Haseyama et al. (2018)	R_sS^5	I
			R_sS^{12}	I

Supplementary Table S4. Identified S alleles of each plant from five different populations in March 2020

Plant	Class	S allele	Plant	Class	S allele
Tk1		Not determined	Hh1		Not determined
Tk2	I	<i>RsS</i> ²⁸	Hh2	I	<i>RsS</i> ³⁷
Tk3	I	<i>RsS</i> ³³	Hh3	I	<i>RsS</i> ³²
Tk4	I	<i>RsS</i> ¹²	Hh4	I	<i>RsS</i> ²³
Tk5	II	<i>RsS</i> ²²	Hh5	I	<i>RsS</i> ¹³
Tk6	II	<i>RsS</i> ¹¹	Ts1	I	<i>RsS</i> ¹⁴
Tk7	I	<i>RsS</i> ⁷	Ts2	I	<i>RsS</i> ¹³
Tk8	II	<i>RsS</i> ²²		I	<i>RsS</i> ²⁵
Tk9	I	<i>RsS</i> ⁷	Ts3	II	<i>RsS</i> ³⁴
	II	<i>RsS</i> ²²	Ts4	II	<i>RsS</i> ⁸
Tk10	I	<i>RsS</i> ²⁸		I	<i>RsS</i> ²³
Tk11	II	<i>RsS</i> ²²	Ts5	I	<i>RsS</i> ²⁵
Tk12	II	<i>RsS</i> ¹⁷	Ts6	I	<i>RsS</i> ²⁷
Kr1	I	<i>RsS</i> ²	Ts7	I	<i>RsS</i> ¹³
	II	<i>RsS</i> ²²	Ts8	I	<i>RsS</i> ³⁰
Kr2	II	<i>RsS</i> ⁸	Ts9	I	<i>RsS</i> ¹³
	I	<i>RsS</i> ³³	Ts10	I	<i>RsS</i> ²⁵
Kr3	I	<i>RsS</i> ⁷	Ts11	I	<i>RsS</i> ¹³
	II	<i>RsS</i> ²²	Ts12	I	<i>RsS</i> ²⁸
Kr4	I	<i>RsS</i> ³³	Ts13	I	<i>RsS</i> ²⁵
Kr5	II	<i>RsS</i> ²²	Ts14	I	<i>RsS</i> ¹³
Kr6	I	<i>RsS</i> ¹⁴	Ts15	I	<i>RsS</i> ²⁷
	II	<i>RsS</i> ²²	Ts16	II	<i>RsS</i> ⁸
Kr7	II	<i>RsS</i> ⁸	Ts17	I	<i>RsS</i> ²⁵
	I	<i>RsS</i> ¹²	Ts18	I	<i>RsS</i> ³⁵
Kr8	II	<i>RsS</i> ⁸	Ts19	I	<i>RsS</i> ¹⁴
	II	<i>RsS</i> ²²			
Kr9	I	<i>RsS</i> ⁷			
Kr10	I	<i>RsS</i> ²			
Kr11	II	<i>RsS</i> ²²			
Kr12	I	<i>RsS</i> ⁷			
	II	<i>RsS</i> ²²			
Nk1	II	<i>RsS</i> ¹¹			
	I	<i>RsS</i> ¹⁴			
Nk2	I	<i>RsS</i> ¹⁴			
	II	<i>RsS</i> ²²			
Nk3	I	<i>RsS</i> ¹⁴			
Nk4		Not determined			
Nk5	I	<i>RsS</i> ¹⁴			
Nk6	I	<i>RsS</i> ³⁶			

A

B

C

Supplementary Fig. S1. Naturalized radish plants growing in Yakushima in March 2020. (A) A whole plant *in situ*. (B) Flowers of a naturalized radish plant. (C) Structure of the whole plant Ts6 (see also Fig. 5C).

A

B

C

D

Supplementary Fig. S2. The relationship between S allele distribution and the position of each radish plant in 1 m × 1 m quadrats in Tsukazaki (A), Kurio (B), Nakama (C) and Haruta (D). Red circles indicate plants with flowers (sampled). Blue triangles indicate small plants without flowers (not sampled).

A

SRK-a (GQ121139)	1	GCCLDPDDTKLIYDYIENSGLDYFLFRKKLSSNLNWKDRFSIRSGVAPGLLSRLHSRFR	60
RsSRK35 (Ts18)	1	GCCIDGDEKMLIYEYLENLSLDSHLFGKTRSSKLSWKERFDIVNGVARGLLYLHQDSRFR	60
RsSRK36 (Nk6)	1	GCCIDADEKMLIYEYLENLSLDSYLFGKTQRSKLNWKERFDITNGVARGLLYLHQDSRFR	60
RsSRK37 (Hh2)	1	GCCIEADEMMLIYEYLENSSLDYLFNGTRRSKPNWRERFDIINGVARGLLYLHQDSRFR	60
SRK-a (GQ121139)	61	IIHRDMKAGNILLDKNMIPKISDFGLARIARDQTEASTDTPIGTYGYSPEYAMYGILS	120
RsSRK35 (Ts18)	61	IIHRDLKASNILLDKNMIPKISDFGMARVFADETEVNTKKVVGTYGYSPEYAMHGIFS	120
RsSRK36 (Nk6)	61	IIHRDLKVSNIILLDKNMIPKISDFGMARIFGRDETEANTMKVVGTYGYSPEYAMNGIFS	120
RsSRK37 (Hh2)	61	IIHRDLKVSNIILLDKNMIPKISDFGMARVFAREETQANTMKVVGTYGYSPEYAIHGIFS	120
SRK-a (GQ121139)	121	EKTDVFSFGVIVLEIVTGKRNRGFYQSNPEDNLVICYAWTHWAQGRALEIVDPVIVDSLS-	179
RsSRK35 (Ts18)	121	EKSDIFSFGVIVLEIVTAKRNRGFNNLNIEDNLLNYAWSNWEEGRALEIIDPDIIVDSLS-	179
RsSRK36 (Nk6)	121	EKSDVFSFGVIVLEIVTGKRNRGFYNLNKNNFLSYAWSHWKEGRALEIIDPVITDSFSS	180
RsSRK37 (Hh2)	121	EKSDVFSFGVIVLEIVTGKRNRGFNNLNIEDNLLNYAWRHWKEGRALEFVDPVIVDSLS	180
SRK-a (GQ121139)	180	--STFQPKFVLKCIQIGLLCIQERAEHRPTMSS----	210
RsSRK35 (Ts18)	180	-----QEVLKCVQIGLLCVQDLAEHRPTMSSVVG	209
RsSRK36 (Nk6)	181	LSSTFQRQFVLKCIQIGLLCVQELAEHRPT-----	210
RsSRK37 (Hh2)	181	LPSTFQPQFVLKCIQIGLLCVQELQSTDQ-----	209

B

SRK-15 (LC341214)	1	YEYIENLSIDSHLFDETRSCMLNWQMRFDIINGIARGLLYLHQDSRFRIIHRDLKASNVL	60
RsSRK34 (Ts3)	1	YEYIENLSIDSHLFDETRSCMLNWQMRFDIINGIARGLLYLHQDSRFRIIHRDLKASNVL	60
SRK-15 (LC341214)	61	LDKDMTPNISDFGMARIFGRDETEADTRKVVGTYGYSPEYAMNGTFSMKSDVFSFGVLL	120
RsSRK34 (Ts3)	61	LDKDMTPKISDFGMARIFGRDETEADTRKVVGTYGYSPEYAMNGTFSMKSDVFSFGVLL	120
SRK-15 (LC341214)	121	LEIISGKRNGKFCDSNSNLNLLGCVWRNWKEGQGLEIVDRIVIDSSSPTFRPCEILRCIQ	180
RsSRK34 (Ts3)	121	LEIISGKRNGKFCDSNSNLNLLGCVWRNWKEGQGLEIVDRVIDSSSPTFRPREILRYMQ	180
SRK-15 (LC341214)	181	IGLLCVQERVEEDRPMMSSV	199
RsSRK34 (Ts3)	181	IGLVCVQERVEEDRPMMSSL	199

Supplementary Fig. S3. Alignment of deduced partial amino acid sequences of the novel SRKs found in samples from Yakushima. (A) Deduced partial amino acid sequences of the class-I alleles of SRK (RsSRK³⁵, RsSRK³⁶ and RsSRK³⁷) aligned with that of RsSRK-a (accession no. GQ121139), a class-I SRK deposited in the DDBJ/EMBL/GenBank databases. (B) Deduced partial amino acid sequence of the class-II allele of SRK (RsSRK³⁴) aligned with that of RsSRK-15 (accession no. LC341214), a class-II SRK deposited in the DDBJ/EMBL/GenBank databases.