Note

Classification of "nabana" (*Brassica rapa*) cultivars and landraces based on simple sequence repeat markers

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Brassica rapa or *B. napus* vegetables for eating as young inflorescences and stalks are called "nabana". Japanese nabana includes "flower-bud type" and "stem-and-leaf type". Chinese and European types are also known (cai-xin, zicaitai, and broccoletto). We classified nabana belonging to *B. rapa* and other *B. rapa* vegetables. In a simple sequence repeat-based phylogram, 49 ingroup samples were classified into four groups (I–IV). Flower-bud and stem-and-leaf types were separated into groups I and III, respectively, with a slight overlap in group II. Cai-xin and non-heading Chinese cabbages were included in group IV. Broccoletto was placed in group III, close to turnips. Zicaitai cultivars were included in group II. We tested for clubroot resistance (CR) and its marker genotypes in nabana because of their agronomical importance. Ten cultivars were resistant to group 4 pathogen but not to group 2. Most of the CR cultivars had heterozygous resistance alleles in the *CRb* and *Crr1* loci, consistent with inoculation tests. Our results suggest that Japanese nabana lines and foreign types were differentiated according to their consumption parts and cultivar origins, respectively. This study elucidates the relationships and CR properties of nabana and provides valuable information for the breeding of nabana cultivars.

Key Words: *Brassica rapa*, classification, clubroot resistance (CR), nabana, simple sequence repeat (SSR).

Introduction

"Nabana" is the Japanese name for *Brassica* vegetables—except for broccoli and cauliflower—used for their edible young inflorescences and stalks. This type of vegetable includes two species, *B. rapa* and *B. napus* (see Ishida 2004 for a review), in which the former species is more popular. In Kyoto, nabana is called "hanana" because it was formerly planted for cut flowers (Takashima 2003). Nabana or hanana is currently regarded as an associated heirloom vegetable of Kyoto (Kyo-yasai). According to the parts mainly used for

consumption, two types exist in Japanese nabana: "flower-bud type" mainly used for young stalks with flower buds; and "stem-and-leaf type" used for stems including tender young leaves and small flower buds. Chinese and European types of such vegetable are also known and are often called flowering Chinese cabbage. They include "cai-xin", "zicaitai" ("kosaitai" in Japanese), and "broccoletto" (and many other designations for each), all of which are members of *B. rapa* (Bonnema *et al.* 2011, see Cheng *et al.* 2016b for their morphotypes). Several nabana cultivars have been released from seed companies, and landraces are locally maintained from northeastern to western parts of Japan (Vegetable and Ornamental Crops Research Station 1980). However, reports on their relationships are quite limited. Aoba (1964) measured several morphological traits in Japanese non-heading *B. rapa* vegetables including local

varieties of nabana and showed a correlation between seed coat type and geographical distribution. Takuno *et al.* (2007) included a nabana landrace for classification of *B. rapa* vegetables. Previous studies indicated that cai-xin and broccoletto were close to pak choi and turnips, respectively (Pino Del Carpio *et al.* 2011, Zhao *et al.* 2005). Cheng *et al.* (2016a, 2016b) re-sequenced hundreds of *B. rapa* and *B. oleracea* germplasms, including several cai-xin and zicaitai lines. They classified germplasm based on single nucleotide polymorphism markers to show that the pak choi, wutacai, cai-xin, and zicaitai varieties form a single group (Cheng *et al.* 2016a). The relationship of zicaitai to other *B. rapa* vegetables is unclear because its closest type differed in previous reports (Cheng *et al.* 2016a , Pino Del Carpio *et al.* 2011, Zhao *et al.* 2005).

Nabana is mainly grown in Chiba Prefecture and the western part of Japan (Kansai and Shikoku regions). In these cultivation areas, clubroot disease caused by the soil-borne, obligate parasite *Plasmodiophora brassicae* is one of the most serious diseases. Multiple pathotypes or races of this species with different pathogenicity are found in the field (see Hirani and Li 2015 for a review). Hatakeyama *et al.* (2004) classified Japanese field isolates into four pathotypes (groups 1–4) based on their pathogenicity to a clubroot resistant (CR) Chinese cabbage cultivar set. Group 2 and 4 pathotypes have been found in nabana cultivation fields in Chiba and Kyoto Prefectures (Kubo *et al.* 2017, Oshikiri *et al.* 2014). Concerning the CR trait, some of the European fodder turnips are highly resistant to clubroot disease and their responsible CR loci have been identified in part (see Hirani and Li 2015 for a review). For example, the *CRb* locus derived from a Chinese cabbage cultivar 'CR Shinki', whose resistance source might be a CR fodder turnip 'Gelria R' (Hirai 2006), is effective against group 3 and 4 pathotypes in a dominant manner (Kato *et al.* 2012). *Crr1* from a CR fodder turnip 'Siloga' is an incompletely dominant locus conveying resistance to group 2 and 4 pathotypes (Hatakeyama *et al.* 2013, Suwabe *et al.* 2006). *Crr2*, another locus from 'Siloga', provides high resistance under the co-existence of homozygous resistance alleles at both *Crr1* and *Crr2* loci (Suwabe *et al.* 2003, 2006). *Crr3, CRc*, and *CRk* loci derived from CR fodder turnips 'Milan white' and 'Debra' have also been identified (Hirai *et al.* 2004, Sakamoto *et al.* 2008). Several CR cultivars have been bred in *B. rapa* vegetables from CR fodder turnips and such cultivars are also available in Japanese nabana. To date, eight CR nabana cultivars have been tested to show that they are resistant to group 3 and 4 pathotypes but are susceptible to group 2 pathotype (Kuginuki 2001, Oshikiri *et al.* 2014). However, genetic information on CR traits is unknown for Japanese nabana except for 'Hanamusume', whose resistance source is 'Gelria R' (Tomikawa 1997).

Molecular markers are useful tools for genetic analyses such as classification, linkage mapping, and positional cloning of genetic loci. Many kinds of molecular markers have been developed and used in *B. rapa*. Of these, simple se-quence repeats (SSRs) are DNA repeats consisting of 1–6 nucleotide repeat units. SSRs are frequently used as molecular markers in many eukaryotic organisms because of their merits: abundance in eukaryotic genomes; high rates of polymorphism and stability; and relatively easy detection of different alleles (see Merritt *et al.* 2015 for a review).

In this study, we conducted a classification of nabana cultivars and landraces with other *B. rapa* vegetables (neep greens, turnips, heading and non-heading Chinese cabbages). Tests for CR and detection of CR alleles were also performed in nabana cultivars because of their agronomical importance. Genetic relationships among nabana lines and CR properties of nabana cultivars are discussed.

Materials and Methods

Plant materials

Thirty-nine lines of Japanese nabana cultivars and landraces were used in this study (**Table 1**; **Supplemental Table 1**), all of which are members of *B. rapa*. No nabana line belonging to *B. napus* was investigated because its amphidiploid genome could produce more than two alleles per SSR locus. For Chinese and European types, five (one cai-xin, three zicaitai (kosaitai), and one broccoletto) cultivars were used. Six cultivars of other *B. rapa* vegetables (neep greens, turnips, non-heading and heading Chinese cabbages) were also included in this study. Approximately 20 individuals per line were investigated for SSR analysis.

DNA extraction, data analysis, and construction of a phylogram

Genomic DNA was extracted from fresh leaves using the CTAB method (Murray and Thompson 1980) or DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) with slight modifications. Eight Chinese cabbage SSR markers (Suwabe *et al.* 2002, 2006) were selected from preliminary experiments based on polymorphisms (**Supplemental Table 2**). SSR fragments were amplified by polymerase chain reaction (PCR) with fluorescence-labeled primers (Sigma-Aldrich, St Louis, MO, USA) and analyzed on a CEQ8000 DNA sequencer (Sciex, Vaughan, Canada) as reported previously (Kubo *et al.* 2009).

Numbers of alleles per locus (A), allelic richness (A_R, a measure of the numbers of alleles independent of sample size) (Petit *et al.* 1998), expected (H_E) and observed heterozygosities (H_O), and fixation index (F_{IS}) (**Table 1**) were calculated with GENEPOP 4.2 (Rousset 2008) and FSTAT 2.9.3 softwares (Goudet 1995, 2001). Deviation of F_{IS} from Hardy-Weinberg equilibrium (HWE) was tested with FSTAT 2.9.3. A population-based neighbor-joining (NJ) phylogram was constructed using Populations 1.2.32 software (Langella 2011). A Chinese cabbage cultivar 'Muso' was used as an outgroup of the phylogram. Bootstrap analysis was performed from 1,000 replications.

Table 1. Genetic diversity of 50 *B. rapa* lines analyzed in this study

Type	Cultivar or line name[a]	N[b]	$H_O{}^c$	$H_E{}^d$	$F_{IS}{}^e$	Note
Japanese nabana, "flower-bud type"						
	CR Hanakanzashi	19	0.2961	0.1711	−0.7308*	Hybrid CR cultivar
	CR Hanamatsuri	20	0.4500	0.2444	−0.8412*	Hybrid CR cultivar
	CR Hananomai	17	0.3750	0.2822	−0.3290*	Hybrid CR cultivar
	CR Kyobare	17	0.3162	0.2544	−0.2430	Hybrid CR cultivar
	CR Kyonoharu	19	0.3816	0.2712	−0.4070*	Hybrid CR cultivar
	Eika	18	0.2639	0.1640	−0.6090*	Hybrid CR cultivar
	Hanamusume	19	0.8224	0.4433	−0.8549*	Hybrid CR cultivar
	Shunka	13	0.3365	0.2881	−0.1683	Hybrid CR cultivar
	Shunrai	15	0.4000	0.2119	−0.8876*	Hybrid CR cultivar
	88 go no. 20	20	0.4375	0.2352	−0.8601*	Hybrid cultivar
	Hanaguruma	18	0.6042	0.3505	−0.7238*	Hybrid cultivar
	Hanakazari	20	0.5312	0.3059	−0.7366*	Hybrid cultivar
	Kanzaki 21 go	20	0.3563	0.2569	−0.3867*	Hybrid cultivar
	Shuka	20	0.3125	0.2053	−0.5224*	Hybrid cultivar
	Soyo 1 go	20	0.3250	0.3105	−0.0466	Hybrid cultivar
	Toka	18	0.5139	0.3288	−0.5627*	Hybrid cultivar
	Ezuki	19	0.4803	0.4549	−0.0558	Non-hybrid cultivar
	Kaneki hanana	19	0.5066	0.4846	−0.0452	Non-hybrid cultivar
	Kanzaki natane	14	0.5625	0.4753	−0.1835	Non-hybrid cultivar
	Kurokawa kanzaki	20	0.3875	0.3903	0.0072	Non-hybrid cultivar
	Kyoto Fushimi kanzaki	19	0.4605	0.4958	0.0711	Non-hybrid cultivar
	Nabana (kanzaki hanana)	19	0.4671	0.4635	−0.0079	Non-hybrid cultivar
	Shokuyo nanohana	20	0.4313	0.4979	0.1338	Non-hybrid cultivar
	Shunyo	20	0.0187	0.0638	0.7062	Non-hybrid cultivar
	Soshun nabana	20	0.2687	0.2789	0.0366	Non-hybrid cultivar
	Awa zairai	19	0.3092	0.3876	0.2023	Landrace
	Nagaokakyo	20	0.0750	0.1082	0.3070	Landrace
Japanese nabana, "stem-and-leaf type"						
	Fukitachina (kasamai-kei)	20	0.6188	0.6128	−0.0097	Non-hybrid cultivar
	Himeji wakana	20	0.3563	0.3768	0.0546	Non-hybrid cultivar
	Kukitachina	18	0.5833	0.5729	−0.0182	Non-hybrid cultivar
	Nagaokana	20	0.4437	0.4595	0.0344	Non-hybrid cultivar
	Orina	19	0.5461	0.5475	0.0027	Non-hybrid cultivar
	Wakana	18	0.5139	0.5118	−0.0040	Non-hybrid cultivar
	Wakana (kurona)	22	0.5739	0.5974	0.0394	Non-hybrid cultivar
	Fukidachi (kukidachi)	18	0.5000	0.5139	0.0270	Landrace
	Katsuyama mizuna 1 go	16	0.2812	0.2599	−0.0822	Landrace
	Natane G	16	0.5469	0.4870	−0.1230	Landrace
	Orina (fukitachi)	20	0.6375	0.5868	−0.0863	Landrace
	Sangatsuna	20	0.4062	0.4977	0.1837	Landrace
Chinese flowering cabbage, "zicaitai"						
	Hon tsai tai	19	0.1842	0.1776	−0.0370	Non-hybrid cultivar
	Kosaitai	19	0.3355	0.3330	−0.0077	Non-hybrid cultivar
	Kosaitai (beni nabana)	20	0.2938	0.3089	0.0490	Non-hybrid cultivar
Chinese flowering cabbage, "cai-xin"						
	Wasekei saishin	20	0.2812	0.3285	0.1437	Non-hybrid cultivar
European flowering cabbage, "broccoletto"						
	Cima di rapa	20	0.5000	0.5360	0.0672	Non-hybrid cultivar
Other *B. rapa* vegetables						
	Shiroguki hatakena	19	0.5855	0.6513	0.1010	Non-hybrid cultivar
	CR Omasa	19	0.3750	0.1875	−1.0000*	Hybrid CR cultivar
	Golden ball	17	0.3897	0.4058	0.0396	Non-hybrid cultivar
	Pak choi	20	0.4562	0.5166	0.1168	Non-hybrid cultivar
	Taasai	19	0.3553	0.3359	−0.0577	Non-hybrid cultivar
	Muso	17	0.0588	0.0460	−0.2800	Hybrid cultivar

[a] Cultivars are sorted according to their categories (CR/non-CR and hybrid/non-hybrid) (see **Supplemental Table 1** for details).

[b] Sample number.

[c] Observed heterozygosity.

[d] Expected heterozygosity.

[e] Fixation index. Asterisk: significant deviation from Hardy-Weinberg equilibrium expectations after Bonferroni corrections ($p < 0.05$) based on 400,000 randomisations.

Population structure analysis

Detection of a hierarchical genetic population structure was performed with STRUCTURE 2.3.4 software (Hubisz *et al.* 2009) with 50,000 burn-in steps and 1,000,000 Markov chain Monte Carlo steps after burn-in. Suitable number of subpopulations (K) was determined based on the ΔK values (Evanno *et al.* 2005) (**Supplemental Table 3**) with STRUCTURE HARVESTER 0.6.94 software (Earl and vonHoldt 2012). Bar plots at determined K value were drawn with CLUMPAK 1.1 software (Kopelman *et al.* 2015).

Tests for CR and CR marker analysis of nabana cultivars

Inoculation tests were performed according to Kuginuki *et al.* (1999). *P. brassicae* isolates Ng2 and Ng9, whose pathotypes are groups 4 and 2, respectively (Kubo *et al.* 2017), were inoculated to 10 CR and two non-CR nabana cultivars (**Table 2**), in which approximately 20 individuals were tested for each cultivar. A Chinese cabbage cultivar set with differential pathogenicity (Hatakeyama *et al.* 2004) was also inoculated as a control.

Six markers linked to four known CR loci (*CRb* and *Crr1-3*) were tested by PCR amplification using 10 CR and one non-CR nabana cultivars (four individuals per cultivar) (**Supplemental Table 4**) according to previous reports (Hirai *et al.* 2004, Kato *et al.* 2013, Matsumoto *et al.* 2017, Suwabe *et al.* 2006). DNAs of three CR lines ('CR Shinki', G004, and N-WMR-3) (Hirai *et al.* 2004, Suwabe *et al.* 2003) were used for positive controls of *CRb*, *Crr1/Crr2*, and *Crr3* resistance alleles, respectively. Alleles were detected using a DNA sequencer with a post-labeling method (Shimizu and Yano 2011) or by agarose gel electrophoresis.

Results

Polymorphisms of SSR markers and genetic diversity of nabana lines

In eight SSR markers used in this study, A and A_R values ranged from 3 to 12 and from 2.915 to 7.995, respectively (**Supplemental Table 2**). The average value of A in this study (8.75) was similar to that in a previous report on turnips (8.72) (Takahashi *et al.* 2016). H_E ranged from 0.0638 to 0.4979 and from 0.2599 to 0.6128 in flower-bud and stem-and-leaf types of Japanese nabana, respectively (**Table 1**). The latter type showed higher values than the former. F_{IS} values of non-hybrid cultivars and landraces in any type of *B. rapa* did not differ significantly from HWE. In contrast, F_{IS} values were significantly deviated from HWE in hybrid cultivars of 13 flower-bud types of nabana and a turnip (**Table 1**, asterisks).

Relationships of nabana cultivars and landraces based on an NJ phylogram

According to the SSR data, a population-based NJ phylogram was constructed using a heading Chinese cabbage cultivar 'Muso' as an outgroup (**Fig. 1**). The ingroup 49
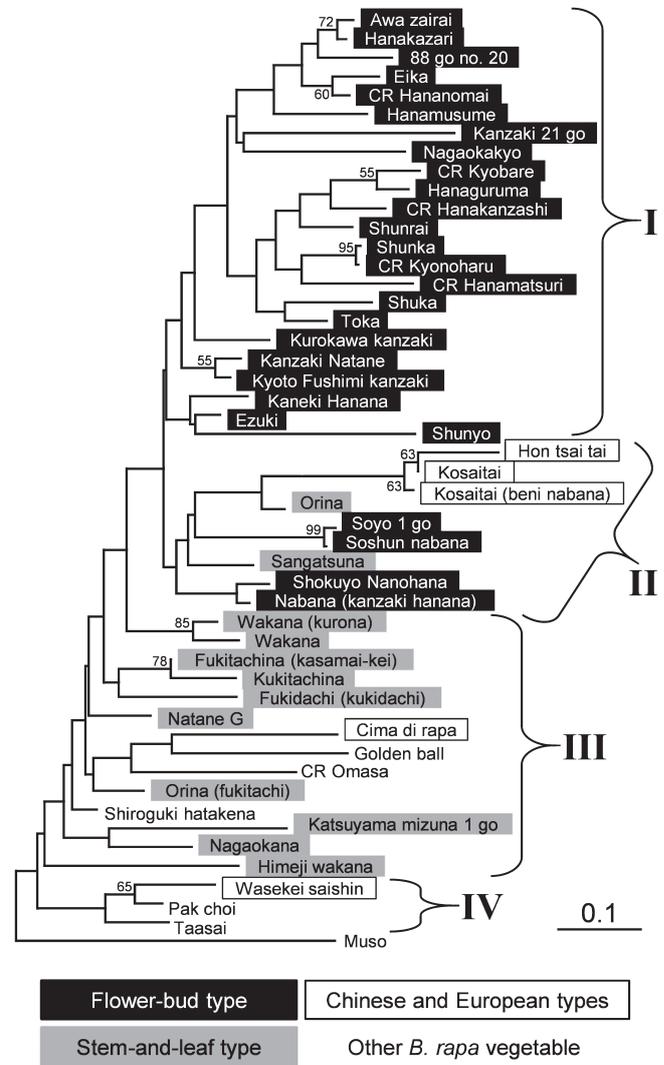
Fig. 1. A neighbor-joining phylogram of nabana based on eight *B. rapa* SSR loci. "Flower-bud type", "stem-and-leaf type", Chinese and European types, and other types of *B. rapa* cultivars (neep greens, turnips, non-heading and heading Chinese cabbages) are indicated with filled, gray, and open boxes, and plain typeface, respectively. Four potential groups are indicated with roman numbers (I–IV). Numbers on nodes are bootstrap values from 1,000 replicates (≥50%). The scale bar indicates the genetic distance D_A (Nei *et al.* 1983).

lines could be classified into four groups (I–IV). Group I included 23 flower-bud types of Japanese nabana (21 cultivars and two landraces) (**Fig. 1**, filled boxes). Group II was a mixture of four flower-bud types, two stem-and-leaf types, and three zicaitai cultivars (**Fig. 1**, filled, gray, and open boxes). Group III comprised of 10 stem-and-leaf types of nabana lines, one broccoletto ('Cima di rapa'), two turnips, and one neep greens (**Fig. 1**, gray and open boxes, and plain typeface). Group IV contained one cai-xin ('Wasekei saishin') and two non-heading Chinese cabbage cultivars (**Fig. 1**, open box and plain typeface).

Population structure of nabana lines

We investigated the population structures of the 50 *B. rapa* lines. The most suitable value for *K* was obtained at *K* = 2 (**Supplemental Table 3**, red text) based on Δ*K* values (Evanno *et al*. 2005) after the calculation of *K* values from 1 to 7. According to the estimated two subpopulations, clusters 1 and 2 represented flower-bud type and the other *B. rapa* vegetables, respectively (**Supplemental Fig. 1**, light blue and orange colors). Exceptions were observed in Awa zairai and 'Shunyo', which were more derived from cluster 2 than the other flower-bud types, and for 'Sangatsuna', 'Wakana' and 'Wakana (kurona)', which were more derived from cluster 1.

Tests for CR in nabana cultivars

We tested for CR traits in Japanese nabana cultivars because these are the most important targets for the breeding of *B. rapa* vegetables. Ten CR and two non-CR nabana cultivars were inoculated with pathogens of group 2 and 4 pathotypes. Non-CR cultivars were susceptible to both pathogens, as expected. All of the 10 CR cultivars were resistant to group 4 pathotype of mild virulence (**Table 2**, gray boxes) whereas they showed no resistance to more virulent pathotype from group 2. This result was consistent with the previous reports for eight CR cultivars (Kuginuki 2001, Oshikiri *et al*. 2014).

Genotypes of CR loci in nabana cultivars

Next, we analyzed genotypes of Japanese nabana cultivars with six markers linked to *CRb* and *Crr1-3* (Hirai *et al*.

Table 2. Test for clubroot resistance (CR) of nabana cultivars

Cultivar[a]	Pathogen	Ng2 (group 4) ID[b]	Ng9 (group 2) ID[b]
Chinese cabbage (control)	SCR Hioroki	0.0 (−)	0.1 (−)
	CR Ryutoku	0.0 (−)	2.0 (+)
	Muso	3.0 (+)	3.0 (+)
Non-CR nabana	Hanakazari	3.0 (+)	3.0 (+)
	88 go no. 20	2.8 (+)	3.0 (+)
CR nabana	CR Hanakanzashi	0.0 (−)	1.9 (±)
	CR Hanamatsuri	0.0 (−)	2.2 (+)
	CR Hananomai	0.0 (−)	2.3 (+)
	CR Kyobare	0.0 (−)	2.1 (+)
	CR Kyonoharu	0.0 (−)	2.2 (+)
	Eika	0.0 (−)	2.1 (+)
	Kanzaki 21 go	0.0 (−)	2.2 (+)
	Shunka	0.0 (−)	2.0 (+)
	Shunrai	0.0 (−)	2.3 (+)
	Hanamusume	0.2 (−)	3.0 (+)

[a] Three different cultivars of Chinese cabbage used for classification based on the system of Hatakeyama *et al*. (2004). Two CR cultivars, 'CR Ryutoku' and 'Super CR Hiroki', showed different resistance responses depending on clubroot pathogen. A non-CR cultivar 'Muso' susceptible to any clubroot pathogen was used as a positive control.

[b] Mean disease index according to Kuginuki *et al*. (1999). −, ±, and + represent resistant (ID ≤ 1.0), intermediate (1.0 < ID < 2.0), and susceptible (ID ≥ 2.0), respectively. Resistance is indicated with a gray box.

2004, Kato *et al*. 2013, Matsumoto *et al*. 2017, Suwabe *et al*. 2006). The 10 CR cultivars tested had a resistance allele with the *CRb* marker B1210 (**Supplemental Table 4**, red text). Nine CR cultivars (except for 'Hanamusume') showed a resistance allele with another *CRb* marker (KB59N06) and two *Crr1* markers (BSA7 and B359). By contrast, only 'Hanamusume' had a resistance allele with the *Crr2* marker 523A1R. Most of the resistance loci were heterozygous in the CR nabana cultivars (**Supplemental Table 4**, two numbers connected with slashes) except for the B1210 locus, whose genotype might have been detected as homozygous because of low levels of polymorphisms. There was no nabana cultivar with a resistance allele carrying the *Crr3* marker OPC11-2S.

Discussion

Genetic diversity of nabana lines

In this study, we classified Japanese nabana cultivars and landraces, foreign types of cultivars, and other *B. rapa* vegetables based on SSR markers. Our data suggest that the stem-and-leaf type lines were under less stringent selection pressure because they are maintained as local crops (Vegetable and Ornamental Crops Research Station 1980). Non-hybrid cultivars and landraces in our samples could be treated as randomly mating populations based on their F_{IS} values without significant deviation from HWE, although the Nagaokakyo landrace might be inbred judging from its low H_E value (0.1082). In contrast, many hybrid cultivars deviated from HWE probably because of artificial selection in breeding programs.

Classification of nabana lines

In the SSR-based NJ phylogram, 49 ingroup samples could be classified into four groups (I–IV). Although a few terminal nodes were supported with relatively high bootstrap values, no large group was supported by ≥50% bootstrap value. This could be because many of the lines were closely related and because some cultivars might be derived from intercrossing between distantly related lines. The flower-bud and stem-and-leaf types of Japanese nabana strains were separated into groups I and III, respectively, with a slight overlap in group II (**Fig. 1**). This result suggests that Japanese nabana lines were differentiated according to the parts used for consumption (flower buds or young stem-and-leaf portions). The separation of flower-bud type from stem-and-leaf type was also supported by the population structure analysis (**Supplemental Fig. 1**).

Chinese and European types formed three clusters and were located in groups II–IV. Of these, 'Wasekei saishin' and 'Cima di rapa' were close to non-heading Chinese cabbage ('Pak choi' and 'Taasai') and turnip cultivars ('CR Omasa' and 'Golden Ball'), respectively (**Fig. 1**). This finding was similar to previous reports (Pino Del Carpio *et al*. 2011, Zhao *et al*. 2005), confirming their close relationships and their cultivar origins. A cluster composed of three

zicaitai cultivars ('Hon tsai tai', 'Kosaitai', and 'Kosaitai (beni nabana)') was included in group II, unlike either of the previous reports that showed relationships to wutacai (Zhao *et al*. 2005), turnips (Pino Del Carpio *et al*. 2011), and a group comprising pak choi, wutacai, and cai-xin (Cheng *et al*. 2016a). Therefore, the relationship of zicaitai in *B. rapa* vegetables remains unclear.

CR properties of nabana cultivars

Based on the previous and present inoculation tests to clubroot pathogens, the 10 CR nabana cultivars analyzed here could have the same resistance property (**Table 2**), although they might show partial resistance depending on the population density of the clubroot pathogen and the composition of its pathotypes in soil. Results from CR markers suggest that nine CR nabana cultivars ('CR Hanakanzashi', 'CR Hanamatsuri', 'CR Hananomai', 'CR Kyobare', 'CR Kyonoharu', 'Eika', 'Kanzaki 21 go', 'Shunka', and 'Shunrai') have *CRb* and *Crr1* resistance alleles, potentially as heterozygotes (**Supplemental Table 4**). Unlike these cultivars, 'Hanamusume' could have *CRb* and *Crr2* resistance alleles. Because 'Hanamusume' showed a different allelic pattern in the four markers (KB59N06, BSA7, B359, and 523A1R) compared with the nine other CR cultivars, this cultivar might have an unknown resistance allele from its resistance source 'Gelria R'. That the CR nabana cultivars had a *CRb* resistance allele effective against group 3 and 4 pathotypes was consistent with the results of previous inoculation tests (Kuginuki *et al*. 2001, Oshikiri *et al*. 2014), in which the CR nabana cultivars were resistant to both groups. Although we did not test for group 3 pathogens among the 10 CR cultivars, eight were resistant to them (Kuginuki 2001, Oshikiri *et al*. 2014), and the remaining two might have the same resistance property judging from their similar allelic patterns (**Supplemental Table 4**). The heterozygous *Crr1* allele might be ineffective in these nabana cultivars because it acts in an incompletely dominant manner. In fact, its heterozygous allele was not sufficient to show the complete resistance to group 4 pathotype (Hatakeyama *et al*. 2013).

Effectiveness of molecular marker-based analysis for nabana cultivars and landraces

In conclusion, we have elucidated the hitherto unknown genetic relationships of nabana cultivars and landraces. In addition, the properties of CR nabana cultivars have been shown by inoculation tests and genotyping of CR markers. The cultivar 'Hanamusume' might have unique characteristics with regard to its genotypes of resistance loci, compared with the other CR nabana cultivars analyzed. The information obtained in this study will provide valuable information for the breeding of nabana cultivars.

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