Development of an SSR marker set for efficient selection for resistance to black spot disease in pear breeding

Shingo Terakami*, Yoshihiko Adachi, Yukie Takeuchi, Norio Takada, Sogo Nishio, Toshihiro Saito and Toshiya Yamamoto

Institute of Fruit Tree and Tea Science, NARO, 2-1 Fujimoto, Tsukuba, Ibaraki 305-8605, Japan

Black spot disease, which is caused by *Alternaria alternata* (Fries) Keissler Japanese pear pathotype, is one of the most harmful diseases in Japanese pear cultivation. Because of the potential harm of fungicides to consumers and the environment, resistant cultivars are desired. In this study, to enable efficient marker-assisted selection in pear breeding, we conducted comprehensive inoculation tests and genotyping with 207 pear cultivars. We identified a marker set (Mdo.chr11.27 and Mdo.chr11.34) suitable for selection for black spot resistance. In most susceptible cultivars, Mdo.chr11.27 amplified a 220-bp band and Mdo.chr11.34 amplified a 259-bp band. The genotype of Mdo.chr11.34 corresponds perfectly to the estimated genotype of Japanese pears susceptible to black spot disease. Using linkage analysis, we identified the positions of the gene for susceptibility to black spot disease in Chinese pear. Mdo.chr11.27 and Mdo.chr11.34 were tightly linked to susceptibility in Chinese pear, and the susceptibility gene was mapped at the top of linkage group 11, similar to that in Japanese pear. This marker set and the accumulation of phenotypic data will enable efficient marker-assisted breeding for black spot resistance in pear breeding.

Key Words: *Alternaria alternata*, black spot disease, *Pyrus pyrifolia*, *Pyrus ussuriensis*, marker-assisted selection.

Introduction

Pears (*Pyrus* spp.) belong to the family Rosaceae, subfamily Spiraeoideae, tribe Pyreae. Pears have been grown in East Asia, Europe, and North America for more than 3000 years and are among the most important fruit trees in more than 50 temperate regions worldwide (Bell 1990, Bell *et al.* 1996). This genus is believed to have originated during the Paleocene (65–55 million years ago) in what is now the mountainous area of western and southwestern China and spread east and west from there. The Japanese pear (*Pyrus pyrifolia* Nakai), the European pear (*P. communis* L.), and the Chinese pear (*P. bretschneideri* Rehd. and *P. ussuriensis* Maxim.) are the major edible species grown commercially for fruit production (Bell *et al.* 1996). The Japanese and Chinese pears are grown in East Asia, while European pears are grown in Europe, North America, and temperate regions of the Southern Hemisphere. All *Pyrus* species are inter-cressable, and there are no major incompatibility barriers to interspecific hybridization in this genus (Westwood and Bjornsta 1971).

Japanese pear is vulnerable to many bacterial and fungal diseases, such as pear scab induced by the fungus *Venturia nashicola* and fire blight induced by the proteobacterium *Erwinia amylovora*. Black spot disease is caused by *Alternaria alternata* (Fries) Keissler Japanese pear pathotype (previously, *A. kikutiana* Tanaka). Infected pears suffer from leaf and fruit necrosis and early defoliation, resulting in reduced productivity and fruit quality. This disease is one of the most severe diseases of Japanese pears, such as ‘Nijisseiki’, grown in Asia, but has not been reported in North America or Europe (Sanada *et al.* 1988). Spraying fungicides to prevent infection by this pathogen is very costly (Kozaki 1973), and fungicides pose potential harm to consumers and the environment (Donald *et al.* 2002, Reis *et al.* 2007). As in many other crops, the breeding of disease-resistant cultivars is the most effective and economical method of control. A single dominant gene, designated *A*, controls susceptibility to black spot disease (Kozaki 1973). Susceptible cultivars are heterozygous (*Aa*), but no homozygous (*AA*) cultivars have been identified (Kozaki 1973). Inactivation of *A* has been attempted to obtain resistant mutants, and moderately resistant cultivars, ‘Gold Nijisseiki’, ‘Osa Gold’, and ‘Kotobukishinsui’, were selected after chronic γ-ray irradiation (Kitagawa *et al.* 1999, Masuda *et al.* 1998, Sanada *et al.* 1993).

Traditional plant breeding methods are based on
phenotypic selection, but phenotypic evaluation is environmentally dependent, unreliable, and labor-intensive. Plant breeding using molecular markers avoids these problems because molecular markers appear to be independent of the environment, unaffected by plant growth conditions, and detectable at all stages of plant growth. This approach, referred to as “marker-assisted breeding”, uses genetic selection instead of phenotypic selection. The development of easy-to-use marker selection systems and the accumulation of genotype data of cultivars are essential for its success. Several groups have reported the development of DNA markers linked to A. The first markers, random amplified polymorphic DNA (RAPD) markers, were reported in Japanese pears ‘Osa Nijisseiki’ and ‘Kinchaku’ (Banno et al. 1999, Iketani et al. 2001). However, these reports did not identify the position of the susceptibility gene on genetic linkage maps; therefore, little information on molecular markers linked to the gene was available. In our previous studies (Terakami et al. 2007, 2016), we constructed linkage group (LG) 11 of each of the Japanese pear cultivars ‘Osa Nijisseiki’, ‘Nansui’, and ‘Kinchaku’ and mapped the susceptible gene at the top of each cultivar LG. Fine mapping localized the susceptibility gene of ‘Kinchaku’ within a 1.5-cM region between the simple sequence repeat (SSR) markers Mdo.chr11.28 and Mdo.chr11.34 (Terakami et al. 2016). The physical size of this region was estimated to be 107 kb in the draft Chinese pear genome (Wu et al. 2013). SSR markers tightly linked to and co-segregating with the gene were identified (Terakami et al. 2016), but the polymorphism of these markers, i.e., their suitability for marker-assisted selection (MAS), has not been tested.

The aim of the present study was to develop an efficient system for MAS of seedlings resistant to black spot disease. We conducted spore inoculation tests on large-scale pear genetic resources and collected genotype data for markers linked to the susceptibility gene. We constructed a genetic map of LG 11 and identified the exact position of the susceptibility gene in Chinese pears. Lastly, we discuss the susceptibility to A. alternata in pear and apple.

Materials and Methods

**Plant materials and DNA extraction**

For black spot inoculation tests and genotyping by DNA markers (Table 1), we used 207 pear cultivars: 165 Japanese pears (*P. pyrifolia* Nakai), 35 Chinese pears (*P. bretschneideri* Rehd. and *P. ussuriensis* Maxim.), 4 interspecific hybrid cultivars, and 3 other cultivars. ‘Babaucchiagnashi’ and ‘Iwate Tamenashi’ are native cultivar and wild species collected in Japan, respectively. ‘Cheung Dang No Ri’ is introduced from Korea. The name and the species of each cultivar are given in Table 1 according to the registration with the Genebank project, NARO (https://www.gene.affrc.go.jp/index_en.php). All trees were propagated by grafting and maintained in the orchard at the Institute of Fruit Tree and Tea Science, NARO (NIFTS; Tsukuba, Ibaraki, Japan). Five *F₁* progeny were used for genetic linkage analysis of susceptibility to black spot disease (Table 2). Four of them were derived from interspecific crosses between the Japanese pear ‘Kousui’ (synonym ‘Kosui’; resistant to black spot) and Chinese pears (‘Xiang Ya Li’, ‘Mi Li Cui’, ‘Tai Huang Li’, and ‘Huang Li’; susceptible to black spot). The other population was derived from a cross between Japanese pear ‘Housui’ (synonym ‘Hosui’; resistant) and ‘Kinchaku’ (susceptible). Ungrafted seedlings were grown in plastic pots (18 cm diameter × 16 cm height) and maintained at NIFTS (Tsukuba).

Frozen young leaves (30–40 mg) were homogenized by strong shaking for 30 s in a Shake Master Auto (Bio Medical Science). Genomic DNA was extracted using a NucleoMag Plant (Macherey-Nagel) according to the manufacturer’s instructions with a slight modification in the lysis buffer MC1 (2-mercaptoethanol was added to a final concentration of 2%). The purified genomic DNA was quantified with a Qubit 3.0 fluorometer (Thermo Fisher Scientific) and Qubit dsDNA BR assay kit (Thermo Fisher Scientific) and then diluted to 2.5 ng/μL for PCR analysis.

**Evaluation of black spot susceptibility or resistance**

Responses to black spot disease were evaluated using the spore inoculation test (Hayashi et al. 1990, Terakami et al. 2016). All cultivars and *F₁* plantlets were inoculated with spores of the virulent isolate No.15A of *A. alternata*, which was kindly provided by Dr. T. Tsuge (Chubu University). The isolate was cultured in potato dextrose broth without shaking for 10–14 days at 25°C. Mycelial mats were washed with tap water to remove culture medium and maintained at 25°C in the dark. The spores formed were collected, suspended in distilled water, and diluted to approximately 1 × 10⁵/mL. The spore suspension was sprayed onto three young leaves with a glass atomizer. The second or third young leaves of each plant were detached and used for inoculation test. The inoculated leaves were incubated in a moist chamber at 25°C for 66 h in the dark. Leaves were classified into two groups: resistant (no disease symptoms) and susceptible (necrotic symptoms). Black necrotic spots occur only in susceptible cultivars and black spots does not occur in resistant cultivars (Nishimura and Kohimoto 1983). All inoculation tests with all cultivars and *F₁* progeny were performed in duplicate.

**SSR analysis and evaluation of the usefulness of the markers for MAS**

We determined the genotypes of 4 SSR markers (Terakami et al. 2016; Mdo.chr11.27, Mdo.chr11.28, Mdo.chr11.30, and Mdo.chr11.34) around *A* and examined the relationship between marker genotypes and susceptibility to black spot disease in the 207 cultivars (Fig. 1). SSR-PCR analysis was performed using the one-tube, single-reaction nested PCR method (Schuelke 2000), in a total
### Table 1. Response to black spot disease and genotypes of SSR markers linked to *Aki* in pear cultivars

| Cultivar                | Species                  | JP accession number in NARO Genebank | Putative genotype<sup>a</sup> | Susceptibility to black spot disease<sup>b</sup> | SSR genotype<sup>c</sup> |
|-------------------------|--------------------------|--------------------------------------|-------------------------------|-----------------------------------------------|--------------------------|
| Abumi                   | *P. pyrifolia* Nakai     | 113559                               | a/a                           | R                                             | 246/256, 256/272         |
| Aikansui                | *P. pyrifolia* Nakai     | 118522                               | R                             | 230/230, 284/284                             |                          |
| Aiuchi                  | *P. pyrifolia* Nakai     | 113560                               | a/a                           | R                                             | 230/230, 286/286         |
| Akaho                   | *P. pyrifolia* Nakai     | 113561                               | a/a                           | R                                             | 230/230, 284/284         |
| Akiakari                | *P. pyrifolia* Nakai     | 118536                               | R                             | 230/230, 284/284                             |                          |
| Akiabae                 | *P. pyrifolia* Nakai     | 238254                               | R                             | 230/230, 284/284                             |                          |
| Akizuki                 | *P. pyrifolia* Nakai     | 118538                               | R                             | 230/230, 284/284                             |                          |
| Amanogawa               | *P. pyrifolia* Nakai     | 113562                               | a/a                           | R                                             | 228/230, 274/283         |
| Aoyagi                  | *P. pyrifolia* Nakai     | 113567                               | a/a                           | R                                             | 230/230, 284/284         |
| Asahi                   | *P. pyrifolia* Nakai     | 113568                               | a/a                           | R                                             | 230/230, 272/284         |
| Asahiryuu               | *P. pyrifolia* Nakai     | 113569                               | A/a                           | S                                             | 220/230, 259/284         |
| Atago                   | *P. pyrifolia* Nakai     | 113570                               | R                             | 230/230, 284/284                             |                          |
| Awayuki                 | *P. pyrifolia* Nakai     | 113572                               | R                             | 230/230, 259/284                             |                          |
| Azumanishiki            | *P. pyrifolia* Nakai     | 113573                               | a/a                           | R                                             | 230/230, 284/284         |
| Cheong Sil Ri           | *P. pyrifolia* Nakai     | 113693                               | R                             | 216/234, 276/282                             |                          |
| Chikusui                | *P. pyrifolia* Nakai     | 113716                               | R                             | 230/230, 284/284                             |                          |
| Chizu                   | *P. pyrifolia* Nakai     | 118524                               | S                             | 220/230, 259/284                             |                          |
| Chouju                  | *P. pyrifolia* Nakai     | 113575                               | R                             | 230/230, 284/284                             |                          |
| Choujuuruou             | *P. pyrifolia* Nakai     | 113574                               | a/a                           | R                                             | 230/230, 272/284         |
| Chousen                 | *P. pyrifolia* Nakai     | 113576                               | a/a                           | R                                             | 230/230, 284/284         |
| Doitsu                  | *P. pyrifolia* Nakai     | 113577                               | A/a                           | S                                             | 220/220, 259/284         |
| Echigonishiki           | *P. pyrifolia* Nakai     | 115740                               | R                             | 230/230, 274/284                             |                          |
| Edoya                   | *P. pyrifolia* Nakai     | 113578                               | A/a                           | S                                             | 220/258, 259/272         |
| Fukushima               | *P. pyrifolia* Nakai     | 113579                               | R                             | 234/258, 259/272                             |                          |
| Geishun                 | *P. pyrifolia* Nakai     | 113581                               | a/a                           | R                                             | 230/230, 274/284         |
| Gion                    | *P. pyrifolia* Nakai     | 113582                               | a/a                           | R                                             | 230/230, 284/284         |
| Gold Nijisseiki         | *P. pyrifolia* Nakai     | 110823                               | S                             | 220/220, 259/284                             |                          |
| Gozennashi              | *P. pyrifolia* Nakai     | 113583                               | a/a                           | R                                             | 230/230, 282/282         |
| Hakataoo                | *P. pyrifolia* Nakai     | 113584                               | A/a                           | S                                             | 220/220, 259/284         |
| Hakkou                  | *P. pyrifolia* Nakai     | 113585                               | a/a                           | R                                             | 230/230, 284/284         |
| Hakuteiryuu             | *P. pyrifolia* Nakai     | 113586                               | a/a                           | R                                             | 230/230, 282/284         |
| Han Hemung Li Kou       | *P. pyrifolia* Nakai     | 113727                               | a/a                           | R                                             | 216/230, 264/276         |
| Han Heung Li Otsu       | *P. pyrifolia* Nakai     | 113728                               | a/a                           | R                                             | 230/230, 284/284         |
| Harikonatsu             | *P. pyrifolia* Nakai     | 113587                               | a/a                           | R                                             | 230/234, 272/275         |
| Hatsuaki                | *P. pyrifolia* Nakai     | 113588                               | a/a                           | R                                             | 230/230, 284/284         |
| Hatsumaru               | *P. pyrifolia* Nakai     | –                                    | R                             | 230/230, 284/284                             |                          |
| Hatsushima              | *P. pyrifolia* Nakai     | 113589                               | a/a                           | R                                             | 228/230, 274/284         |
| Hattatsu                | *P. pyrifolia* Nakai     | 113590                               | a/a                           | R                                             | 230/230, 284/284         |
| Hayatama                | *P. pyrifolia* Nakai     | 113591                               | A/a                           | S                                             | 220/220, 259/284         |
| Heishi                  | *P. pyrifolia* Nakai     | 113592                               | A/a                           | S                                             | 220/220, 259/284         |
| Heiwa                   | *P. pyrifolia* Nakai     | 113593                               | a/a                           | R                                             | 230/230, 272/284         |
| Higashino               | *P. pyrifolia* Nakai     | 113594                               | A/a                           | S                                             | 220/220, 259/284         |
| Hoe Ryng Saibai         | *P. pyrifolia* Nakai     | 113729                               | R                             | 216/230, 264/276                             |                          |
| Hokkainashi             | *P. pyrifolia* Nakai     | 113596                               | a/a                           | R                                             | 230/234, 286/286         |
| Hokkan                  | *P. pyrifolia* Nakai     | 143889                               | S                             | 220/230, 259/284                             |                          |
| Hokushin                | *P. pyrifolia* Nakai     | 238257                               | R                             | 230/230, 284/284                             |                          |
| Hoshiakari              | *P. pyrifolia* Nakai     | –                                    | R                             | 230/230, 284/284                             |                          |
| Hougetsu                | *P. pyrifolia* Nakai     | 113720                               | R                             | 230/230, 284/284                             |                          |
| Hougyoku                | *P. pyrifolia* Nakai     | 113595                               | R                             | 230/236, 275/279                             |                          |
| Housui (Hosui)          | *P. pyrifolia* Nakai     | 113598                               | a/a                           | R                                             | 230/230, 284/284         |
| Ichihara Wase           | *P. pyrifolia* Nakai     | 113599                               | a/a                           | R                                             | 228/258, 272/274         |
| Imamuraaki              | *P. pyrifolia* Nakai     | 113600                               | a/a                           | R                                             | 228/230, 274/284         |
| Imamuranatsu            | *P. pyrifolia* Nakai     | 113601                               | a/a                           | R                                             | 228/230, 274/283         |

<sup>a</sup> SSR genotype

<sup>b</sup> Susceptibility to black spot disease

<sup>c</sup> SSR genotype
Table 1.  (continued)

| Cultivar             | Species       | JP accession number in NARO Genebank | Putative genotype<sup>a</sup> | Susceptibility to black spot disease<sup>b</sup> | SSR genotype<br><br>Mdo.chr11.27 | Mdo.chr11.34 |
|----------------------|---------------|-------------------------------------|------------------------------|---------------------------------|-------------------------------|----------------|
| Inagi                | *P. pyrifolia* Nakai | 113602                              | R                            | 230/230                         | 284/284                      |
| Inugoroshi           | *P. pyrifolia* Nakai | 113607                              | R                            | 228/228                         | 274/282                      |
| Isai                 | *P. pyrifolia* Nakai | 118528                              | S                            | 220/230                         | 259/284                      |
| Ishii Wase           | *P. pyrifolia* Nakai | 113603 i/a                          | R                            | 230/230                         | 284/284                      |
| Ishinashi            | *P. pyrifolia* Nakai | 239688                              | S                            | 224/234                         | 272/274                      |
| Iyohikari            | *P. pyrifolia* Nakai | 113604 A/a                          | S                            | 220/220                         | 259/284                      |
| Izunohomare          | *P. pyrifolia* Nakai | 113605 i/a                          | R                            | 230/230                         | 272/284                      |
| Jouhana              | *P. pyrifolia* Nakai | 113606 A/a                          | S                            | 220/230                         | 259/284                      |
| Kamenashi            | *P. pyrifolia* Nakai | 113608                              | R                            | 220/228                         | 259/282                      |
| Kansai Asaryu        | *P. pyrifolia* Nakai | 113609 i/a                          | R                            | 230/230                         | 272/284                      |
| Kansai Ichii         | *P. pyrifolia* Nakai | 113610 i/a                          | R                            | 230/230                         | 282/284                      |
| Kanta                | *P. pyrifolia* Nakai | –                                   | R                            | 230/230                         | 284/284                      |
| Kikusui              | *P. pyrifolia* Nakai | 113611                              | R                            | 220/230                         | 284/284                      |
| Kimizukawase         | *P. pyrifolia* Nakai | 113612 A/a                          | S                            | 220/230                         | 259/284                      |
| Kinchaku             | *P. pyrifolia* Nakai | 113613 A/a                          | S                            | 220/230                         | 259/284                      |
| Kiraseiki            | *P. pyrifolia* Nakai | 118529                              | R                            | 230/230                         | 284/284                      |
| Kisui                | *P. pyrifolia* Nakai | 238258                              | S                            | 220/230                         | 259/284                      |
| Kiyosumi             | *P. pyrifolia* Nakai | 113614                              | S                            | 220/230                         | 259/284                      |
| Kokuchou             | *P. pyrifolia* Nakai | 113621 i/a                          | R                            | 230/230                         | 284/284                      |
| Konpeitou            | *P. pyrifolia* Nakai | 113617 i/a                          | R                            | 228/230                         | 274/284                      |
| Kotobukishinsui      | *P. pyrifolia* Nakai | 110824                              | S                            | 220/230                         | 259/284                      |
| Kougetsu             | *P. pyrifolia* Nakai | 113615 A/a                          | S                            | 220/230                         | 259/284                      |
| Kougiku              | *P. pyrifolia* Nakai | 116285                              | R                            | 230/230                         | 284/284                      |
| Kounowatashi         | *P. pyrifolia* Nakai | 113616 i/a                          | R                            | 230/230                         | 282/284                      |
| Koushu               | *P. pyrifolia* Nakai | 113618 i/a                          | R                            | 230/230                         | 272/284                      |
| Kousui (Kosui)       | *P. pyrifolia* Nakai | 113619 i/a                          | R                            | 230/230                         | 284/284                      |
| Kozan                | *P. pyrifolia* Nakai | 118530                              | R                            | 234/258                         | 272/272                      |
| Kouzou               | *P. pyrifolia* Nakai | 113620 i/a                          | R                            | 230/230                         | 284/284                      |
| Koyuki               | *P. pyrifolia* Nakai | 113622                              | R                            | 230/230                         | 274/284                      |
| Kumoii               | *P. pyrifolia* Nakai | 113623 i/a                          | R                            | 230/230                         | 284/284                      |
| Kunitomi             | *P. pyrifolia* Nakai | 113624 A/a                          | S                            | 220/230                         | 259/284                      |
| Kuroki               | *P. pyrifolia* Nakai | 113625 i/a                          | R                            | 230/242                         | 284/284                      |
| Kwankinbe            | *P. pyrifolia* Nakai | 118531                              | R                            | 230/230                         | 284/284                      |
| Meigetsu             | *P. pyrifolia* Nakai | 113626 A/a                          | S                            | 220/230                         | 259/284                      |
| Mishirazu            | *P. pyrifolia* Nakai | 113627                              | R                            | 230/234                         | 286/286                      |
| Musashi              | *P. pyrifolia* Nakai | 221165 i/a                          | R                            | 230/230                         | 284/284                      |
| Nangetsu             | *P. pyrifolia* Nakai | 238261                              | R                            | 230/230                         | 284/284                      |
| Nansui Chabo         | *P. pyrifolia* Nakai | 115741                              | R                            | 230/230                         | 272/284                      |
| Nansui               | *P. pyrifolia* Nakai | 115742                              | S                            | 220/230                         | 259/284                      |
| Narumi               | *P. pyrifolia* Nakai | –                                   | R                            | 230/230                         | 284/284                      |
| Natsushizuku         | *P. pyrifolia* Nakai | 230439                              | R                            | 230/230                         | 284/284                      |
| Nekogoroshi          | *P. pyrifolia* Nakai | 113628                              | R                            | 228/234                         | 256/282                      |
| Niihatanashi         | *P. pyrifolia* Nakai | 113629 i/a                          | R                            | 230/230                         | 270/283                      |
| Niihakii             | *P. pyrifolia* Nakai | 113630 i/a                          | R                            | 230/230                         | 284/284                      |
| Nijisseki            | *P. pyrifolia* Nakai | 113631 A/a                          | S                            | 220/230                         | 259/284                      |
| Nikki               | *P. pyrifolia* Nakai | 118540                              | R                            | 230/230                         | 284/284                      |
| Okukouzou            | *P. pyrifolia* Nakai | 113632 i/a                          | R                            | 230/230                         | 284/284                      |
| Okuroko              | *P. pyrifolia* Nakai | 113633 i/a                          | R                            | 230/230                         | 284/284                      |
| Okusankichi          | *P. pyrifolia* Nakai | 113634                              | R                            | 230/230                         | 284/284                      |
| Onba                 | *P. pyrifolia* Nakai | 113636                              | R                            | 230/230                         | 272/284                      |
| Ooishiromaru         | *P. pyrifolia* Nakai | 113637 i/a                          | R                            | 230/230                         | 284/284                      |
| Ookoga               | *P. pyrifolia* Nakai | 113638                              | R                            | 230/230                         | 284/284                      |
| Ootani               | *P. pyrifolia* Nakai | 113639 A/a                          | S                            | 220/230                         | 259/284                      |
Table 1. (continued)

| Cultivar          | Species            | JP accession number in NARO Genebank | Putative genotype<sup>a</sup> | Susceptibility to black spot disease<sup>b</sup> | SSR genotype<sup>c</sup> |
|-------------------|--------------------|--------------------------------------|--------------------------------|---------------------------------------------|--------------------------|
| Osa Gold          | *P. pyrifolia* Nakai | 110825                               |                                | S                                          | 220/230  259/284         |
| Osa Nijisseiki    | *P. pyrifolia* Nakai | 113640                               |                                | S                                          | 220/230  259/284         |
| Oushuo            | *P. pyrifolia* Nakai | 118539                               |                                | R                                          | 230/230  282/284         |
| Rikiya            | *P. pyrifolia* Nakai | 113641                               | a/a                            | R                                          | 230/230  284/284         |
| Rinka             | *P. pyrifolia* Nakai | –                                    |                                | R                                          | 230/230  284/284         |
| Rokugatsu         | *P. pyrifolia* Nakai | 113642                               |                                | S                                          | 220/230  259/284         |
| Ruisannashi       | *P. pyrifolia* Nakai | 113643                               |                                | R                                          | 230/260  266/282         |
| Sagami            | *P. pyrifolia* Nakai | 113644                               | a/a                            | R                                          | 230/230  284/284         |
| Saizounashi       | *P. pyrifolia* Nakai | 113645                               | a/a                            | R                                          | 230/230  274/284         |
| Segawa            | *P. pyrifolia* Nakai | 113646                               | A/a                            | S                                          | 220/258  259/272         |
| Seigyoku          | *P. pyrifolia* Nakai | 113647                               | a/a                            | R                                          | 230/230  284/284         |
| Seika             | *P. pyrifolia* Nakai | 113648                               | A/a                            | R                                          | 220/248  259/292         |
| Seiryuu           | *P. pyrifolia* Nakai | 113649                               | a/a                            | R                                          | 228/260  272/274         |
| Seikaichi         | *P. pyrifolia* Nakai | 113650                               | A/a                            | R                                          | 220/230  259/284         |
| Sekiryuu          | *P. pyrifolia* Nakai | 113651                               | a/a                            | R                                          | 230/230  274/284         |
| Senryou           | *P. pyrifolia* Nakai | 113652                               | a/a                            | R                                          | 230/234  286/286         |
| Shihykakume       | *P. pyrifolia* Nakai | 113653                               | a/a                            | R                                          | 230/230  284/284         |
| Shikishima        | *P. pyrifolia* Nakai | 113654                               | a/a                            | R                                          | 230/230  284/284         |
| Shimokatsuginashi | *P. pyrifolia* Nakai | 113662                               |                                | R                                          | 230/234  284/284         |
| Shimonashi        | *P. pyrifolia* Nakai | 113661                               |                                | R                                          | 224/228  274/274         |
| Shinchuu          | *P. pyrifolia* Nakai | 113656                               | a/a                            | R                                          | 230/230  284/284         |
| Shinkou           | *P. pyrifolia* Nakai | 113657                               | a/a                            | R                                          | 230/230  274/284         |
| Shimsei           | *P. pyrifolia* Nakai | 113694                               | a/a                            | R                                          | 228/230  274/284         |
| Shinsenki         | *P. pyrifolia* Nakai | 113658                               |                                | R                                          | 230/230  284/284         |
| Shinsetsu         | *P. pyrifolia* Nakai | 113659                               |                                | R                                          | 230/230  284/284         |
| Shinsui           | *P. pyrifolia* Nakai | 113660                               | A/a                            | S                                          | 220/230  259/284         |
| Shirayuki         | *P. pyrifolia* Nakai | 113663                               | a/a                            | R                                          | 228/230  274/283         |
| Shugyoku          | *P. pyrifolia* Nakai | 113707                               |                                | R                                          | 230/230  284/284         |
| Shuurei           | *P. pyrifolia* Nakai | 118537                               |                                | R                                          | 230/230  284/284         |
| Shuusui           | *P. pyrifolia* Nakai | 116286                               |                                | R                                          | 230/230  284/284         |
| Sotoorihime       | *P. pyrifolia* Nakai | 113664                               | a/a                            | R                                          | 234/240  272/274         |
| Suisei            | *P. pyrifolia* Nakai | 113665                               | a/a                            | R                                          | 230/230  284/284         |
| Suishiu           | *P. pyrifolia* Nakai | 118541                               |                                | R                                          | 230/230  284/284         |
| Taihaku           | *P. pyrifolia* Nakai | 113666                               |                                | R                                          | 230/230  284/284         |
| Taihei            | *P. pyrifolia* Nakai | 113667                               |                                | R                                          | 228/256  272/282         |
| Tama              | *P. pyrifolia* Nakai | 113668                               |                                | R                                          | 230/230  284/284         |
| Tamotoyabure      | *P. pyrifolia* Nakai | 113695                               |                                | R                                          | 230/230  284/284         |
| Tanponashi        | *P. pyrifolia* Nakai | 113699                               |                                | R                                          | 228/230  282/284         |
| Tanzawa           | *P. pyrifolia* Nakai | 116287                               | a/a                            | R                                          | 230/230  274/284         |
| Tenyuu            | *P. pyrifolia* Nakai | 113670                               |                                | S                                          | 220/230  259/284         |
| Tosajou           | *P. pyrifolia* Nakai | 113672                               | a/a                            | R                                          | 230/230  282/284         |
| Tosajounishiki    | *P. pyrifolia* Nakai | 113673                               | a/a                            | R                                          | 228/230  274/284         |
| Tosanashi         | *P. pyrifolia* Nakai | 113674                               |                                | R                                          | 230/230  272/284         |
| Tosanishiki       | *P. pyrifolia* Nakai | 113675                               | a/a                            | R                                          | 228/230  274/284         |
| Touhou            | *P. pyrifolia* Nakai | 113671                               |                                | S                                          | 220/234  259/272         |
| Tsugaruao         | *P. pyrifolia* Nakai | 113676                               |                                | R                                          | 216/216  276/276         |
| Tsukutounashi     | *P. pyrifolia* Nakai | 113677                               |                                | R                                          | 228/228  272/274         |
| Wase Kouzou       | *P. pyrifolia* Nakai | 113682                               | a/a                            | R                                          | 230/230  284/284         |
| Wase Taichou      | *P. pyrifolia* Nakai | 113684                               | A/a                            | S                                          | 220/230  259/284         |
| Waseaka           | *P. pyrifolia* Nakai | 113678                               | a/a                            | R                                          | 228/230  274/284         |
| Yabase            | *P. pyrifolia* Nakai | 118542                               |                                | S                                          | 220/230  259/284         |
| Yachiyo           | *P. pyrifolia* Nakai | 113686                               | a/a                            | R                                          | 230/230  284/284         |
| Yagoemon          | *P. pyrifolia* Nakai | 113687                               |                                | R                                          | 230/234  272/284         |
| Cultivar              | Species                        | JP accession number in NARO Genebank | Putative genotype<sup>a</sup> | Susceptibility to black spot disease<sup>b</sup> | SSR genotype<sup>c</sup> |
|----------------------|--------------------------------|-------------------------------------|-------------------------------|---------------------------------|------------------------|
| Yahatanishiki        | *P. pyrifolia* Nakai           | 113690                              | a/a                           | R                               | Mdo.chr11.27 230/230 284/284 |
| Yakumo               | *P. pyrifolia* Nakai           | 113688                              | a/a                           | R                               | 230/230 284/284 |
| Yasato               | *P. pyrifolia* Nakai           | 113718                              | R                             |                                 | 230/230 284/284 |
| Yokogoshi            | *P. pyrifolia* Nakai           | 113691                              | S                             |                                 | 220/228 259/274 |
| Yoshikaori           | *P. pyrifolia* Nakai           | 118543                              | R                             |                                 | 230/230 284/284 |
| Yoshino              | *P. pyrifolia* Nakai           | 113692                              | R                             |                                 | 230/230 284/284 |
| Bai Li               | *P. bretschneideri* Rehd.      | 113526                              | R                             |                                 | 216/230 275/284 |
| Agenoshou Shinanashi | *P. ussuriensis* Maxim.        | 113730                              | R                             |                                 | 224/230 274/284 |
| Ba Li Xiang          | *P. ussuriensis* Maxim.        | 113749                              | R                             |                                 | 222/246 274/282 |
| Baozhuli             | *P. ussuriensis* Maxim.        | 118544                              | R                             |                                 | 240/244 274/277 |
| Bei Jin Bai Li       | *P. ussuriensis* Maxim.        | 113731                              | a/a                           | R                               | 230/236 275/284 |
| Cang Xi Li           | *P. ussuriensis* Maxim.        | 113752                              | R                             |                                 | 248/274 274/282 |
| Chang Xi Li          | *P. ussuriensis* Maxim.        | 113751                              | R                             |                                 | 236/236 272/310 |
| Da Tou Huang Li      | *P. ussuriensis* Maxim.        | 113747                              | R                             |                                 | 222/222 264/272 |
| En Li                | *P. ussuriensis* Maxim.        | 113732                              | a/a                           | R                               | 228/248 282/292 |
| Hong Li              | *P. ussuriensis* Maxim.        | 113733                              | a/a                           | R                               | 216/216 276/276 |
| Hong Xiao Li         | *P. ussuriensis* Maxim.        | 113734                              | R                             |                                 | 216/226 272/276 |
| Huang Li             | *P. ussuriensis* Maxim.        | 113750                              | S                             |                                 | 214/230 280/284 |
| Huang Shi Li         | *P. ussuriensis* Maxim.        | 113748                              | R                             |                                 | 216/234 276/282 |
| Jian Ba Li           | *P. ussuriensis* Maxim.        | 113735                              | R                             |                                 | 224/236 276/276 |
| Lai Yang Ci Li       | *P. ussuriensis* Maxim.        | 113736                              | a/a                           | R                               | 228/248 282/292 |
| Lunanhuangli         | *P. ussuriensis* Maxim.        | 118545                              | R                             |                                 | 228/246 282/282 |
| Ma Ke Zao Li         | *P. ussuriensis* Maxim.        | 113744                              | R                             |                                 | 228/260 266/282 |
| Ma Ti Huang          | *P. ussuriensis* Maxim.        | 113746                              | R                             |                                 | 222/222 264/272 |
| Mi Li                | *P. ussuriensis* Maxim.        | 113753                              | R                             |                                 | 216/226 272/276 |
| Mi Li Cui            | *P. ussuriensis* Maxim.        | 113759                              | S                             |                                 | 228/230 280/282 |
| Ping Li              | *P. ussuriensis* Maxim.        | 113754                              | R                             |                                 | 230/230 280/284 |
| Qiu Bai Li           | *P. ussuriensis* Maxim.        | 113737                              | a/a                           | R                               | 228/248 282/292 |
| Su Hyang Ri          | *P. ussuriensis* Maxim.        | 113738                              | a/a                           | R                               | 216/230 264/276 |
| Tai Huang Li         | *P. ussuriensis* Maxim.        | 113760                              | S                             |                                 | 216/230 276/280 |
| Wo Wo Li             | *P. ussuriensis* Maxim.        | 113739                              | a/a                           | R                               | 224/228 280/282 |
| Xiang Ya Li          | *P. ussuriensis* Maxim.        | 113756                              | S                             |                                 | 228/230 280/282 |
| Xie Hua Tian         | *P. ussuriensis* Maxim.        | 113755                              | R                             |                                 | 230/230 280/284 |
| Xuehua Li            | *P. ussuriensis* Maxim.        | 245604                              | R                             |                                 | 228/230 280/282 |
| Ya Gua Li            | *P. ussuriensis* Maxim.        | 113740                              | a/a                           | R                               | 230/248 284/292 |
| Ya Li                | *P. ussuriensis* Maxim.        | 113741                              | a/a                           | R                               | 230/230 284/284 |
| Yang Nai Xiang       | *P. ussuriensis* Maxim.        | 116297                              | R                             |                                 | 230/236 274/278 |
| Yin Bai Li           | *P. ussuriensis* Maxim.        | 113757                              | R                             |                                 | 222/230 284/296 |
| Yuan Ba Li           | *P. ussuriensis* Maxim.        | 113742                              | a/a                           | R                               | 230/246 268/284 |
| Zaosu Li             | *P. ussuriensis* Maxim.        | 245605                              | R                             |                                 | 230/230 264/284 |
| Zhu Zui Li           | *P. ussuriensis* Maxim.        | 113743                              | a/a                           | R                               | 216/248 256/276 |
| Ninomiya             | *P. pyrifolia × P. communis*   | 113781                              | R                             |                                 | 228/230 284/284 |
| Ooharabeni           | *P. pyrifolia × P. communis*   | 113780                              | R                             |                                 | 230/230 284/284 |
| Taiheiyou            | *P. pyrifolia × P. communis*   | 113782                              | R                             |                                 | 230/248 272/284 |
| Ninomiya Bai Li      | *P. ussuriensis × P. pyrifolia*| 113784                              | a/a                           | R                               | 230/230 284/284 |
| Babaucciaginashi     | *P. babauttiaginashi* Koidz.   | 113763                              | R                             |                                 | 228/228 274/274 |
| Cheung Dang No Ri    | *Pyrus* sp.                    | 113828                              | R                             |                                 | 216/234 272/276 |
| Iwate Tanenashi      | *Pyrus* sp.                    | 113802                              | R                             |                                 | 234/260 272/272 |

<sup>a</sup> Proposed in Kozaki (1973). a/a: heterozygote susceptible to black spot; a/a: resistant; blank cell: not tested.

<sup>b</sup> S, necrotic symptoms (susceptible); R, no disease symptoms (resistant).

<sup>c</sup> Numbers separated by “/” indicate the estimated size (bp) of the alleles of the same locus.
volume of 5 μL containing 2.5 μL of 2 × GoTaq G2 Hot Start Green Master Mix (Promega), 0.3 μM forward primer with a tail at the 5'-end, 0.5 μM reverse primer, 0.2 μM 6-FAM-labeled universal primer (Thermo Fisher Scientific), and 2.5 ng of genomic DNA. The original M13(-21) tail sequence was modified to the 20-bp (5'- GCTACGGACTGACCTCGGAC-3') universal sequence. A 7-bp pigtail sequence (5'-GTTTCTT-3') (Brownstein et al. 1996) was added at the 5'-end of each reverse primer to improve genotyping accuracy. DNA was amplified in a GeneAmp PCR system 9700 (Thermo Fisher Scientific) with an initial denaturation step at 95°C for 2 min; 40 cycles at 95°C for 30 sec (denaturation), 55°C for 30 sec (annealing), and 72°C for 45 sec (extension); and a final extension at 72°C for 5 min.

Amplified DNA fragments were separated and detected using an Applied Biosystems 3130xl Genetic Analyzer (Thermo Fisher Scientific) with a 36 cm-capillary array, POP-7 polymer, and an internal size standard (GeneScan HD 400 ROX; Thermo Fisher Scientific). Data were collected and analyzed in GeneMapper v. 5.0 software (Thermo Fisher Scientific).

Fine mapping of Aki and linkage analysis of the susceptibility gene of Chinese pear

For fine mapping of the black spot susceptibility gene of ‘Kinchaku’, Aki, 1061 F_1 plants obtained from a cross between ‘Hosui’ and ‘Kinchaku’ were genotyped with the four markers (Fig. 1). Because A were located in the same region of LG 11 in Japanese pear (Terakami et al. 2007, 2016), we suspected that the susceptibility gene of Chinese pear might also be in that region. To examine this possibility, we analyzed all F_1 plantlets derived from four different mapping populations (‘Kosui’ × ‘Xiang Ya Li’, ‘Kosui’ × ‘Mi Li Cui’, ‘Kosui’ × ‘Tai Huang Li’, and ‘Kosui’ × ‘Huang Li’) using Mdo.chr11.27 and Mdo.chr11.34 (Fig. 1), which show significant linkage to A. SSR-PCR analyses were performed as described above.

Statistical analysis (χ² test) was performed in R v. 3.5.1 software using the chisq.test function (R Core Team 2018). Linkage analysis was performed in JoinMap v. 4.1 software (Van Ooijen 2006, 2011), and a pseudo-testcross strategy was used to create genetic linkage maps (Grattapaglia and Sederoff 1994). An independence logarithm of odds (LOD) threshold of 10.0 was used to define linkage groups. To construct a linkage group, the regression mapping algorithm was selected with the following parameters: recombination frequency ≤0.40, a LOD ≥1.0, goodness-of-fit jump threshold for removal of loci = 5.0, number of added loci after which to perform a ripple = 1, and third round = “No”. Map distances were calculated according to Kosambi’s mapping function (Kosambi 1944). The linkage map was drawn in MapChart v. 2.3 software (Voorrips 2002).

Map integration

An integrated linkage map was constructed in JoinMap v. 4.1 software assuming that the candidate genes of the four Chinese pear cultivars (‘Xiang Ya Li’, ‘Mi Li Cui’, ‘Tai Huang Li’, and ‘Huang Li’) were the same and using combined data from the four mapping populations. First, a map of each population was constructed to determine the coupling phase linked to the susceptibility gene. Then the

### Table 2. Mapping populations used in this study and segregation of susceptibility and resistance to black spot disease

| Female (Resistant) | Male (Susceptible) | Number of progeny | Expected ratio | χ² | P-value |
|-------------------|--------------------|-------------------|----------------|-----|--------|
| Hosui             | Kinchaku           | 526               | 535            | 1:1 | 0.076  | 0.782  |
| Kosui             | Xiang Ya Li        | 72                | 50             | 1:1 | 3.967  | 0.046* |
| Kosui             | Mi Li Cui          | 31                | 28             | 1:1 | 0.153  | 0.696  |
| Kosui             | Tai Huang Li       | 40                | 43             | 1:1 | 0.108  | 0.742  |
| Kosui             | Huang Li           | 20                | 24             | 1:1 | 0.364  | 0.546  |

*P-values indicate fit to the expected ratio (1:1). Distorted segregation is indicated by a significant p-value of the χ² test: *, p < 0.05.
groups were combined by applying the “Combine Groups for Map Integration” function from the JoinMap menu.

Results

Fine mapping of Aki and precise determination of the marker position

In our previous study, genetic linkage analysis of Aki was conducted using 621 F₁ progeny (Terakami et al. 2016). Here, we performed fine mapping of Aki and constructed a more detailed linkage map using 1061 F₁ plantlets of a ‘Hosui’ × ‘Kinchaku’ cross. In the tests for susceptibility or resistance to black spot, 526 plantlets showed necrotic symptoms and 535 showed no symptoms (Table 2); identical results were obtained in all duplicate tests. The segregation ratio of resistant to susceptible plants fitted the expected ratio of 1:1 in the chi-squared test (Table 2; χ² = 0.076, p = 0.782).

Fine mapping of Aki was performed with four SSR markers (Mdo.chr11.27, Mdo.chr11.28, Mdo.chr11.30, and Mdo.chr11.34) developed from the apple genome sequence (Terakami et al. 2016). These four markers show scorable polymorphism, i.e., a heterozygous genotype in ‘Kinchaku’ and polymorphic band patterns between ‘Hosui’ and ‘Kinchaku’, and show significant linkage to Aki (Terakami et al. 2016). Aki was located within a 1.1-cM region between Mdo.chr11.28 and Mdo.chr11.34 (Fig. 1). Previously we mapped Mdo.chr11.27 and Mdo.chr11.28 to the same position (Terakami et al. 2016), but a more accurate mapping with more progeny showed that the distance between Aki and Mdo.chr11.27 was 0.4 cM and that between Aki and Mdo.chr11.28 was 0.3 cM (Fig. 1). In the mapping population, we detected recombination between Mdo.chr11.27 and Aki in four plantlets, between Mdo.chr11.28 and Aki in three, and between Mdo.chr11.34 and Aki in nine. No double recombination events between Mdo.chr11.27 and Mdo.chr11.34 were detected. Mdo.chr11.30 co-segregated with Aki in all 1061 F₁ plantlets. The segregation ratio was not distorted at any locus.

Evaluation of black spot susceptibility or resistance

Of the 207 cultivars tested, 43 were susceptible (39 Japanese and 4 Chinese) and 164 were resistant to black spot disease (Table 1). Susceptible and resistant cultivars could be clearly distinguished because no cultivars showed an intermediate response. The results of the 101 cultivars that had been previously tested (Kozaki 1973) were consistent with those previous results (Table 1); 22 susceptible cultivars (18 Japanese and 4 Chinese) were newly identified. Disease symptoms were observed on the entire surface of inoculated leaves, with no differences among the susceptible cultivars, including ‘Gold Nijisseiki’, ‘Osa Gold’, and ‘Kotobukishinsui’, which reportedly have medium disease resistance (Kitagawa et al. 1999, Masuda et al. 1998, Sanada et al. 1993).

Relationship between SSR genotype and susceptibility to black spot disease

We genotyped the 207 cultivars to investigate the polymorphism of the four Aki-linked SSR markers and their correspondence with phenotypes (Table 1). Mdo.chr11.27 and Mdo.chr11.34 showed amplification of specific bands in most Japanese cultivars that were susceptible in the inoculation test.

Mdo.chr11.27 showed a 220-bp band in most susceptible cultivars (Tables 1, 3, Fig. 2). Most of the susceptible Japanese cultivars were heterozygous, whereas ‘Doitsu’, ‘Higashino’, and ‘Iyohikari’ were homozygous for the 220-bp band. ‘Fukushima’ was susceptible, but no 220-bp allele was detected. For Mdo.chr11.34, the presence or absence of the 259-bp band in Japanese cultivars was completely consistent with the results of the inoculation test (Tables 1, 3, Fig. 2). All susceptible Japanese cultivars were heterozygous for the 259-bp allele. However, no amplification of specific bands could be related to the inoculation test results was observed in the Chinese cultivar. The 230-bp and 280-bp bands were commonly amplified in susceptible Chinese cultivars with Mdo.chr11.27 and Mdo.chr11.34, respectively, but were also observed in several resistant Chinese pear cultivars.

Markers Mdo.chr11.28 and Mdo.chr11.30 amplified multiple loci, as 1–6 bands were identified (Supplemental Table 1). Therefore, we examined the presence of the 255-bp band for Mdo.chr11.28 and the 186-bp band for Mdo.chr11.30, both of which were linked to Aki of ‘Kinchaku’. The 255-bp band was detected in all susceptible cultivars, but also in many resistant cultivars. The presence of the 186-bp band in the Japanese cultivars was completely consistent with susceptibility in the inoculation test. There was no correlation between the amplification of the 186-bp band and the results of the inoculation test in Chinese pear.

Inheritance mode of the susceptibility gene in Chinese pear

As a prerequisite to mapping the susceptibility gene to black spot disease in Chinese pear, we evaluated the inheritance mode of susceptibility. We obtained four F₁ segregating populations from crosses between resistant and susceptible cultivars and evaluated plantlets for resistance or susceptibility to black spot (Table 2). Resistant and susceptible plantlets could be clearly differentiated; no plantlets showed an intermediate response. Identical results were obtained in all duplicate tests.

Of 122 F₁ plantlets of the ‘Kosui’ × ‘Xiang Ya Li’ cross, 72 showed necrotic symptoms and were judged as susceptible (Table 2). The other 50 were resistant (no symptoms). The ratio of susceptible to resistant plants (72:50) showed a slight distortion from the expected 1:1 ratio at the 5% level in the chi-squared test (χ² = 3.967, p = 0.046). Of 59 F₁ ‘Kosui’ × ‘Mi Li Cui’ plantlets, 31 were susceptible and 28 were resistant (Table 2; χ² = 0.153, p = 0.696). Of 83 F₁
## Table 3. Summary of pear cultivars and band pattern of Mdo.chr11.27 and Mdo.chr11.34 markers

| Species                                | Susceptibility to black spot disease | Band pattern of Mdo.chr11.27 and Mdo.chr11.34 markers<sup>a</sup> |
|----------------------------------------|-------------------------------------|---------------------------------------------------------------|
| **P. pyrifolia** Nakai                 | Resistant                           | P_P, N_P, N_N                                                 |
| **P. bretschneideri** Rehd.            | Resistant                           | Fukuishima                                                    |
| **P. ussuriensis** Maxim.              | Resistant                           |                                                           |
| **P. pyrifolia × P. communis**         | Susceptible                         | P_N, with 220-bp band of Mdo.chr11.27 and 259-bp band of Mdo.chr11.34; N_P, without 220-bp band of Mdo.chr11.27 and with 259-bp band of Mdo.chr11.34; N_N, without 220-bp band of Mdo.chr11.27 and 259-bp band of Mdo.chr11.34. |
| **P. ussuriensis × P. pyrifolia**      | Resistant                           | N_P                                                           |
| **P. babauttiaginashi** Koidz.         | Resistant                           |                                                                 |
| **Pyrus** sp.                          | Resistant                           |                                                                 |

<sup>a</sup> P_N, with 220-bp band of Mdo.chr11.27 and 259-bp band of Mdo.chr11.34; N_P, without 220-bp band of Mdo.chr11.27 and with 259-bp band of Mdo.chr11.34; N_N, without 220-bp band of Mdo.chr11.27 and 259-bp band of Mdo.chr11.34.
‘Kosui’ × ‘Tai Huang Li’ plantlets, 40 were susceptible and 43 were resistant (**Table 2**; \( \chi^2 = 0.108, p = 0.742 \)). Of 44 \( F_1 \) ‘Kosui’ × ‘Huang Li’ plantlets, 20 were susceptible and 24 were resistant (**Table 2**; \( \chi^2 = 0.364, p = 0.546 \)). The segregation ratio of susceptible to resistant plants fitted the expected ratio of 1:1 in three mapping populations (‘Kosui’ × ‘Mi Li Cui’, ‘Kosui’ × ‘Tai Huang Li’, and ‘Kosui’ × ‘Huang Li’). These results indicate that a single dominant gene might control susceptibility to black spot disease in Chinese pears, and that the four Chinese pears are heterozygous for this gene. We designated the genes responsible for susceptibility in the Chinese cultivars as follows: ‘Xiang Ya Li’, \( Axi \); ‘Mi Li Cui’, \( Ami \); ‘Tai Huang Li’, \( Ata \); and ‘Huang Li’, \( Ahu \), because the pedigree of each cultivar was unknown.

**Mapping of the loci conferring susceptibility to black spot disease in Chinese pear**

To identify the loci conferring susceptibility in Chinese pear, we tested Mdo.chr11.27 and Mdo.chr11.34, which showed significant linkage to susceptibility to black spot disease in Japanese pear, in the four mapping populations. Both SSRs showed scorable polymorphisms in all four populations, i.e., a heterozygous genotype in ‘Xiang Ya Li’, ‘Mi Li Cui’, ‘Tai Huang Li’, and ‘Huang Li’, and polymorphic band patterns in ‘Kosui’ (**Table 1**). From linkage analysis, the 230-bp allele of Mdo.chr11.27 showed significant linkage to \( Axi \) (genetic distance, 2.5 cM; LOD score, 30.39), \( Ami \) (5.1 cM; 12.66), \( Ata \) (1.2 cM; 22.89), and \( Ahu \) (9.2 cM; 7.45) (**Fig. 3**). The 280-bp allele of Mdo.chr11.34 co-segregated with \( Axi \) (LOD score, 35.86), \( Ami \) (17.73), and \( Ahu \) (13.17), and showed significant linkage to \( Ata \) (genetic distance, 1.2 cM; LOD score, 22.92) (**Fig. 3**). Markers Mdo.chr11.28 and Mdo.chr11.30 were excluded from the analysis because these markers amplified multiple loci (**Supplemental Table 1**).

An integrated linkage map was constructed, assuming that the candidate genes of the four Chinese cultivars (‘Xiang Ya Li’, ‘Mi Li Cui’, ‘Tai Huang Li’, and ‘Huang Li’) were the same gene, denoted as \( Ac \). \( Ac \) was located within a 3.9-cM region between Mdo.chr11.27 and Mdo.chr11.34 (**Fig. 1**). Thus, the susceptibility gene of Chinese pear, \( Ac \), was located at the top of LG 11, very similar to that of \( Aki \) in Japanese pear.

**Useful marker set for the breeding of pear resistant to black spot disease**

We conclude that the marker set Mdo.chr11.27 and Mdo.chr11.34 would be useful for MAS. Both markers amplified a single locus, and the correspondence of the alleles to resistance and susceptibility was clear. Except in ‘Fukushima’, the 220-bp and 259-bp bands were amplified in susceptible Japanese pear cultivars with Mdo.chr11.27 and Mdo.chr11.34 (**Table 1**). In Chinese pears, no specific bands were found in susceptible cultivars, but linkage analysis determined a coupling phase to the susceptibility gene. Non-specific amplification, e.g., a 215-bp band in ‘Hosui’ (**Fig. 2**), was no longer detected after we switched to a pre-labeled marker (data not shown). Although some cultivars had overlapping size ranges for each marker, multiplex analysis is possible by switching to the pre-labeled dye.

**Discussion**

In this study, we conducted comprehensive inoculation tests and genotyping on large-scale pear genetic resources. We newly identified 18 Japanese and 4 Chinese cultivars susceptible to black spot disease. For the 101 cultivars previously tested by Kozaki (1973), the results of our...
inoculation test were consistent with the reported data, indicating that the evaluation was stable and accurate. Four SSR markers tightly linked to the gene for susceptibility were used to investigate the genotypes. All markers amplified specific bands in most of the susceptible cultivars (Table 1, Supplemental Table 1). In particular, Mdo.chr11.27 and Mdo.chr11.34 amplified a single locus and were highly consistent with the phenotype, so we consider these markers useful for MAS (Table 3). Progeny test showed that the susceptible cultivars were all heterozygous (Kozaki 1973). Crosses between susceptible cultivars showed a 1:3 ratio of resistant to susceptible seedlings, so susceptible homozygous plantlets (A/A) were present at the early seedling stage (Kozaki 1973). We also obtained dominant homozygous (A/A) seedlings from two crosses (‘Doitsu’ × ‘Nansui’ and ‘Shinsui’ × ‘Nijisseiki’). Each seedling was genotyped with two SSRs (Mdo.chr11.27 and Mdo.chr11.34), confirming the presence of dominant homozygous (A/A) seedlings. Dominant homozygous seedlings showed no difference in appearance from other seedlings but died within a few months (data not shown).

We conclude that the extant susceptible cultivars are heterozygous (A/a), indicating that the genotype of Mdo.chr11.34 corresponds perfectly to the estimated genotype of Japanese pears susceptible to black spot disease.

Kozaki (1973) inoculation-tested 11 Chinese pear cultivars, all of which proved resistant to black spot disease (Table 1). Here, we inoculated 35 Chinese pear cultivars, 4 of which were found to be susceptible (Table 1). This is the first report of susceptible Chinese pear cultivars. We crossed these four cultivars with the resistant Japanese cultivar ‘Kosui’ to create four populations for genetic linkage analysis and to confirm the inheritance mode, and mapped the loci of the susceptibility genes in Chinese pear. The segregation ratio of resistant and susceptible progeny fitted the expected ratio of 1:1 in the chi-squared test. This result is in good agreement with the report by Kozaki (1973) that a single dominant gene controls susceptibility to black spot in pear. Linkage analysis revealed that Chinese pears’ susceptibility genes are strongly linked to Mdo.chr11.27 and Mdo.chr11.34 (Fig. 3). In an integrated linkage map, the susceptibility gene of Chinese pear, Ac, was located within a 3.9-cM region between Mdo.chr11.27 and Mdo.chr11.34 (Fig. 1). The order of the markers and the susceptibility gene was the same in the original maps and integrated linkage map, indicating the accuracy of the inoculation tests and SSR analysis. These two markers have been mapped at the top of LG 11 and are strongly linked to the susceptibility gene of Japanese pear (Terakami et al. 2007, 2016). The current study is the first to identify the inheritance mode and to map the position of the gene conferring susceptibility to black spot disease in Chinese pear.

Different pathotypes of A. alternata, which produce host-selective toxins, cause similar diseases among the Rosaceae, e.g., black spot disease of Japanese and Chinese pears, Alternaria blotch of apple, and black spot disease of strawberry (Akimitsu et al. 2014, Tsuge et al. 2013). Susceptibility of those host plants to the disease is controlled by a dominant gene (Kozaki 1973, Saito and Takeda 1984, Yamamoto et al. 1985). In apple, the susceptibility gene Alt has been mapped on chromosome 11 between markers Mdo.chr11.30 and Mdo.chr11.34, and the candidate genes have been identified (Moriya et al. 2019). In three different linkage maps, the susceptibility gene of Japanese pear have been mapped to LG 11 between Mdo.chr11.28 and Mdo.chr11.34 (Terakami et al. 2007, 2016). The black spot susceptibility gene of Chinese pear have also been mapped at the top of LG 11, indicating that these genes are orthologous to those of apple and Japanese pear. Although detailed analysis has not been carried out in strawberry, these results suggest that the genes for susceptibility to A. alternata are conserved among Rosaceous hosts. Cloning of the susceptibility genes of Japanese and Chinese pears would further elucidate the mechanism of susceptibility in the Rosaceae.

Genotypes of old native and present cultivars derived from them show that the Mdo.chr11.27 and Mdo.chr11.34 marker set would be useful for breeding pears resistant to black spot disease. Susceptible ‘Kincho’ and ‘Osa Nijisseiki’ have very useful traits in pear breeding. Pear scab is one of the most harmful diseases of pears, especially Japanese and Chinese pears. It is pathogenic to the major commercial Japanese pear cultivars (Bell et al. 1996, Ishii et al. 1992), but no scab symptoms have been observed on ‘Kincho’ or indigenous Japanese pear (Abe and Kotobuki 1998, Ishii et al. 1992). To achieve stable fruit set without the need for artificial pollination, self-compatibility, which is controlled by multiple S haplotypes at a single locus, has become an important objective in Japanese pear breeding programs (Saito 2016). ‘Osa Nijisseiki’ (a mutant of the self-incompatible cultivar ‘Nijisseiki’) is self-compatible and is used as a parent for the breeding of self-compatible cultivars in Japan (Saito 2016). When ‘Kincho’ or ‘Osa Nijisseiki’ is used as a parent for breeding, about half of the progeny are susceptible to black spot disease. The Mdo.chr11.27 and Mdo.chr11.34 marker set could efficiently and accurately select black spot–resistant seedlings. MAS is more efficient when the marker set is combined with DNA markers linked to pear scab resistance and self-compatibility (Okada et al. 2008, Terakami et al. 2006). Pear genetic resources that have useful traits for breeding but are susceptible to black spot disease may be found in the future. Phenotypic data from the spore inoculation test and the genotyping data of markers linked to the susceptibility gene will be useful for pear breeding by MAS.

Author Contribution Statement

ST conducted genetic experiments and inoculation test, analyzed data, and wrote the initial draft of the manuscript. YA and YT performed inoculation test. NT and SN provided the experimental materials. TS and TY contributed to
the preparation of the final version of the manuscript. All authors reviewed and approved the manuscript.

Acknowledgments

We are grateful to Mss. N. Yagihashi, H. Takahashi, M. Tsukamoto, and N. Minagawa for their technical assistance. The virulent isolate No. 15A of A. alternata was provided by Dr. Takashi Tsuge, Chubu University, Japan. This work was partially supported by a grant from the Ministry of Agriculture, Forestry, and Fisheries of Japan (Genomics-based Technology for Agricultural Innovation, HOR-2001).

Literature Cited

Abe, K. and K. Kotobuki (1998) Inheritance of high resistance to Venturia nashicola Tanaka et Yamamoto in Japanese pear (Pyrus pyrifolia Nakai) and Chinese pear (P. ussuriensis Maxim.). J. Japan. Soc. Hort. Sci. 67: 677–680.

Akimitsu, K., T. Tsuge, M. Kodama, M. Yamamoto and H. Otani (2014) Alternaria host-selective toxins: determinant factors of plant disease. J. Gen. Plant Pathol. 80: 109–122.

Banno, K., H. Ishikawa, Y. Hamauzu and H. Tabira (1999) Identification of a RAPD marker linked to the susceptible gene of black spot disease in Japanese pear. J. Japan. Soc. Hort. Sci. 68: 476–481.

Bell, R.L. (1990) Pears (Pyrus). In: Moore, J.N. and J.R. Ballington Jr. (eds.) Genetic resources of temperate fruit and nut crops I, International Society for Horticultural Science, Wageningen, The Netherlands, pp. 655–697.

Bell, R.L., H.A. Quamme, R.E.C. Layne and R.M. Skirvin (1996) Pears. In: Janick, J. and J.N. Moore (eds.) Fruit Breeding, vol. I: Tree and Tropical Fruits, John Wiley & Sons, London, pp. 441–514.

Brownstein, M.J., J.D. Carpenter and J.R. Smith (1996) Modulation of non-templated nucleotide addition by Tag DNA polymerase: Primer modifications that facilitate genotyping. BioTechniques 20: 1004–1010.

Donald, T.M., L. Elen, J. Auham and M. Leaky (2002) Identification of resistance gene analogs linked to a powdery mildew resistance locus in grapevine. Theor. Appl. Genet. 104: 610–618.

Grattapaglia, D. and R. Sederoff (1994) Genetic-linkage maps of Eucalyptus grandis and Eucalyptus urophylla using a pseudo-testcross: mapping strategy and RAPD markers. Genetics 137: 1121–1137.

Hayashi, N., K. Tanabe, T. Tsuge, S. Nishimura, K. Kohimoto and H. Otani (1990) Determination of host-selective toxin production during spore germination of Alternaria alternata by high-performance liquid chromatography. Phytopathology 80: 1088–1091.

Iketani, H., K. Abe, T. Yamamoto, K. Kotobuki, Y. Sato, T. Saito, O. Terai, N. Matsuta and T. Hayashi (2001) Mapping of disease-related genes in Japanese pear using a molecular linkage map with RAPD markers. Breed. Sci. 51: 179–184.

Ishi, H., H. Udagawa, S. Nishimoto, T. Tsuda and H. Nakashima (1992) Scab resistance in pear species and cultivars. Acta Phytopathol. Entomol. Hung. 27: 293–298.

Kitagawa, K., M. Nagara, M. Uchida, K. Inoue, K. Murata, T. Terakami, Adachi, Takeuchi, Takada, Nishio, Saito and Yamamoto

Masuda, T. Yoshioka and K. Kotobuki (1999) A new Japanese pear cultivar ‘Kotobuki Shinsui’. Bull. Tottori Hortic. Exp. Stn. 3: 1–13 (in Japanese with English summary).

Kosambi, D.D. (1944) The estimation of map distances from recombination values. Ann. Eugen. 12: 172–175.

Kozaki, I. (1973) Black spot disease resistance in Japanese pear. I. Heredity of the disease resistance. Bull. Hortic. Res. Stn. (Minist. Agric. For.) Ser. A 12: 17–27 (in Japanese with English summary).

Masuda, T., T. Yoshioka, T. Sanada, K. Kotobuki, M. Nagara, M. Uchida, K. Inoue, K. Murata, K. Kitagawa and A. Yoshida (1998) A new Japanese pear cultivar ‘Osa Gold’, resistant mutant to the black spot disease of Japanese pear (Pyrus pyrifolia Nakai) induced by chronic irradiation of gamma-rays. Bull. Natl. Inst. Agrobiol. Resour. 12: 1–11 (in Japanese with English summary).

Moriya, S., S. Terakami, K. Okada, T. Shimizu, Y. Adachi, Y. Katayose, H. Fujisawa, J.Z. Wu, H. Kanamori, T. Yamamoto et al. (2019) Identification of candidate genes responsible for the susceptibility of apple (Malus ×domestica Borkh.) to Alternaria blotch. BMC Plant Biol. 19: 132.

Nishimura, S. and K. Kohmoto (1983) Host-specific toxins and chemical structures from Alternaria species. Annu. Rev. Phytopathol. 21: 87–116.

Okada, K., N. Tonaka, Y. Moriya, N. Norioka, Y. Sawamura, T. Matsumoto, T. Nakasone and T. Takasaki-Yasuda (2008) Deletion of a 236 kb region around S4-RNase in a stilar-part mutant S4sm-haplotype of Japanese pear. Plant Mol. Biol. 66: 389–400.

Reis, R.F., T.F. de Almeida, E.S. Stuchi and A. de Goes (2007) Susceptibility of citrus species to Alternaria alternata, the causal agent of the Alternaria brown spot. Sci. Hortic. 113: 336–342.

Saito, K. and K. Takeda (1984) Genetic analysis of resistance to Alternaria blotch (Alternaria maliformis Roberts) in apple: studies on the breeding of the apple. VIII. Japan. J. Breed. 34: 197–209 (in Japanese with English summary).

Saito, T. (2016) Advances in Japanese pear breeding in Japan. Breed. Sci. 66: 46–59.

Sanada, T., T. Nishida and F. Ikeda (1988) Resistant mutant to black spot disease of Japanese pear ‘Nijissiki’ induced by gamma-rays. J. Japan. Soc. Hort. Sci. 57: 159–166.

Sanada, T., K. Kotobuki, T. Nishida, H. Fujita and F. Ikeda (1993) A new Japanese pear cultivar ‘Gold Nijissiki’, resistant mutant to black spot disease of Japanese pear. Japan. J. Breed. 43: 455–461 (in Japanese with English summary).

Schuelke, M. (2000) An economic method for the fluorescent labeling of PCR fragments. Nat. Biotechnol. 18: 233–234.

Terakami, S., M. Shoda, Y. Adachi, T. Gonai, M. Kasumi, Y. Sawamura, H. Iketani, K. Kotobuki, A. Patocchi, C. Gessler et al. (2006) Genetic mapping of the pear scab resistance gene Vnk of Japanese pear cultivar Kinchaku. Theor. Appl. Genet. 113: 743–752.

Terakami, S., Y. Adachi, H. Iketani, Y. Sato, Y. Sawamura, N. Takada, C. Nishitani and T. Yamamoto (2007) Genetic mapping of genes for susceptibility to black spot disease in Japanese pears. Genome 50: 735–741.

Terakami, S., S. Moriya, Y. Adachi, M. Kunihisa, C. Nishitani, T. Saito, K. Abe and T. Yamamoto (2016) Fine mapping of the gene for susceptibility to black spot disease in Japanese pear (Pyrus pyrifolia Nakai). Breed. Sci. 66: 271–280.

Tsuge, T., Y. Harimoto, K. Akimitsu, K. Ohtani, M. Kodama, Y.
Akagi, M. Egusa, M. Yamamoto and H. Otani (2013) Host-selective toxins produced by the plant pathogenic fungus Alternaria alternata. FEMS Microbiol. Rev. 37: 44–66.
Van Ooijen, J.W. (2006) JoinMap 4, Software for the calculation of genetic linkage maps in experimental populations. Kyazma B.V., Wageningen, Netherlands.
Van Ooijen, J.W. (2011) Multipoint maximum likelihood mapping in a full-sib family of an outbreeding species. Genet. Res. 93: 343–349.
Voorrips, R.E. (2002) MapChart: Software for the graphical presentation of linkage maps and QTLs. J. Hered. 93: 77–78.
Westwood, M.N. and H.O. Bjornsta (1971) Some fruit characteristics of interspecific hybrids and extent of self-sterility in Pyrus. Bull. Torrey Bot. Club 98: 22–24.
Wu, J., Z.W. Wang, Z.B. Shi, S. Zhang, R. Ming, S.L. Zhu, M.A. Khan, S.T. Tao, S.S. Korban, H. Wang et al. (2013) The genome of the pear (Pyrus bretscheideri Rehd.). Genome Res. 23: 396–408.
Yamamoto, M., F. Namiki, S. Nishimura and K. Kohmoto (1985) Studies on host-specific AF-toxins produced by Alternaria alternata strawberry pathotype causing Alternaria black spot of strawberry. 3. Use of toxin for determining inheritance of disease reaction in strawberry cultivar Morioka-16. Japanese Journal of Phytopathology 51: 530–535.