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Research Paper

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-crossable, and there are no major incompatibility barriers to interspecific hybridization in this genus (Westwood and Bjornsta 1971).

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*Corresponding author (e-mail: terakami@affrc.go.jp)

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 (*A*/*a*), but no homozygous (*A*/*A*) 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. 'Babaucchiaginashi' and 'Iwate Tanenashi' 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 F_1 mapping populations 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 F_1 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 1×10^5/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 Kohmoto 1983). All inoculation tests with all cultivars and F_1 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[a]	Susceptibility to black spot disease[b]	SSR genotype[c]	
					Mdo.chr11.27	Mdo.chr11.34
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
Akibae	*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		S	220/230	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
Choujuurou	*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		S	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	220/230	259/284
Gozennashi	*P. pyrifolia* Nakai	113583	*a*/*a*	R	230/230	282/282
Hakataao	*P. pyrifolia* Nakai	113584	*A*/*a*	S	220/230	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 Henung 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
Hatsushimo	*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	220/230	259/284
Heishi	*P. pyrifolia* Nakai	113592	*A*/*a*	S	220/230	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

Table 1. (continued)

Cultivar	Species	JP accession number in NARO Genebank	Putative genotype[a]	Susceptibility to black spot disease[b]	SSR genotype[c]	
					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	*a*/*a*	R	230/230	284/284
Ishinashi	*P. pyrifolia* Nakai	239688		R	224/234	272/274
Iyohikari	*P. pyrifolia* Nakai	113604	*A*/*a*	S	220/220	259/284
Izunohomare	*P. pyrifolia* Nakai	113605	*a*/*a*	R	230/230	272/284
Jouhana	*P. pyrifolia* Nakai	113606	*A*/*a*	S	220/230	259/284
Kamenashi	*P. pyrifolia* Nakai	113608		S	220/228	259/282
Kansai Asaryuu	*P. pyrifolia* Nakai	113609	*a*/*a*	R	230/230	272/284
Kansai Ichi	*P. pyrifolia* Nakai	113610	*a*/*a*	R	230/230	282/284
Kanta	*P. pyrifolia* Nakai	–		R	230/230	284/284
Kikusui	*P. pyrifolia* Nakai	113611		R	230/230	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	*a*/*a*	R	230/230	284/284
Konpeitou	*P. pyrifolia* Nakai	113617	*a*/*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	*a*/*a*	R	230/230	282/284
Koushuu	*P. pyrifolia* Nakai	113618	*a*/*a*	R	230/230	272/284
Kousui (Kosui)	*P. pyrifolia* Nakai	113619	*a*/*a*	R	230/230	284/284
Kouzan	*P. pyrifolia* Nakai	118530		R	234/258	272/272
Kouzou	*P. pyrifolia* Nakai	113620	*a*/*a*	R	230/230	284/284
Koyuki	*P. pyrifolia* Nakai	113622		R	230/230	274/284
Kumoi	*P. pyrifolia* Nakai	113623	*a*/*a*	R	230/230	284/284
Kunitomi	*P. pyrifolia* Nakai	113624	*A*/*a*	S	220/230	259/284
Kuroki	*P. pyrifolia* Nakai	113625	*a*/*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	*a*/*a*	R	230/230	284/284
Nangetsu	*P. pyrifolia* Nakai	238261		R	230/230	284/284
Nansei 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
Niigatanashi	*P. pyrifolia* Nakai	113629	*a*/*a*	R	230/230	270/283
Niitaka	*P. pyrifolia* Nakai	113630	*a*/*a*	R	230/230	284/284
Nijisseiki	*P. pyrifolia* Nakai	113631	*A*/*a*	S	220/230	259/284
Nikkori	*P. pyrifolia* Nakai	118540		R	230/230	284/284
Okukouzou	*P. pyrifolia* Nakai	113632	*a*/*a*	R	230/230	284/284
Okuroku	*P. pyrifolia* Nakai	113633	*a*/*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
Oohiromaru	*P. pyrifolia* Nakai	113637	*a*/*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[a]	Susceptibility to black spot disease[b]	SSR genotype[c]	
					Mdo.chr11.27	Mdo.chr11.34
Osa Gold	*P. pyrifolia* Nakai	110825		S	220/230	259/284
Osa Nijisseiki	*P. pyrifolia* Nakai	113640		S	220/230	259/284
Oushuu	*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*	S	220/248	259/292
Seiryuu	*P. pyrifolia* Nakai	113649	*a*/*a*	R	228/260	272/274
Sekaiichi	*P. pyrifolia* Nakai	113650	*A*/*a*	S	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
Shihyakume	*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
Shinsei	*P. pyrifolia* Nakai	113694	*a*/*a*	R	228/230	274/284
Shinseiki	*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
Shuugyoku	*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
Suishuu	*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	113669		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

Table 1. (continued)

Cultivar	Species	JP accession number in NARO Genebank	Putative genotype[a]	Susceptibility to black spot disease[b]	SSR genotype[c]	
					Mdo.chr11.27	Mdo.chr11.34
Yahatanishiki	*P. pyrifolia* Nakai	113690	*a*/*a*	R	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.	113536		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
Babaucchiaginashi	*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

[a] Proposed in Kozaki (1973). *A*/*a*: heterozygote susceptible to black spot; *a*/*a*: resistant; blank cell: not tested.
[b] S, necrotic symptoms (susceptible); R, no disease symptoms (resistant).
[c] Numbers separated by "/" indicate the estimated size (bp) of the alleles of the same locus.

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	χ^2	*P*-value[a]
		Susceptible	Resistant			
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

[a] *P*-values indicate fit to the expected ratio (1:1). Distorted segregation is indicated by a significant *p*-value of the χ^2 test: *, $p < 0.05$.

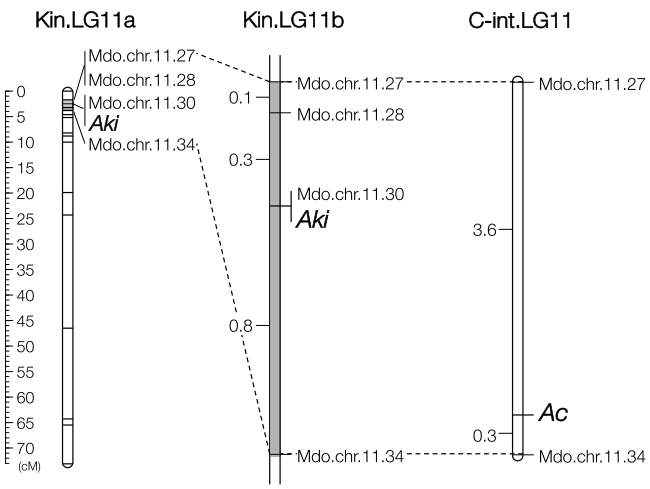

Fig. 1. Comparison of linkage maps of Japanese and Chinese pears. Linkage group (LG) 11 in the Japanese pear 'Kinchaku' (Terakami *et al.* 2016; Kin.LG11a) is shown on the left. *Aki* is the black spot susceptibility gene of 'Kinchaku'. The scale shows genetic distance (cM) from top marker of the linkage group. Kin.LG11b shows fine mapping of *Aki* and flanking regions. The numbers between markers indicate genetic distance. C-int.LG11 is the integrated LG 11 of black spot–susceptible Chinese pears; *Ac*, the black spot susceptibility gene in Chinese pears. In Kin.LG11b and C-int.LG11, numbers indicate genetic distance (cM) between markers.

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′-GCTACGGACTG ACCTCGGAC-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 3130*xl* 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 (χ^2 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

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 F_1 progeny (Terakami *et al.* 2016). Here, we performed fine mapping of *Aki* and constructed a more detailed linkage map using 1061 F_1 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**; $\chi^2 = 0.076$, $p = 0.782$).

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 F_1 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 that 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 F_1 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 F_1 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 ($\chi^2 = 3.967$, $p = 0.046$). Of 59 F_1 'Kosui' × 'Mi Li Cui' plantlets, 31 were susceptible and 28 were resistant (**Table 2**; $\chi^2 = 0.153$, $p = 0.696$). Of 83 F_1

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[a]		
		P_P	N_P	N_N
P. pyrifolia Nakai	Resistant			Abumi, Aikansui, Aiuchi, Akaho, Akiakari, Akibae, Akizuki, Amanogawa, Aoyagi, Asahi, Atago, Azumanishiki, Cheong Sil Ri, Chikusui, Chouju, Choujuurou, Chousen, Echigonishiki, Geishun, Gion, Gozennashi, Hakkou, Hakuteiryuu, Han Henung Li Kou, Han Heung Li Otsu, Harikonatsu, Hatsuaki, Hatsumaru, Hatsushimo, Hattatsu, Heiwa, Hoe Ryng Saibai, Hokkainashi, Hokushin, Hoshiakari, Hougetsu, Hougyoku, Hosui, Ichihara Wase, Imamuraaki, Imamuranatsu, Inagi, Inugoroshi, Ishii Wase, Ishinashi, Izunohomare, Kansai Asaryuu, Kansai Ichi, Kanta, Kikusui, Kiraseiki, Kokuchou, Konpeitou, Kougiku, Kounowatashi, Koushuu, Kosui, Kouzan, Kouzou, Koyuki, Kumoi, Kuroki, Kwankinbe, Mishirazu, Musashi, Nangetsu, Nansei Chabo, Narumi, Natsushizuku, Nekogoroshi, Niigatanashi, Niitaka, Nikkori, Okukouzou, Okuroku, Okusankichi, Onba, Oohiromaru, Ookoga, Oushuu, Rikiya, Rinka, Ruisannashi, Sagami, Saizounashi, Seigyoku, Seiryuu, Sekiryuu, Senryou, Shihyakume, Shikishima, Shimokatsuginashi, Shimonashi, Shinchuu, Shinkou, Shinsei, Shinseiki, Shinsetsu, Shirayuki, Shuugyoku, Shuurei, Shuusui, Sotoorihime, Suisei, Suishuu, Taihaku, Taihei, Tama, Tamotoyabure, Tanponashi, Tanzawa, Tosajou, Tosajounishiki, Tosanashi, Tosanishiki, Tsugaruao, Tsukutounashi, Wase Kouzou, Waseaka, Yachiyo, Yagoemon, Yahatanishiki, Yakumo, Yasato, Yoshikaori, Yoshino
	Susceptible	Asahiryuu, Awayuki, Chizu, Doitsu, Edoya, Gold Nijisseiki, Hakataao, Hayatama, Heishi, Higashino, Hokkan, Isai, Iyohikari, Jouhana, Kamenashi, Kimizukawase, Kinchaku, Kisui, Kiyosumi, Kotobukishinsui, Kougetsu, Kunitomi, Meigetsu, Nansui, Nijisseiki, Ootani, Osa Gold, Osa Nijisseiki, Rokugatsu, Segawa, Seika, Sekaiichi, Shinsui, Tenyuu, Touhou, Wase Taichou, Yabase, Yokogoshi	Fukushima	
P. bretschneideri Rehd.	Resistant			Bai Li
P. ussuriensis Maxim.	Resistant			Agenoshou Shinanashi, Ba Li Xiang, Baozhuli, Bei Jin Bai Li, Cang Xi Li, Chang Xi Li, Da Tou Huang Li, En Li, Hong Li, Hong Xiao Li, Huang Shi Li, Jian Ba Li, Lai Yang Ci Li, Lunanhuangli, Ma Ke Zao Li, Ma Ti Huang, Mi Li, Ping Li, Qiu Bai Li, Su Hyang Ri, Wo Wo Li, Xie Hua Tian, Xuehua Li, Ya Gua Li, Ya Li, Yang Nai Xiang, Yin Bai Li, Yuan Ba Li, Zaosu Li, Zhu Zui Li
	Susceptible			Huang Li, Mi Li Cui, Tai Huang Li, Xiang Ya Li
P. pyrifolia × *P. communis*	Resistant			Ninomiya, Ooharabeni, Taiheiyou
P. ussuriensis × *P. pyrifolia*	Resistant			Ninomiya Bai Li
P. babauttiaginashi Koidz.	Resistant			Babaucchiaginashi
Pyrus sp.	Resistant			Cheung Dang No Ri, Iwate Tanenashi

[a] 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.

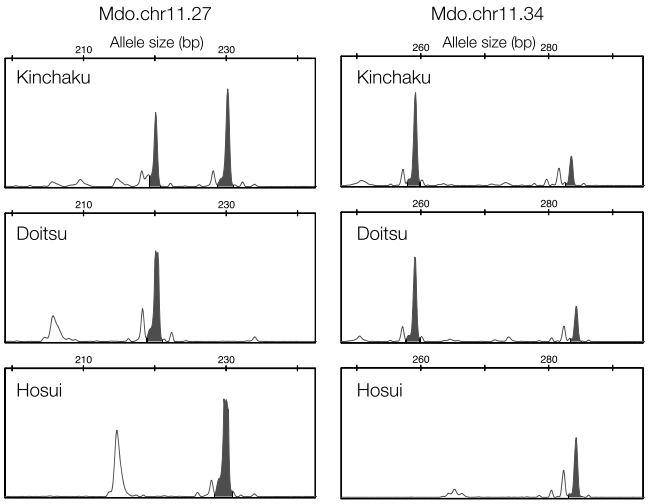

Fig. 2. Peak profiles of capillary gel electrophoresis output of fluorescently labeled SSR marker fragments. The target peaks are highlighted.

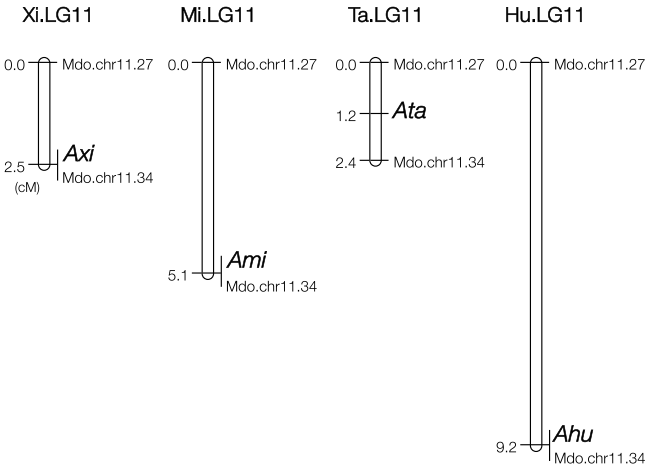

Fig. 3. Linkage groups 11 of Chinese pear cultivars 'Xiang Ya Li' (Xi.LG11), 'Mi Li Cui' (Mi.LG11), 'Tai Huang Li' (Ta.LG11), and 'Huang Li' (Hu.LG11). The respective genes for susceptibility to black spot are denoted as *Axi*, *Ami*, *Ata*, and *Ahu*. The numbers on the left of each marker show genetic distance (cM) from Mdo.chr11.27.

'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, respectively (**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 'Kinchaku' 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 'Kinchaku' 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 'Kinchaku' 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.

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