IDENTIFICATION OF MOLECULAR MARKERS LINKED TO X-DISEASE RESISTANCE IN CHOKECHERRY (*PRUNUS VIRGINIANA* L.)

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ABSTRACT

X-disease, caused by phytoplasmas, is one of the destructive diseases in stone fruit trees, causing yield loss and poor fruit quality. So far no effective methods are available to control X-disease. X-disease resistance has been first discovered in chokecherry (*Prunus virginiana*, 2n=4x=32), which is a native woody species of North America. To identify molecular markers linked to X-disease resistance, simple sequence repeat (SSR) markers were used to construct genetic linkage maps for chokecherry and to identify markers associated with X-disease resistance in chokecherry. In this research, three segregating populations of chokecherry were developed by crossing one X-disease resistant (C_L) with three susceptible chokecherry lines (a, c, and d), of which the progenies were 101, 177, and 82, respectively. In order to construct a genetic map for chokecherry, 108 pairs of SSR primers were employed from other *Prunus* species. Additionally, a set of 246 SSRs were developed from chokecherry sequencing by Roche 454 sequencing technology. A total of 354 pairs of SSR primers were used to screen individuals of all three populations. Two software programs, TetraploidMap and JoinMap, were used to construct linkage map based on single-dose restriction fragments (SDRFs) and two parental linkage maps were generated for each population from both software programs. Bulked segregant analysis (BSA) was applied for identification of X-disease resistance markers. As a result, one SSR marker was found to be linked to the X-disease resistance. The set of 246 chokecherry SSRs was later used to test transferability among another 11 rosaceous species (sour cherry, sweet cherry, wild cherry, peach, apricot, plum, apple, crabapple, pear, june berry, and raspberry). As a result, chokecherry SSR primers can be transferable in *Prunus* species or other rosaceous species. An average of 63.2% and 58.7% of amplifiable chokecherry primers amplified DNA from cherry and other *Prunus* species, respectively, while 47.2% of amplifiable

chokecherry primers can be transferable to other rosaceous species. The genetic information, including genetic map, disease linked marker, chokecherry sequence, and confirmed transferability of the identified chokecherry SSRs to other species, will benefit the genetic research in *Prunus* and other rosaceous species.

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CHAPTER 1. LITERATURE REVIEW

1. X-disease, a unique phytoplasma disease in stone fruit species

1.1 Phytoplasma disease

Phytoplasmas are specialized plant pathogens that are obligate parasites in host plants and transmitting insect vectors (Olivier et al., 2009). Phytoplasmas were originally thought to be viruses because they can be transmitted by insect vectors and cannot be cultured in the artificial medium. In 1967, phytoplasma pathogens were discovered in ultrathin sections of plant phloem tissue based on electron-microscopy observation and termed as mycoplasma-like organisms (MLOs) because of their similarity to mycoplasma in animal tissues (Doi et al., 1967). With the application of molecular technology, particularly, DNA-based techniques, to diagnoses the disease, the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes classified mycoplasma-like organisms as phytoplasmas. Later, the genus name *Phytoplasma* was adopted and they are now at Candidatus status (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes, 1993). Like other Mollicutes, phytoplasmas lack cell walls and are surrounded by a triple layered membrane. Phytoplasmas have more than 50 phylogenetic groups and cause diseases of over 200 economically important plants worldwide (Lee et al., 2000).

Phytoplasmas have small repeat-rich genomes, and the repeated DNAs are organized into large clusters of potential mobile units (PMUs) unique to phytoplasmas (Bai et al., 2006). The different mechanisms of recombination of phytoplasmas allow them to adapt a diverse environment in plants and insects. It was showed that the proteins produced by phytoplasmas targeted the nuclei and might affect gene expression of their hosts.

Phytoplasmas are mainly transmitted from infected plants to healthy plants by insects in the families of Cicadelidea (leafhoppers), Fulgoridea (planthoppers), and Psyllidea (jumping plant lice) (Weintraub and Beanland, 2006). The feeding habit of the insect vector determines the range of plant hosts of phytoplasma pathogens. For example, the polyphagous aster leafhopper feeds on and transmits Aster Yellows (AY) to about 200 plant species, while the monophagous pear psylla mostly feeds on pear and transmits pear decline within pear species (Olivier et al., 2009). Phytoplasma can be transmitted by other vectors as well. For instance, in order to obtain new plant sources for the research of alder yellows (ALY), Marcone and others transmitted the alder yellows phytoplasma to the experimental host periwinkle via dodder bridges (Marcone et al., 1997). Natural root grafting was confirmed to play a role for the spread of apple proliferation (AP) phytoplasma in older orchards and between trees on vigorous rootstocks as well (Baric et al., 2008). In vitro grafting and top grafting are applied to examine phytoplasma for fruit trees and other experimental plant hosts as well (Jarausch et al., 1999; Kaminska and Korbin, 1999). Phytoplasmas are mostly found in the phloem tissue; however, they have been detected in other plant organs. Jiang et al. (2004) reported that the mulberry dwarf (MD) phytoplasma was detected in reproductive organs, such as flowers, fruits, seed coats and overwintering roots in the cold region, indicating that phytoplasmas survive in roots, buds, and stems during the winter with re-entry into phloem in the spring.

The general symptoms of phytoplasma infection include virescence (development of green flowers and loss of normal flower pigments), phyllody (conversion of floral parts into leafy structures), abnormal internode elongation, stunting, witches' broom, smaller leaves, growth reduction, and reduced/no flower or seed production (Lee et al., 2000; Olivier et al., 2009). For example, peach rosette is one of the typical diseases caused by phytoplasma, which is

characterized by abnormal short internodes with large number of leaves. This symptom is caused by a lack of shoot elongation with compacted dormant buds (Scott and Zimmerman, 2001). Virescent flower is the first sign of infection of phytoplasma pathogen. Davey et al. (1981) found that the development of virescence was related to the level of endogenous cytokinins. Junqueira et al. (2004) reported an increase of protein and decrease of sugar and phenolic compounds in maize infected by maize bushy stunt phytoplasma. They also found a reduction of chlorophyll content, which indicated phytoplasma may decrease photosynthesis and speed up senescence in the leaf tissue.

It was a challenge to diagnose phytoplasma disease due to their inability to grow in vitro and their low and variable titers in plants (Olivier et al., 2009). Detection and identification of phytoplasmas were always carried out based on their biological properties (vector specificity and host range), symptomatology, and microscopic observations. In the 1980s, ELISA (enzyme-linked immunosorbent assay) and PCR (polymerase chain reaction) based methods were developed for detection and classification of phytoplasmas. These methods appear to be faster and more accurate than the methods mentioned above. For examples, Loi et al. (2002) used ELISA to detect apple proliferation phytoplasma. Phytoplasma DNA was specifically amplified using Nested-PCR in which both universal and group-specific oligonucleotide primers were designed based on the highly conserved 16S rRNA gene sequences of phytoplasmas (Boudon-Padieu et al., 1989; Deng and Hiruki, 1991; Ahrens and Seemuller, 1992; Lee et al., 1993). Recently, quantitative real time polymerase chain reaction (Q-PCR) and bioimaging techniques have been developed to assess the level of phytoplasma in infected plant tissues (Christensen et al., 2004). Based on the 16S rRNA restriction fragment length polymorphism (RFLP) patterns and calculated similarity coefficients, Wei et al. (2007) suggested that phytoplasma strains can be

classified into 28 groups (from 16SrI to 16SrXXVIII), including Aster Yellow (AY) group, Peanut witches-broom (WB) group, and X-disease group.

So far, no effective methods have been reported to control phytoplasma diseases. Several aspects need to be considered for management of phytoplasma diseases: insect vectors, source of diseases, and host plants. Insect vectors are commonly controlled by applying insecticides, but their effectiveness is affected by factors such as the population of vectors and their mobility. Other non-chemical methods have been attempted to manage these diseases. For example, managing vectors' alternative host plants, building borders around the field, or introducing natural enemies of vectors are useful to control diseases. Diseased trees can serve as reservoirs for pathogen acquisition by insect vectors; therefore, removing infected plants is considered as an efficient way to control the disease spreading. Reports also showed that regulation of planting densities can reduce the incidence of phytoplasma disease. However, utilization of disease-resistant cultivars is still the most promising means to control phytoplasma diseases.

1.2 X-disease in stone fruit species

Phytoplasmas cause many diseases in stone fruit species, such as cherry albino, peach yellows, European stone fruit yellows, and X-disease (Ogawa et al., 1995). Among those diseases, X-disease is the most destructive disease of temperate fruits and causes economic losses. X-disease pathogen belongs to the RFLP group 16Sr III of phytoplasma, which is the second largest group after AY (aster yellows). Ten ribosomal subgroups have been identified among this group (Bertaccini, 2007; Olivier et al., 2009). Two major strains of X-disease phytoplasmas, eastern (Canadian peach) X-disease (CX) and western X-disease (WX), have been found in North America based on their geographic distribution, host range, and insect vectors. These two strains are transmitted by different insect vectors: eastern strain is predominantly

transmitted by *Paraphlesius irroratus* (Say), while western strain is mainly transmitted by *Colladonus montanus* (Van Duzee). Additionally, other insect vectors of X-disease include *Fieberiella florii* (Stal), *Colladonus clitellarius* (Say), *Colladonus geminates* (Van Duzee), *Gyponana expanda* (DeLong), *Macropsis trimaculata* (Fitch), *Norvellina seminude* (Say), *Osbornellus borealis* (DeLong and Mohr), and *Scaphytopius acutus* (Say) (Lee et al. 1992; Olivier et al., 2009). X-disease can also be transmitted by budding, and grafting, as well as natural root grafting between adjacent trees.

1.3 Host range of X-disease

Many stone fruit species, such as peach (*Prunus persica*), sweet cherry (*P. avium*), sour cherry (*P. cerasus*), nectarine (*P. persica* var. nectarina), and chokecherry (*P. virginiana*) can be easily infected by X-disease phytoplasmas (Guo et al., 1996). X-disease is also observed on almond (*P. amygdalus*), mahaleb cherry (*P. mahaleb*), Korean cherry (*P. japonica*), bitter cherry (*P. emarginata*), hollyleaf cherry (*P. ilicifolia*), and several plum hybrids (*P. armericana*) (Verma and Sharma, 1999). Besides the stone fruits, some herbaceous plant species can be infected by X-disease phytoplasma as well. For example, milk weed (*Asclepias syriaca*) is known to carry X-disease phytoplasma under natural conditions (Guo et al., 1998).

1.4 Diagnosis of X-disease

1.4.1 Morphological or symptom-based method

Phytoplasmas have usually been differentiated by symptomology, host range, and vector-pathogen relationships (Anfoka et al., 2003). In general, X-disease symptoms are apparent two months after infection. Infected leaves curl inward and develop irregular yellow to reddish-purple spots. The typical symptom on chokecherry is stunted (red or yellow) leaves and shoots,

deformed and discolored fruits, reduced hardiness of current shoots and dieback of branches and stems. Diseased trees will eventually die in 3-5 years.

1.4.2 Serological method

Using serological methods to identify and detect X-disease phytoplasmas is generally quite specific. Monoclonal and polyclonal antibodies usually react with the phytoplasma strains specifically. Monoclonal antibodies against the aster yellows agent were developed by Chan et al. in 1985. These specific monoclonal antibodies were produced from infected leafhopper vectors. With the help of monoclonal antibodies, the aster yellow agent will be specifically identified by ELISA in lettuce and periwinkle (Chan et al., 1985).

Polyclonal antibodies were applied to identify X-disease by using phytoplasmas purified from infected chokecherry in 1996 (Guo et al., 1996). These polyclonal antibodies only react with diseased plants, thus uncertainty regarding the substantial cross-reactivity with healthy host antigens is avoided.

Further research by Guo et al. (1998) for serological identification of X-disease using a monoclonal antibody was repeated. The monoclonal antibody was produced using an enriched antigen from an infected chokecherry plant. It was more sensitive than previously developed polyclonal antibody, because it did not need to absorb the monoclonal antibody with healthy antigen, as was required for use of the polyclonal antibodies. The unlimited supply of the monoclonal antibody allowed the application of ELISA for detection of the X-disease phytoplasma in a large scale (Guo et al., 1998).

1.4.3 Molecular method

The advances in polymerase chain reaction (PCR) technology enabled researchers to amplify any genomic DNA sequences from an organism using specific primers. The method of

PCR has been applied for classification and differentiation of phytoplasmas based on the genetic variations in 16S rRNA genes, two ribosomal protein (*rp*) genes, 23S rRNA genes, and the 16S/23S spacer region (Guo et al., 2000). Although no difference among 43 ChX phytoplasma isolates was detected based on the RFLP patterns of 16S rRNA, 23S rRNA, and the *rp* genes, variations were observed at four positions when compared sequences of the 441-bp 16S/23S spacer region in these isolates (Guo et al., 2000). Availability of these phytoplasma-specific gene sequences has greatly facilitated the detection and phylogenetic analysis of phytoplasmas.

Comparative analysis of the amplified 16S rRNA genes from various phytoplasmas has become a foundation for establishing phytoplasmal taxonomy (Zhang et al., 2004). Classification of phytoplasmas can be achieved based primarily on sequence variations in the 16S rRNA gene. The other two rRNA genes in phytoplasmas, encoding the 23S and 5S rRNAs, have not been cloned and used for phytoplasmal taxonomy or diagnosis. Because the size of 23S rRNA gene is about twice that of the 16S rRNA gene, it may have potential ability to provide more information for studying phylogenic relationships, genetics, and diagnosis of phytoplasmas (Guo et al., 2000).

Nested PCR, a modification of PCR, has been developed to reduce the contamination in products due to the amplification of unexpected primer binding sites. Two different pairs of PCR primers were used to amplify a fragment. The logic of this strategy is that if the wrong loci are amplified by mistake, the probability is very low that it would also be amplified at a second time by a second pair of primers. Nested-PCR can be used to amplify the 16S rRNA gene because single-step PCR does not reliably result in a visible band. Gundersen et al. (1996) noticed that nested-PCR assays using two universal primer pairs, R16mF2/R1 and R16F2n/R2, could increase the detection sensitivity of phytoplasma pathogen from all woody hosts and insect hosts.

Additionally, RFLP analysis of the nested-PCR products identified the primary phytoplasma associated with each tissue sample. With the application of RFLP analysis of the 16S ribosomal DNA sequence amplified with the nested-PCR, chokecherry X-disease was firstly confirmed in the Great Plains in 1996 (Guo et al., 1996). RFLP analysis of PCR-amplified 16S rRNA, 23S rRNA and the *rp* genes was used to characterize and assess genetic variability within ChX phytoplasma isolated from North Dakota (Guo et al., 2000).

1.5 Management of X-disease in cherry and peach species

Damage of X-disease is known to cause economic losses associated with reduction of yield and fruit quality, cost of removing and replacing infected trees, and the subsequent 3 to 4 years wait for another commercial crop. The symptoms of the infected plants are variable depending upon the strain, host plants, rootstocks, and environmental conditions.

1.5.1 X-disease in cherry

Symptoms in cherry species appear first in the inoculated place and the entire tree may be systemically infected within two or more years. Leaves on infected limbs are smaller compared to normal ones and develop a red or orange tinge along the mid-vein at mid-growing season. Terminal shoot growth is reduced and dieback of twigs and branches also occurs. Symptoms for fruits include late or delayed maturation, small-sized fruits, and incomplete color development. Glenn et al. (1984) researched the spread and damage of western X-disease of chokecherry in eastern Nebraska and reported that after artificial inoculation, the western X-disease pathogen can spread rapidly from infected chokecherries to healthy ones. More than 60% and 80% of chokecherry trees showed the symptoms within 3 and 5 years, respectively. The mortality of infected chokecherries was more than 50% and 80% within 8 and 15 years, respectively. As a

control, none of the American plum trees had been killed or damaged by the pathogen within 9 years being infected.

1.5.2 X-disease in peach

Symptoms in peach first appear in the infected scaffold. Leaves of diseased trees are normal in appearance at the start of the growing season, but suddenly show the symptoms by mid-summer. The symptoms of the infected leave include rolling along the mid-vein, red, irregular blotches, and prematurely falling down. Fruits on the infected scaffolds develop later and are less numerous, smaller, and lack flavor. The branches show dieback after infestation. Mortality of diseased trees varies as older trees may survive for several years, while younger trees may die within 1-3 years after first symptoms appear (Ogawa et al., 1995; Verma and Sharma, 1999).

1.5.3 Management of X-disease

X-disease is spread by leafhoppers that feed on diseased plant hosts; therefore, the management practice may focus on treating the orchard for leafhopper vectors and managing nearby leafhopper hosts. Leafhopper vectors can be controlled by spraying X-disease infected trees with insecticide before rouging (Weintraub and Beanland, 2006). For X-disease plant hosts, since the pathogen is sensitive to oxytetracycline antibiotics, trunk or scaffold limb injection of antibiotics can improve tree vigor and partially restore fruit production. Lacy (1982) reported that X-diseased scaffolds of peach produced nearly normal yields after application of oxytetracycline hydrochloride (OTC). Besides that, identifying and removing the infected plants is the most important and cost-effective way to manage the X-disease (Ogawa et al., 1995; Verma and Sharma, 1999). Because chokecherry is an important reservoir host of X-disease, it should be removed within 200 m of an orchard of other *Prunus* species.

2. Chokecherry and chokecherry X-disease

2.1 Chokecherry

The genus *Prunus*, belonging to the family of Rosaceae, is comprised of more than 200 species of trees and shrubs, including plum (*P. americana*), peach (*P. persica*), apricot (*P. armeniaca*), almond (*P. dulcis*), sweet cherry (*P. avium*), and sour cherry (*P. cerasus*) (Mason, 1913; Browicz and Zohary, 1996; Bortiri et al., 2006). Many species in this genus are important producers of commercial fruit, valuable sources of wildlife food, or used for ornamental and erosion-control plantings. The fruit is a drupe with a relatively large hard seed, so members in the genus *Prunus* are called stone fruits. In North America, *Prunus* has 40 or more species, in which 14 species of wild cherries are native to the United States, including black cherry (*P. serotina*), bitter cherry (*P. emarginata*), pin cherry (*P. pennsylvanica*), and chokecherry (*P. virginiana*).

Chokecherry, a native woody species to North America, is a small tree or large shrub and distributed throughout the central and northern U.S. It can be divided into two varieties, the eastern chokecherry (*P. virginiana* var. virginiana) and the western chokecherry (*P. virginiana* var. demissa) (Dirr, 1998). Chokecherry is tolerant to many environmental stresses, such as drought, cold, and alkaline soil. Chokecherry has an extensive root system and the seedlings can sprout in clusters either from seeds or roots of established plants. The leaves are oval, with a coarsely serrated margin and usually 3-10 cm long. The panicle inflorescence is developed into 10-15 racemes with 5-10 flowers per raceme in late spring. The fruit is about 1 cm in diameter with the color from bright red to black and ripen in the late summer or early fall. Chokecherry fruits are edible with sour flavor when fully matured and commonly used to make jellies, juices, sauces, wine, and jams. Chokecherry is also an important tree species for windbreaks, watershed

protection, diverse species for habitats, and highway beautification. The purple-leaved selection is a popular landscape plant.

Chokecherry is known as a high level source of antioxidant pigment compounds. Li et al. (2008, 2009) found that chokecherry had the highest antioxidant capacity and phenolic acids, including caffeic acid, *p*-counmaric acid, protocatechuic acid, ferulic acid, gallic acid and *trans*-cinnamic acid, which played a protective role against oxidative damage of diseases, compared with berry fruits and sea-buckthorns (*Hippophae* L.). However, chokecherry is also toxic to humans and animals because of two kinds of the cyanogenic glycoside (amygdalin and prunasin), from which cyanide can be released when digested by enzymes in the stomach. The poisoning can occur with new growth, bruised, wilted, or dried foliage (Knight and Walter, 2001; Soto-Blanco et al., 2008).

Chokecherry can be propagated with seeds, division, and stem cutting. The formation of adventitious roots from stem cuttings is affected by genotype, developmental stage of cuttings, and other environmental conditions. Chokecherry can also be vegetatively propagated using micropropagation method (Zhang et al., 2000; Dai et al., 2004).

2.2 Chokecherry, a unique *Prunus* species for X-disease research

X-disease was first found on peach and cherry in 1931 and 1933 in California and Connecticut, respectively. In the genus of *Prunus*, chokecherry is considered as the source of X-disease since it is an important reservoir host of leafhopper, by which the X-disease phytoplasma is vectored. In that case, removal of chokecherry plants within a radius of 500 feet is considered an effective way to reduce the spread of X-disease (Stoddard et al., 1951). Besides that, the X-disease pathogen can overwinter both in buds and roots of chokecherry (Gilmer et al., 1954). Rosenberger and Jones (1977) conducted grafting study with diseased buds of chokecherry and peach to

compare the infectivity of X-disease between these two species. They found that the infectivity of peach buds declined during the late summer to fall and the winter-inoculated chokecherry had a higher percentage of seedlings developing X-disease than peach during the same period. Moreover, they also reaffirmed previous observations of more irregular distribution and lower diseased populations in peach than in chokecherry.

Chokecherry can be infected by both eastern X-disease and western X-disease (Reeves et al., 1951; Stoddard et al., 1951). Newly infected chokecherries begin growth later in the spring than normal ones. The symptoms are first seen as a slight yellowing or reddening of the foliage. Later, the symptoms may become more obvious year after year and show vivid red or yellow leaves and a distinct resetting at the tip of the branches. The infected chokecherry may blossom, but the ovaries abort at the early developmental stage. The retained fruits do not mature at the end of the season and have undeveloped flesh and always dead seeds in the pits. Diseased chokecherries often die within 1-3 years after the appearance of symptoms (Reeves et al., 1951; Stoddard et al., 1951).

2.3 Current research on X-disease in chokecherry

As early as 1983, a chokecherry seed source provenance planting was established by the U.S. Dept. of Agriculture Natural Resources Conservation Service (NRCS) Plant Materials Center near Bismarck, ND. The germplasms were collected from Minnesota, North Dakota, and South Dakota, which consisted of over 3,000 plants from 179 accessions, with the purpose to select potential X-disease resistant materials to manage this severe disease (USDA-NRCS, 1993). The X-disease symptoms were detected in 1987, and by 1994, 44% of the plants were dead. X-disease phytoplasma was present in all 1,792 surviving plants, including 1% that had no

symptoms and 4% that had symptoms but little damage. These plants with light or no symptoms may be resistant or highly tolerant to X-disease (Walla et al., 1996).

In addition to fluorescence microscopy, which was utilized by Douglas in 1986, Guo et al. (1996a, b; 1998a, b) used polyclonal antibody, monoclonal antibody, and molecular method to detect X-disease. Using these diagnosis technologies, Guo and Cheng (1998) did a large-scale screening in chokecherry plantings using a three step screening strategy by combing symptomology, serology, and PCR technology. First of all, 1,737 (97%) plants were identified to be infected based on disease ratings. Eighty percent of remained plants without symptoms were diagnosed as positive for the X-disease phytoplasma using monoclonal antibody as the second step. For the last step, the authors used nested-PCR, which was the most sensitive and time consuming method, and found all of the 11 samples that were IF (immunofluorescence staining) negative or questionable contained the X-disease phytoplasma.

Nested-PCR and RFLP (restriction fragment length polymorphism) analyses were used to examine the relationships among X-diseased chokecherry strain (ChX) and the eastern (CX) and western (WX) strains of phytoplasmas (Guo et al., 1996b). They found that the fragment pattern of ChX phytoplasma was different from the other two phytoplasmas (CX and WX), indicating that these three strains of X-disease phytoplasmas were not genetically identical and the ChX phytoplasma was relatively uniform among the samples collected in ND. In order to closely characterize ChX phytoplasma, Guo et al. (1996b) did another experiment to verify CX, WX, and goldenrod yellows (GR1) phytoplasma groups and found that ChX was closely related to CX and WX and easily verified from GR1.

3. Molecular markers in plant genome analysis

3.1 DNA based molecular markers

Many important agronomic traits of crops, such as yield, disease resistance, and stress tolerance, are controlled by more than one major or a few minor genes (Collard et al., 2005). It is a challenge to identify the contributed genes only based on the phenotypic evaluation. Advancement of molecular genetics, genomics, and biotechnology has greatly enhanced our understanding of the structure and function of plant genomes and facilitated plant breeding (Semagn et al., 2006). In plant breeding, screening of breeding populations for targeted traits is the most time and labor consuming process. Conventional breeders often use phenotypic characters, such as color, seed shape, height, yield, quality, and symptoms caused by biotic or abiotic stresses, etc. to select elite progenies/lines.

In 1950s, isozyme markers were used to associate phenotypic traits with protein markers (Semagn et al., 2006). Since 1980s, DNA based molecular markers have been intensively used to screen breeding populations at the DNA level (Collard et al., 2005). Molecular markers can be divided into three classes: hybridization-based, such as RFLP (Restriction fragment length polymorphism), polymerase chain reaction (PCR)-based, such as RAPD (Random amplified polymorphic DNA), and DNA sequence-based, such as SSR (Simple sequence repeat) (Agarwal et al., 2008).

3.1.1 Restriction fragment length polymorphism (RFLP)

RFLP is the most widely used hybridization-based DNA marker. It was first used for virus study in 1975 (Grodzicker et al., 1975) and later for human and plant genome mapping (Botstein et al., 1980; Helentjaris et al., 1986). RFLP is based on characterization of restriction enzymes that can recognize a specific nucleotide sequence (restriction site) and cut DNA at that

site. The digested DNA is separated and hybridized with a chemically labeled DNA probe to a Southern blot to detect DNA polymorphisms with differential DNA fragment profile. In general, RFLP can detect nucleotide substitutions or DNA rearrangements, such as insertion, deletion, or single nucleotide polymorphisms. Thus, RFLP markers are relative high polymorphic, co-dominant, and highly reproducible. However, it has some disadvantages though: it is time consuming and labor intensive, requiring expensive and radioactive or toxic reagents and large amount of high quality genomic DNA. It also needs prior sequence information for probe preparation (Winter and Kahl, 1995; Staub et al., 1996; Agarwal et al., 2008).

3.1.2 Random amplified polymorphic DNA (RAPD)

RAPD was first described by Williams et al. in 1990 in human research. It is based on PCR amplification of random genomic DNA segments using single arbitrary nucleotide primers that are usually 9-12 nucleotides long (Semagn et al., 2006). Since RAPD does not require prior genome information, it can be applied across species by using universal primers. RAPD is highly polymorphic and can detect single base changes in genomic DNA. The major drawback is the low reproducibility, because of its dependence on reaction conditions (Winter and Kahl, 1995; Staub et al., 1996; Agarwal et al., 2008).

3.1.3. Simple sequence repeat (SSR)

SSR, also called microsatellite or short tandem repeat (STR), is a repeating sequence of 1-6 base pairs of DNA. The repeated sequence consists of two, three, or four or more nucleotides (called di-, tri-, or tetra-nucleotide repeats, respectively) and can be repeated 10 to 100 times. The excision or addition in the number of tandemly repeated units is caused by the strand slippage during DNA replication (Agarwal et al., 2008). The DNA sequences flanking SSRs are conserved and can be used to design PCR primers that are used to amplify the intervening SSR.

SSR technology was first used in plants in 1992 by Akkaya for soybean genome analysis (Akkaya, 1992). Since then, it has become one of the most favorable molecular markers due to its multiallelic nature, reproducibility, co-dominant inheritance, high abundance and extensive genome coverage (Gupta and Varshey, 2000).

3.1.4. Amplified fragment length polymorphism (AFLP)

AFLP was developed by Vos et al. in 1995 by using virus, bacteria, yeast, and several plant DNAs including tomato, Arabidopsis, maize, cucumber, lettuce, and brassica. AFLP combines the advance of RFLP with the flexibility of polymerase chain reaction (PCR). The principle of AFLP is that the primer-recognition sequences (adaptors) are ligated to the restricted DNA fragments from a digest of total genomic DNA. The molecular genetic polymorphisms are identified by the presence or absence of the DNA fragments (Vos et al., 1995; Semagn et al., 2006; Agarwal et al., 2008). Usually, 50-100 restriction fragments can be amplified per assay and detected on a denaturing polyacrylamide gel. AFLP is considered as a novel molecular fingerprinting technology and extensively used in plant genome analysis for the development of high-resolution genetic maps and the positional cloning of genes of interest (Jones et al., 1997; Blears et al., 1998; Agarwal et al., 2008).

3.1.5. Sequence characterized amplified region (SCAR)

A SCAR marker is a DNA fragment that is identified from PCR amplification product (Semagn et al., 2006). A single locus that is identified by arbitrary marker analysis (RAPD) is cloned and sequenced and the two ends of the sequence is used to design specific primer pairs of 15-30 bp. Using such a primer pair, a single major band with the similar size of that cloned fragment can be amplified from the genome (Paran and Michelmore, 1993; Mcdermott et al., 1994). Polymorphism of SCAR markers can be either retained as presence or absence of the

band or appear as size polymorphisms as co-dominant marker. SCAR markers are usually developed to bridge the gap between the markers and the gene of interest in a short time. Since SCARs are defined based on other genetic marker system, they can be utilized as genetic markers and physical landmarks in the genome. The co-dominance character of SCARs is even more informative for genetic mapping (Staub et al., 1996; Semagn et al., 2006; Agarwal et al., 2008).

3.1.6. Target region amplification polymorphism (TRAP)

TRAP was developed based on the expressed sequence tag (EST) database information (Hu and Vick, 2003). Of its two primers, one is called the fixed primer that is designed from the targeted EST sequence in the database; the other one is arbitrary primer with either an AT- or GC- rich core for annealing to an intron or exon. The TRAP marker is useful for genotyping germplasm and generating markers linked to desirable agronomic traits for crop species (Hu et al., 2005). It is extensively applied for fingerprinting, estimating genetic diversity and QTL mapping (Hu et al., 2005; Liu et al., 2005; Alwala et al., 2006; Agarwal et al., 2008).

3.1.7 Cleaved amplified polymorphic sequence (CAPS)

CAPS (Cleaved amplified polymorphic sequence), known as PCR-RFLP marker, is a PCR based marker system that utilizes the DNA sequences from mapped RFLP markers (Konieczny and Ausubel, 1993; Konori and Nitta, 2005). The polymorphisms of CAPS are presented as the length of DNA fragments that are generated by the restriction digestion of PCR products with one or more restriction enzymes. The primers are synthesized from gene bank database, genomic or cDNA clones, or cloned RAPD bands (Staub et al., 1996; Semagn et al., 2006; Agarwal et al., 2008). The CAPS markers are co-dominant and locus specific and have been applied in many aspects including genotyping, positional or map based cloning, and

molecular identification studies (Konieczny and Ausubel, 1993; Weiland and Yu, 2003; Konori and Nitta, 2005; Spaniolas et al., 2006).

3.1.8 Single nucleotide polymorphism (SNP)

SNP was first developed for the Blue Cone Pigment (BCP) gene in human research (Jordan and Humphries, 1994). It was later applied for map-based cloning in *Arabidopsis* (Drenkard et al., 2000) and then developed rapidly for improving agronomic traits in crops (Semagn et al., 2006; Agarwal et al., 2008; Ayeh, 2008). A SNP is the DNA variation occurring at a single nucleotide (A-, T-, C-, or G-) in the genome within a species or even between paired chromosomes of an individual. SNPs can be detected in coding, and non-coding regions in the whole genome. The abundance of the polymorphic sites and wide distribution make SNP marker an attractive tool for gene identification, genotype fingerprinting, mapping, marker-assisted breeding, and map-based cloning (Gupta et al., 2001; Semagn et al., 2006; Agarwal et al., 2008).

3.2 Genetic mapping in tetraploid plant species

A large number of genetic linkage maps have been available for many diploid plants; however, mapping studies are much less advanced for polyploid species because of their complex inheritance (Luo et al., 2001, 2004). Recently, genetic mapping for some tetraploid species, such as alfalfa, cotton, and sour cherry have been reported (Canli, 2004; Rong et al., 2007; Robins et al., 2008).

3.2.1 Alfalfa

Alfalfa (*Medicago sativa* L.) (2n=4x=32) is an important perennial forage crop in North America. Several genetic linkage maps have been constructed using diploid alfalfa. The cultivated alfalfa is an autotetraploid and only a few genetic maps were published (Yu and Pauls, 1993; Brouwer and Osborn, 1999; Diwan et al., 2000). The first genetic linkage map of alfalfa

was reported in 1993 (Yu and Pauls, 1993). This map was constructed using a F_1 population and composed of 27 RAPD markers. Based on χ^2 analyses of co-segregation for the RAPD markers, only four linkage groups were identified in this study.

Later, the second genetic map was constructed by Brouwer and Osborn (1999), in which four coupling-phase co-segregation groups were detected to be homologous to eight linkage groups of the diploid alfalfa map except the linkage group 7. This map was composed of 88 RFLP loci on seven linkage groups covering 443 cM. Diwan et al. (2000) tried to introduce simple sequence repeat (SSR) markers into both diploid and tetraploid alfalfa populations on the basis of the existing F_2 diploid alfalfa RFLP map. Only 18 alleles from nine out of ten SSR loci were identified to be single-dose (simplex) alleles in the tetraploid population. Within the 18 alleles, just 8 alleles were associated with the loci belonged to three linkage groups. Both of maps were constructed by using MAPMAKER 3.0 and the marker order was generally conserved among those tetraploid and diploid linkage maps.

Since 2003, two genetic maps of tetraploid alfalfa have been constructed using the software TetraploidMap (Julier et al., 2003; Robins et al., 2008). Eight groups of homologous chromosomes per parent with four chromosomes per group were identified in both studies. However, the marker orders were different: Julier et al. (2003) observed the similar marker order to other maps constructed for tetraploid and diploid alfalfa, while Robins et al. (2008) found that TetraploidMap resulted in different marker order comparing with the ones created with JoinMap.

3.2.2 Cotton

Cotton (*Gossypium* ssp.) is the most important fiber crop in the world. Five cotton species have been confirmed as tetraploid (2n=4x=52). The first genetic map for tetraploid cotton was

constructed in 1994 (Reinisch et al., 1994) and 705 RFLP loci were mapped in 41 linkage groups with a total length of 4675 cM. Since then, many genetic maps have been constructed with different molecular markers in different populations (Mei et al., 2004; Rong et al., 2007; Lu et al., 2008; Yang et al., 2008). For example, 325 RFLP loci were positioned on the map constructed by Rong et al. (2007) using an F_2 population, while two genetic maps were constructed by Yang et al. (2008) using a BC_1 and a F_2 population containing 129 and 49 SSR loci, respectively. However, the marker order in different maps was inconsistent. A genome wide comprehensive reference map (CRM) was constructed by Yu et al. (2010) for a tetraploid cotton species and this CRM map contained 7424 markers, including SSR, RFLP, and AFLP markers, and represented 93% of the combined map information from 28 individual public cotton genetic maps.

3.2.3 Sour cherry

Sour cherry (*Prunus cerasus*), an allotetraploid species (AAFF, 2n=4x=32), is cultivated for small fruit production. It is one of the well-studied tetraploid *Prunus* species in genetic linkage mapping. The first published genetic map for sour cherry was constructed by Wang et al. (1998), in which two RFLP genetic maps were created for both parents. The RS ('Rheinische Schattenmorelle') linkage map was composed of 126 single-dose restriction fragment (SDRF) markers in 19 linkage groups and the total length was 461.6 cM, while the EB ('Erdi Botermo') linkage map consisted of 95 SDRF markers covering 279.2 cM in 16 linkage groups. Moreover, 13 sets of homologous linkage groups were identified based on 53 markers mapped in both parents by using JoinMap V2.0. In 2004, Canli developed a second generation genetic linkage map by adding new SSR markers. This map consisted of 161 markers, including 17 SSR markers and covered 442.4 cM in 19 linkage groups (Canli, 2004).

3.3 Application of molecular markers in *Prunus* species

The genus *Prunus* consists of more than 200 species of trees and shrubs. Many species in this genus are important producers of commercial fruits. In recent decades, molecular marker technology has been applied to many aspects of genetic research in the genus of *Prunus* including characterization of genotype identity and genetic relationships, genetic mapping, and marker-assisted selection (Martínez-Gómez et al., 2003, 2005).

3.3.1 Determination of genetic diversity

Traditional methods to identify plant species and cultivars are based on morphological and physiological traits. Molecular marker technology provides a powerful means to study the genetic diversity in plants. For instance, using microsatellites developed from a genomic DNA library of peach, polymorphism was detected in 25 peach cultivars (Aranzana et al., 2002). Wunsch and Hormaza (2002) employed a set of peach SSR primers for characterization of 76 sweet cherry (*P. avium*) genotypes and identified 68 corresponding unique cultivar genotypes and 2 closely related cultivars. Dangl et al. (2009) screened 18 almond (*P. dulcis*) cultivars grown in California with previously published 53 SSR loci from apricot, peach, and sweet cherry and selected 12 of the markers to identify those cultivars. Other molecular markers, such as RAPD, RFLP, AFLP, SCAR, have been also used to determine genetic diversity in other *Prunus* species including mei (*P. mume*), apricot (*P. armeniaca*), and plum (*P. domestica*) (Mariniello et al., 2002; Decroocq et al., 2004; Rohrer et al., 2004; Yang et al., 2008).

3.3.2 Genetic mapping

Several intra-specific and inter-specific genetic maps in the genus *Prunus* have been constructed since 1993 when the first genetic map was developed using RFLP marker for peach (Belthoff et al., 1993). So far, five genetic maps were constructed in peach using morphological

isozyme (Chaparro et al., 1994; Dirlewanger et al., 1998), RAPD (Dirlewanger and Bodo, 1994), SSR (Sosinski et al., 2000), and RFLP markers (Dettori et al., 2001); three genetic maps for almond using isozyme (Viruel et al., 1995) and other molecular markers (Joobeur et al., 2000; Jiang and Ma, 2003); molecular markers were also used for the map construction for apricot (Lamert et al., 2004; Dondini et al., 2007; Lalli et al., 2008), sweet cherry (Stockinger et al., 1996; Olmstead et al., 2008), and sour cherry (Wang et al., 1998; Canli, 2004). Among those maps, the T $\times$E genetic map is mostly used and considered as a reference map for genetic mapping in *Prunus* (Joobeur et al., 1998). This map was constructed using an interspecific F_2 population between almond 'Texas' and peach 'Earlygold' with 11 isozymes and 235 RFLP markers. Lambert et al. (2004) selected 88 RFLP probes and 20 SSR primers from this reference map to construct the map for apricot. The reference map was applied mostly in the research for comparative mapping (Dirlewanger et al., 2004a).

During the past decade, development of molecular markers associated with disease resistance genes has received more and more attention for many important crops. As reviewed by Cheong (2011), several approaches have been used to develop molecular markers linked to disease resistance in tree species, including isoenzymes, RFLPs, RAPDs, AFLPs, and SSRs. Sharka disease, caused by Plum pox virus (PPV), is one of the well-studied virus diseases in *Prunus*, especially in apricot and plum. A resistance map was constructed, in which resistance gene analogs (RGAs) were identified and mapped with the aid of peach BAC library and physical map. A total of 42 resistant regions were positioned into this map without the use of segregating populations (Lalli et al., 2005). Lalli et al. (2008) published a new genetic linkage map for apricot containing 357 loci and found a PPV resistance locus mapped in linkage group 1 and four AFLP markers co-segregating with the PPV resistance trait.

3.3.3 Marker-assisted selection

Marker-assisted selection (MAS) refers to the utilization of identified molecular markers to directly select a trait that is linked to the marker. These traits include resistance or tolerance to biotic and abiotic stresses, morphological characteristics, yield, and quality, and so on (Knapp, 1998). Utilization of MAS can greatly shorten the selection time, particularly, for fruit and other tree species because they have a long juvenile period and some traits, such as flower character and fruit quality do not appear until maturation phase (Martínez-Gómez et al., 2003, 2005). Different approaches have been used for marker-trait association analysis, including the utilization of mapping populations segregating for the traits of interest, bulked segregant analysis (BSA), and linkage disequilibrium (LD) (Testolin, 2003). Many important characters and QTLs have been identified and mapped in *Prunus* species, including flower blooming time (Ballester et al., 2001), self-incompatibility (Ballester et al., 1998; Tobutt et al., 2004), pollen-sterility (Hauck et al., 2002), fruit shape and quality (Dirlewanger et al., 1999), leaf color, pillar and weeping tree architecture (Abbott et al., 1998), and resistance to various pests and diseases, such as root-knot nematodes (Lu et al., 1998), powdery mildew (Dirlewanger et al., 1996), PPV (plum pox virus) resistance (Hurtado et al., 2002). For instance, using the high-density linkage map of peach constructed by Abbott et al. (1998), several agriculturally important traits that controlled fruit quality, tree architecture, and pest resistance were discovered in three segregating populations. Lu et al. (1996) used both RFLP and RAPD markers to screen a cross between peach cultivars 'Nemard' (resistant) and 'Lovell' (susceptible) to map genes for resistance to root-knot nematodes. Lecouls et al. (1999) used RAPD to detect markers linked to the *Ma1* root-knot nematode resistance gene in Myrobalan plum (*P. cerasifera*). A linkage map spanned 14.7 cM and three markers were identified. The nearest markers (OPAL 19_{720} and $OPA16_{1400}$) were

located at 3.7 and 6.7 cM on each side of the nematode resistance gene *Ma1* (Lecouls et al., 1999).

3.4 Bulked segregant analysis technology

Bulked segregant analysis (BSA) is the method used for rapidly identifying markers associated with the trait of interest, especially for monogenic qualitative trait. Two bulked pools are formed from the plant sources with a similar genetic background, but having either high or low expression of the trait. This technology was first developed for identification of markers linked to disease resistance in lettuce (Michelmore et al., 1991) and has been used to identify markers in fruit tree species. Lecouls et al. (1999) used BSA to distinguish RAPD markers linked to the *Ma1* gene for controlling a wide-spectrum resistance to root-knot nematodes for Myrobalan plum. The same strategy was applied to identify the markers linked to resistance to plum pox virus (PPV) in apricot (Salava et al., 2001). BSA technology is utilized not only for disease and pest resistance, but also for identifying markers linked to other traits. For instance, RAPD markers flanking the red-leaf (Gr) and malate dehydrogenase loci were distinguished in a F_2 peach population (Chaparro et al., 1994). Three RAPD markers were confirmed to be associated with a gene controlling delayed blooming time in almond (Ballester et al., 2001). Recently, one SSR marker (EMPaS02) was confirmed to be linked to self-compatibility and located at 3.2 cM on linkage group 3 for sweet cherry by using BSA method (Cachi and Wunsch, 2011).

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CHAPTER 2. GENETIC LINKAGE MAPS AND MOLECULAR MARKERS ASSOCIATED WITH X-DISEASE RESISTANCE IN CHOKECHERRY (*PRUNUS VIRGINIANA* L.)

1. Abstract

X-disease, caused by phytoplasmas, is one of the destructive diseases in stone fruit trees, causing yield loss and poor fruit quality. So far, no effective methods are available to control X-disease. X-disease resistance has been first discovered in chokecherry (*Prunus virginiana*) (2n=4x=32), which is a native woody species to North America. To identify molecular markers linked to X-disease resistance, simple sequence repeat (SSR) markers were used to construct genetic linkage maps and to identify markers associated with X-disease resistance in chokecherry. In this research, three segregating populations were developed by crossing one X-disease resistant line (C_L) with three susceptible chokecherry lines (a, c, and d). These three populations, namely $C_L \times a$, $C_L \times c$, and $C_L \times d$, contained 101, 177, and 82 progenies, respectively. A total of 354 pairs of SSR primers including 246 pairs developed using chokecherry genome sequence data and 108 pairs adopted from other *Prunus* species were used to screen individuals of all three populations. TetraploidMap and JoinMap were used to construct the genetic maps. Using TetraploidMap, 164, 148, and 149 loci were mapped on the map of $C_L \times a$, $C_L \times c$, and $C_L \times d$, respectively. Each map contained two sets of linkage groups, one for parent (C_L) and the other for parent (a, c, or d). Maps constructed using JoinMap were composed of 153, 148, and 132 loci on the three maps, respectively. Five markers (BPPCT002-2, BPPCT017-1, PS12A02-2, PS12A02-3, and PS12A02-4) were found to be associated with X-disease resistance. One SSR marker (C4136) co-segregating with the X-disease susceptible trait,

was identified using bulked segregant analysis (BSA) and mapped on the linkage group 14 of the map of $C_L \times a$ and on the linkage group 1 and 7 of the map of $C_L \times d$. Genetic maps and molecular marker identified in this research will further facilitate genetic research and breeding of X-disease resistance in chokecherry and other *Prunus* species.

2. Introduction

Chokecherry (*Prunus virginiana*), a woody species native to North America, is a small tree or large shrub widely distributed across the United States and Canada. Chokecherry plays an important role as habitats and food sources for wildlife. It is also an important tree species for windbreaks, watershed protection, and highway beautification. Chokecherry is not only used in small fruit production for beverages, jellies, dried fruit products, and wine, but also used as an ornamental plant because of the beautiful white flowers in spring and colorful leaves and fruits in fall. The purple-leaved cultivar (‘Schubert’ or ‘Canada Red’) is a popular landscape plant. Chokecherry can be divided into two varieties, the eastern chokecherry (*P. virginiana* var. virginiana) and the western chokecherry (*P. virginiana* var. demissa) (Dirr, 1998). This special tree species belongs to the *Prunus* genus in the Rosaceae family, in which many species are victims of phytoplasma diseases. The genus *Prunus* is comprised of more than 200 species of trees and shrubs including many important stone fruit species, such as peach, sweet cherry, sour cherry, nectarine, apricot, plum, etc. Chokecherry has the same base chromosome number as other *Prunus* species (x = 8); however, it is a tetraploid, having 32 chromosomes (2n = 4x = 32).

X-disease is the one of destructive diseases caused by phytoplasmas on stone fruit plants. Phytoplasmas are known to cause diseases in more than 300 higher plant species including many economically important food, fiber, forage, fruit, and ornamental plants (Lee et al., 1992). The

pathogen of X-disease belongs to the RFLP group 16SrIII of phytoplasma, which is the second largest group after AY (aster yellow) (Bertaccini 2007; Olivier et al., 2009). X-disease was first found on cherry and peach in 1931 and 1933 in California and Connecticut, respectively (Ogawa, 1991). It is one of the limiting factors for production of many major *Prunus* species (Rosenberger, 1982), and is particularly devastating to peaches, cherries, nectarines, and chokecherries (Gilmer et al., 1954; Rosenberger and Jones, 1977; Peterson, 1984). In the genus of *Prunus*, chokecherry is considered as the source of X-disease since it is an important reservoir host of leafhopper, by which the X-disease phytoplasma is vectored. The research for the X-disease on chokecherry has been focused on the characterization and diagnosis of X-disease pathogen by using polyclonal antibody, monoclonal antibody (Guo et al., 1998a), and molecular methods (Guo et al., 1996; 1998b; Guo and Cheng, 2000). Development of resistant genotypes offers an excellent disease management option. Unfortunately, breeding resistant cultivars for tree species using conventional methods is not only time- and labor-consuming, but also less predictable due to their highly heterozygous genetic background, long juvenile stage, long life cycle, and limited genetic information available.

Since the 1980s, when DNA-based markers were utilized on plant breeding, the advances in molecular genetics, genomics, and biotechnology have greatly enhanced our understanding of the structure and function of plant genomes (Agarwal et al., 2008). Within the molecular markers, SSR (Simple sequence repeat) has become the most favorable due to its multi-allelic nature, reproducibility, co-dominant inheritance, high abundance, and extensive genome coverage (Gupta and Varshey, 2000).

Several intra-specific and inter-specific genetic maps in the genus *Prunus* have been constructed since 1993 when the first genetic map was developed for peach (Belthoff et al., 1993). So far, genetic maps have been constructed for peach (Dettori et al., 2001), almond (Viruel et al., 1995), almond × peach (Jiang and Ma, 2003), apricot (Dondini et al., 2007), sweet cherry (Olmstead et al., 2008), and sour cherry (Cali, 2004) using RAPD, RFLP, AFLP, and SSR markers. Many important characters and QTLs have been identified and mapped in *Prunus* species, including flower blooming time (Ballester et al., 2001), self-incompatibility (Tobutt et al., 2004), pollen-sterility (Hauck et al., 2002), fruit shape and quality (Dirlewanger et al., 1999), leaf color, pillar and weeping tree architecture (Abbott et al., 1998), and resistance to various pests and diseases, such as root-knot nematodes (Lu et al., 1998), powdery mildew (Dirlewanger et al., 1996), and sharka (Hurtado et al., 2002). A saturated genetic linkage map for *Prunus* was constructed using an interspecific cross between almond (cv Texas) and peach (cv Earlygold), designated the T × E map (Joobeur et al., 1998), which is treated as a reference genetic map for the *Prunus* genus and is being added to with more markers from peach and other species (Dirlewanger et al., 2004b). Many major trait loci affecting agronomic characters including fruit flesh and flower color, insect and disease resistance, morphology, and fertility, etc. identified in various *Prunus* species have been anchored on the T × E map (http://www.bioinfo.wsu.edu/gdr/).

A large number of genetic linkage maps have been available for many diploid plants; however, mapping studies are much less advanced for polyploid species because of their complex inheritance (Luo et al., 2001, 2004). Therefore, the majority of linkage maps for polyploid species were constructed using their diploid relatives. The first genetic map constructed for a polyploid species was published by Al-Janabi et al. (1993) using single dose fragment (SDF)

markers (Wu et al., 1992). Recently, genetic mapping for some tetraploid species, such as alfalfa (Robins et al., 2008), cotton (Rong et al., 2007), and sour cherry (Cali, 2004) have been reported. Generally, JoinMap and TetraploidMap are the two major software programs used for map construction of the tetraploid species. TetraploidMap, a powerful computer program, has been developed for constructing a linkage map for the autotetraploid species based on the dominant and co-dominant marker information scored in two parents and their full-sib progeny (Hackett and Luo, 2003). It was used to infer the parental genotypes, identify possible double reductants, assign linkage groups, and to estimate recombination frequencies and accompanying LOD scores. TetraploidMap has been currently extended to TetraploidMap for Windows that is considerably enhanced from its original function of constructing a linkage map and has a new function of performing QTL interval mapping (Hackett et al., 2007). A Window-based interface facilitates data entry and exploration. This software has proved suitable for linkage and QTL analysis in potato (Bradshaw et al., 2004) and alfalfa (Robin et al., 2008). However, no genetic linkage map has been constructed for chokecherry and no molecular markers associated with X-disease resistance have been identified.

The objective of this study was to construct a genetic map for chokecherry and identify SSR markers linked to X-disease resistance. Identification of informative SSR markers may be a benefit to comparative mapping studies in *Prunus* genus. Elucidation of inheritance of X-disease resistance and identification and mapping of genes linked to X-disease resistance will provide an efficient method of marker assisted selection (MAS) to assist breeding/selection of stone fruit trees resistant to X-disease and possibly to other phytoplasma-associated diseases.

3. Materials and methods

3.1 Plant material

3.1.1 Development of segregating populations

3.1.1.1 Selection of parents

To develop highly segregated chokecherry populations in X-disease resistance, crosses were conducted between resistant and susceptible parents. The parental chokecherry lines were selected from a large chokecherry germplasm collection that was established in 1983 by the U.S. Dept. of Agriculture Natural Resources Conservation Service (NRCS) Plant Materials Center in Bismarck, ND and Pierre, SD. These selected lines were rated as highly resistant (5) or susceptible (1 and 2). Three populations were developed from crosses between one resistant, R-II-2010-3 (C_L) and three susceptible, S-V-2077-3 (a), S-I-2009-2 (c), and S-II-3674-1 (d) chokecherry lines in the Bismarck planting.

3.1.1.2 Hybridization

Hybridization was done in May of 2005, 2007, and 2008. Pollen was collected in advance by forcing flowers to bloom indoors and the pollen was stored in a refrigerator (4 ^{o}C) until hybridization. Flowers were emasculated and bagged before they opened. At the early flower stage, emasculated flowers were pollinated twice during one week period and bagged again. The paper bags were removed two weeks after the second pollination. Fruits were harvested and de-pulped in July to August. Hybrid seeds were dried on a paper towel under room temperature and stored in a sealed bottle in a refrigerator (4 ^{o}C).

3.1.1.3 Seed germination

Dried seeds were mixed with moist “Sunshine Mix” (Sun Gro Horticulture Canada Ltd.) in zipper bags and kept in a refrigerator under 4 ℃ for 3 to 4 months for stratification. In the following spring, the seeds were sowed in plastic trays filled with “Sunshine Mix” in the greenhouse. After germination, seedlings were potted in the 3-in containers and then repotted to the 6-in containers at the late growing season. All seedlings were moved from the greenhouse to the outside in late October for natural acclimation before they were moved to the cooler in late November for cold treatment. All chokecherries were moved from the cooler to the greenhouse in the following May.

3.2 X-disease inoculation

3.2.1 Inoculums

Fresh chokecherry branches with typical X-disease symptoms were collected from diseased trees no more than 2 h before grafting inoculation. The strain of the X-disease phytoplasma was determined by sequencing the product of Nested PCR described in 3.2.3.

3.2.2 Grafting inoculation

Side grafting was applied to inoculate chokecherry seedlings with X-disease pathogen in August. Scions (3-5 cm long) with 1-2 buds and two leaves were prepared. One end of the scion was cut into a wedge shape (1-2 cm long). A slanting cut with angle in a downward direction was made near the base of the stem of the rootstock (seedling) as soon as possible after the scion was ready. The scion was inserted into the cut with the thicker side facing the interior of the rootstock so that the phloem of both the scion and rootstock contacted with each other. The scion was tight with a plastic tape. Two scions were employed for each seedling to ensure the success

of inoculation. Each graft was covered with a plastic bag to keep moisture and kept out of light with a paper bag. After 3 to 4 weeks, bags were removed.

3.2.3 Confirmation of inoculation

Nested PCR were used to confirm the successful transmission of X-disease phytoplasma from the scion to the rootstock based the method of Guo et al. (2000). The DNA was extracted from leaves, main veins, and roots separately based on the method described in 3.4.1. Tissue samples were collected every week beginning from the second week after grafting and ending in the 8th week.

Two pairs of PCR primers were used:

(1) Universal primer:

R16 F2: 5'—ACGACTGCTGCTAAGACTGG—3'

R16 R2: 5'—TGACGGGCGGTGTGTACAAACCCCG—3'

(2) X-disease phytoplasma-specific primer:

R16 (III) F2: 5'—AAGAGTGGAAAAACTCCC—3'

R16 (III) R1: 5'—TCCGAACTGAGATTGA—3'

Each PCR reaction consisted of 20 ng of DNA, 1.5 mM $MgCl_2$, $10\times$buffer, 200 µM dNTP, 0.2 pM each primer, and 0.125 U *Taq* DNA polymerase in a final volume of 25 µl. The amplification conditions consisted of denaturing for 1 min at 94 ℃ (2 min for the first cycle), annealing for 3 min at 46 ℃, and extension for 3 min at 72 ℃ (10 min for the final cycle) for 35 cycles. The product of the first PCR was diluted 25-fold with distilled water, and 1 µl of the diluted solution was used as template DNA for the second PCR with the same amplification conditions. The product of the second PCR (10 µl) was examined by electrophoresis through a

1% agarose gel and visualized under UV light after staining in the 0.5 μg/ml ethidium bromide solution. Gel images were captured using software "Alphalmager". Chokecherry seedlings were graft-inoculated again if unsuccessful inoculation occurred based on the result of nested PCR.

3.3 Screening of X-disease resistance

X-disease resistance for population $C_L \times a$ was evaluated in 2008, 2009, 2010, and 2011. Screening of X-disease resistance for populations $C_L \times c$ and $C_L \times d$ were performed in 2010 and 2011. X-disease severity was rated at a scale of 0-5 based on the X-disease symptom and tree vigor including discolored leaves (usually red), stunted foliage and shoots, and reduced plant vigor (length of current year shoots): 0 = whole plant died; 1 =leaves were discolored, most shoots and leaves were very stunted, and very low growth vigor; 2 =leaves were discolored, most shoots and leaves were stunted, and low growth vigor; 3 =leaves were discolored, most shoots and leaves were moderately stunted, moderate growth vigor; 4 = all or part of tree with slight symptoms, high growth vigor; and 5 = no symptoms, high growth vigor.

3.4 Construction of genetic linkage maps

3.4.1 Genomic DNA extraction

Genomic DNA was extracted following the method developed for woody plants in Dr. Dai's lab in the Department of Plant Sciences at North Dakota State University in Fargo, ND. In brief, fresh leaf or root tissue (100-200 mg) was ground in the presence of liquid nitrogen with a mortar and pestle and transferred to a 1.5 ml Eppendorf tube. Pre-heated (600 μl, 65 ℃) extraction buffer that contained 2% CTAB, 0.1 M Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaOH, and 20 mM *β*-mercaptoethanol (added just before use) and 10 mg PVP (Polyphenolpyrollidine) were added to each tube. The tube was incubated at 65 ℃ for 60 min

and shaken every 10 min. After incubation, 600 μl 24:1 solution of chloroform: isoamyl alcohol was added, mixed gently, and centrifuged at 6000 rpm for 15 min at room temperature. The top aqueous phase (about 600 μl) was transferred to a new 1.5 ml Eppendorf tube and emulsified again with 600 μl of 24:1 solution of chloroform: isoamyl alcohol followed by another 15 min of centrifugation at 6000 rpm. The upper phase was transferred to a new 1.5 ml Eppendorf tube and 1/10 volume of 5 M NaCl and 2 volumes of cold 95% ethanol were added. The tube was placed in a freezer (-20 ℃, > 2 h) for DNA precipitation. Precipitated DNA was collected by centrifugation at 12000 rpm for 15 min at room temperature. The pelleted DNA was washed with 70% ethanol in a slow moving shaker for 3-5 h. After another 5 min centrifugation at 12000 rpm, the pellet was washed with cold 70% ethanol and dried at room temperature before being dissolved in 600 μl TE buffer. To remove contaminated RNA and proteins, DNA was digested with 6 μl of RNase A (10 mg/ml) for 60 min and then 6 μl of Proteinase K (1mg/ml) for another 15 min at 37 ℃. The treated DNA was purified using the same volume of 1:1 solution of chloroform: phenol and then pure chloroform. At last, the DNA was precipitated with 1/10 volume of 5 M NaCl and 2 volumes of 95% ethanol in -20 ℃ for > 2 h and then collected by centrifugation at 12000 rpm for 15 min at room temperature. The DNA pellet was rinsed with 70% ethanol, dried, and dissolved in 100 ml of TE buffer. The DNA concentration was determined using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc.) and stored in a refrigerator (4 ℃) until use.

3.4.2 SSR analysis

3.4.2.1 Primers

Primers used in this research came from: 1) other *Prunus* species; 2) designed based on the chokecherry genome sequences.

3.4.2.1.1 Primers from other *Prunus* species

A total of 108 pairs of SSR primers were obtained from six *Prunus* species posted on http://www.bioinfo.wsu.edu/gdr/, the Genome Database for Rosaceae (GDR) website. A total of 45 SSR primers were generated from peach (*P. persica*); 20 from sweet cherry (*P. avium*); 15 from Japanese plum (*P. salicina*); 11 from apricot (*P. armeniaca*); 15 from almond (*P. amygdalus*); and 2 from sour cherry (*P. cerasus*).

3.4.2.1.2 Primers from chokecherry genome sequence

Approximately 20 μg of genomic DNA extracted following the method described in 3.4.1 from chokecherry roots was sent to the Center for Genetic, Proteomic, and Bioinformatic Research at University of Hawaii-Manoa for library preparation and sequencing using the 454 Genome Sequencer FLX Titanium (Roche Applied Science) following emulsion polymerase chain reaction (emPCR). Newbler was used to analyze the sequencer-generated SFF data and assemble the reads into contigs in FASTA format files via a command line (runAssembly), which was kindly provided by Dr. Zheng Jin Tu at the Supercomputing Institute for Advanced Computational Research at the University of Minnesota. All contigs or sequences longer than 100 bp were searched for microsatellites using the software SSRIT (Simple Sequence Repeat Identification Tool) available at www.gramene.org/db/markers/ssrtool. The minimum number of repeat motifs to be considered microsatellites was 12 repeats for a mononucleotide motif and

more than five repeats for the 2-5 bp-nucleotide motifs. Primers were then designed using the online software Primer3 (http://frodo.wi.mit.edu/primer3/).

3.4.2.2 PCR amplification

An 18 μl sample of PCR reaction consisted of 60 ng of template DNA, 2.0 mM $MgCl_2$, 10× buffer, 200 μM dNTP, 0.2 pmol of each primer, and 0.125 U *Taq* DNA polymerase. The amplification was carried out under the condition of denaturing for 30 seconds at 94 ℃ (5 min for the first cycle), annealing for 30 seconds at the temperature ranged from 56 ℃ to 61 ℃, and extension for 30 seconds at 72 ℃ (7 min for the final cycle) for 35 cycles. The PCRs were performed on Programmable Thermal Controller PTC-100™ and Applied Biosystems 2720 Thermal Cycler.

3.4.2.3 Gel electrophoresis

Electrophoresis was carried out through two analysis systems. The products amplified by primers developed from other *Prunus* species were examined on non-denaturing 6% polyacrylamide gels in 1×TBE at 60 voltage (V) for 1 h. Gels were then stained using 0.001% GelRed (Biotium, Inc., Hayward, CA) for 20 min and scanned using a Typhoon 9410 variable mode imager (GE Healthcare, Inc. Waukesha, WI). PCR products amplified using chokecherry primers were separated in a non-denaturing 6% polyacrylamide gel (29:1 acrylamide:bis, J.T. Baker, Mallinckrodt Baker, Inc. NJ). The gel was prepared as follows. A mixture of 8.5 ml 10× TBE buffer, 25.5 ml acrylamide (29:1), 136 ml ddH_2O, 0.12 mg APS (Ammonium Persulfate), and 130 μl TEMED (Tetramethylenediamine) was poured in between two glass plates for polymerization about 50 min and then pre-ran at 350 V in 0.5×TBE buffer for 1 h so that the ethidium bromide can migrate from the buffer into the gel. Samples of PCR products were

loaded into the gel and ran at 250 V for 3 h. The gel was visualized under UV lights and images were captured using software “Alphalmager”.

3.4.3 Scoring, χ^2 analysis and map construction

The polymorphic bands were scored for the absence or presence in the mapping populations. Only informative markers that qualified to be SDRFs (Single Dose Restriction Fragments) at the 5% level were included in this research. It included the fragments presented in one or both parents [(+--- ×----), (---- ×+---), or (+--- ×+---)] with segregation ratio of 1:1, 1:1, or 3:1, respectively] (Canli, 2004).

TetraploidMap and JoinMap software programs were used for construction of chokecherry genetic linkage maps. For the maps constructed using TetraploidMap, genotypes of progenies were scored as 1, 0, and 9 for present, absent, and missed band. The function “Cluster” was used to create linkage groups and “16” was used based on the chromosome number of chokecherry. In each homologous linkage group, marker orders were calculated by combining a two-point linkage analysis with initial-run and Ripple ordering. Linkage phase of markers were considered when LOD (logarithom of odds) scores were larger than 2 (Luo et al., 2001).

The code “CP” was used as the population code when using JoinMap version 4.0 (Stam, 1993) to construct genetic linkage maps. Genotypes of progenies were coded as “ll” and “lm” for the loci heterozygous in the first parent and “nn” and “np” for the loci heterozygous in the second parent. Linked loci were grouped if LOD (logarithom of odds) values were larger than 3. Regression mapping algorithm was used for marker order determination, while map distances were calculated using “Kosambi” map function. Each population was examined separately and maps for the common parent of three populations (C_L) were merged according to the common

markers shared by two or three maps. To merge the linkage groups, "Combine Groups for Map Integration" function was applied and the map function was changed to be "Haldane's" map function with default parameters.

All linkage maps were drawn by the MapChart 2.2 program (Voorrips, 2002).

3.5 Identification of markers linked to X-disease

Bulked segregant analysis (BSA) was performed on the population of $C_L \times a$ because a stable phenotyping result was drawn from four years observations of X-disease resistance. Two resistant bulks and four susceptible bulks were developed by pooling the DNA from ten X-disease resistant (rated as 5) or susceptible (rated as 1 or 2) individuals into each bulk. All SSR primers were used to screen the parents (C_L and a) and bulks. PCR was performed under the same condition as the one for primer analysis described in 3.4.2.2. Amplified products were separated in a non-denaturing 6% polyacrylamide gel and visualized under UV light. Gel images were captured using software "Alphalmager".

At the same time, an analysis for identification of the markers linked to X-disease resistance was performed using the software TetraploidMap using "Marker" function following the software instruction (Luo et al., 2001). Kruskal-Wallis test and ANOVA test were performed for marker identification and both of them are significant with $p < 0.05$.

4. Results

4.1 Development of mapping populations

Hybrid fruits were collected in August when fruits completely matured. Fruits were de-pulped, dried, and stored in a sealed bottle in a 4 ^{o}C refrigerator. Seeds were germinated in the greenhouse after stratification. A total of 101, 177, and 82 hybrid seedlings were obtained from

crosses of $C_L \times a$, $C_L \times c$ (or $c \times C_L$), and $C_L \times d$, respectively. All seedlings were grown in the greenhouse until they were graft-inoculated with X-disease pathogen, then moved to outside of the greenhouse for the remaining growing season.

4.2 Genotyping of the populations using SSR analysis

A total of 108 pairs of primers developed from other *Prunus* species were tested in chokecherry (Table 2.1). Of which, 93 (86.1%) pairs had amplification products. Primers that produced polymorphic bands between parents with 1:1 segregating ratio in progenies and the primers had the same band pattern in both parents with 3:1 segregating ratio in progenies were selected to screen all three populations. As a result, 73 of 93 primers (78.5%) were detected to be qualified for map construction (Table 2.2). A total 246 SSR primers were developed from chokecherry genome sequences (for details, see Chapter 3, 4.1 and 4.2). Of which, 212 primers had PCR products and amplifiable in chokecherry population. Markers amplified from 116 chokecherry primers were qualified to be SDRF and have been mapped in the three populations (Table 2.3). In summary, a total of 354 primers were tested and 189 primers produced markers for map construction in which 134, 134, and 109 primers amplified polymorphic bands that led to identification of 232, 240, and 195 SSR marker loci for $C_L \times a$, $C_L \times c$, and $C_L \times d$ populations, respectively (Table 2.2 and 2.3). An average of 1.7, 1.8, and 1.8 loci per primer set was identified in the three populations, respectively.

Differences in the band patterns were observed between different parent combinations among different populations (Fig. 2.1).

Fig. 2.1. A gel image of chokecherry primer screening. M = 100 bp ladder; 1= C_L; 2= a; 3= c; 4= d; 5-8= individuals in population $C_L \times c$.

Table 2.1. SSR primers used for map construction in chokecherry.

Source species	Primer name	Tested	Amplified	Mapped	% on map	Reference
Peach	BPPCT	41	32	30	71	Dirlewanger et al., 2002
	pchgms	3	3	3	100	Sosinski et al., 2000
Sweet cherry	UCD-CH	15	15	12	80	Struss et al., 2003
	PS	4	3	3	75	Downey and Iezzoni, 2000; Joobeur et al., 2000
	PMS	1	1	1	100	Cantini et al., 2001
Sour cherry	PceGA	2	2	2	100	Cantini et al., 2001
	[AB]	1	1	0	0	Downey and Iezzoni, 2000
Plum	CPSCT	15	14	10	67	Mnejja et al., 2004
Apricot	Pac	9	7	3	33	Decroocq et al., 2003
	aprigms	2	2	1	50	Howad et al., 2005
Almond	CPDCT	8	8	3	50	Mnejja et al., 2005
	UDA	7	5	5	71	Testolin et al., 2004
Chokecherry	C	246	212	116	45	
Total		354	305	189		

Table 2.2. Information for SSR primers from other *Prunus* species that produced polymorphic bands in chokecherry.

Source species	Primer name	Sequences 5' -3'	Annealing temperature	Polymorphism in three populations
Prunus persica	BPPCT001	AATTCCCAAAGGATGTGTATGAG CAGGTGAATGAGCCAAAGC	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	BPPCT002	TCGACAGCTTGATCTTGACC CAATGCCTACGGAGATAAAAGAC	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	BPPCT005	GCTAGCAGGGCACTTGATC ACGCGTGTACGGTGGAT	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	BPPCT006	GCTTGTGGCATGGAAGC CCCTGTTTCTCATAGAACTCACAT	57 ℃	$C_L \times a$, $C_L \times d$
	BPPCT007	TCATTGCTCGTCATCAGC CAGATTTCTGAAGTTAGCGGTA	57 ℃	$C_L \times a$, $C_L \times c$
	BPPCT008	ATGGTGTGTATGGACATGATGA CCTCAACCTAAGACACCTTCACT	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	BPPCT009	ATTCGGGTCGAACTCCCT ACGAGCACTAGAGTAACCCTCTC	57 ℃	$C_L \times a$, $C_L \times d$
	BPPCT010	AAAGCACAGCCCATAATGC GTACTGTTACTGCTGGGAATGC	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	BPPCT012	ACTTCCATTGTCAGGCATCA GGAGCAACGATGGAGTGC	57 ℃	$C_L \times a$, $C_L \times c$
	BPPCT013	ACCCACAAATCAAGCATATCC AGCTTCAGCCACCAAGC	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	BPPCT014	TTGTCTGCCTCTCATCTTAACC CATCGCAGAGAACTGAGAGC	57 ℃	$C_L \times a$
	BPPCT015	ATGGAAGGGAAGAGAAATCG GTCATCTCAGTCAACTTTTCCG	57 ℃	$C_L \times c$
	BPPCT016	GATTGAGAGATTGGGCTGC GAGGATTCTCATGATTTGTGC	57 ℃	$C_L \times a$, $C_L \times d$

Table 2.2. Continued.

Source species	Primer name	Sequences 5' -3'	Annealing temperature	Polymorphism in three populations
Prunus persica	BPPCT017	TTAAGAGTTTGTGATGGGAACC AAGCATAATTTAGCATAACCAAGC	57 ℃	$C_L \times a$, $C_L \times c$
	BPPCT018	CTCAACTGCTGTCCTCACTTC CATGTCTGATCCTAACCCCA	57 ℃	$C_L \times a$, $C_L \times d$
	BPPCT019	TGATACCACCATCCAATCTAGC TTGCTGGGACATGGTCAG	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	BPPCT021	TGCATGAGAAACTTGTGGC CCAAGAGCCTGACAAAGC	57 ℃	$C_L \times a$, $C_L \times d$
	BPPCT024	GAGGAATGTGCCTCTTCTGG CTCCCGTACGCGTTTACC	57 ℃	$C_L \times c$, $C_L \times d$
	BPPCT025	TCCTGCGTAGAAGAAGGTAGC CGACATAAAGTCCAAATGGC	57 ℃	$C_L \times a$, $C_L \times c$
	BPPCT026	ATACCTTTGCCACTTGCG TGAGTTGGAAGAAAACGTAACA	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	BPPCT027	CTCTCAAGCATCATGGGC TGTTGCCCGGTTGTAATATC	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	BPPCT028	TCAAGTTAGCTGAGGATCGC GAGCTTGCCTATGAGAAGACC	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	BPPCT030	AATTGTACTTGCCAATGCTATGA CTGCCTTCTGCTCACACC	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	BPPCT031	CTGGGGAGAAGAAGTGGC GCTTTCATGCCACCTCTCTA	57 ℃	$C_L \times d$
	BPPCT032	TTAAGCCACAACATCCATGAT AATGGTCTAAGGAGCACACG	57 ℃	$C_L \times a$, $C_L \times d$
	BPPCT036	AAGCAAAGTCCATAAAAACGC GGACGAAGACGCTCCATT	57 ℃	$C_L \times a$, $C_L \times c$

Table 2.2. Continued.

Source species	Primer name	Sequences 5' -3'	Annealing temperature	Polymorphism in three populations
Prunus persica	BPPCT037	CATGGAAGAGGATCAAGTGC CTTGAAGGTAGTGCCAAAGC	57 ℃	$C_L \times c$
	BPPCT039	ATTACGTACCCTAAAGCTTCTGC GATGTCATGAAGATTGGAGAGG	57 ℃	$C_L \times a$, $C_L \times d$
	BPPCT040	ATGAGGACGTGTCTGAATGG AGCCAAACCCCTCTTATACG	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	BPPCT042	AACCCTACTGGTTCCTCAGC GACCAGTCCTTTAGTTGGAGC	57 ℃	$C_L \times c$,
	pchpgms1	GGGTAAATATGCCCATTGTGCAATC GGATCATTGAACTACGTCAATCCTC	57 ℃	$C_L \times a$,
	pchpgms2	GTCAATGAGTTCAGTGTCTACACTC AATCATAACATCATTCAGCCACTGC	57 ℃	$C_L \times a$,
	pchpgms3	ACGCTATGTCCGTACACTCTCCATG CAACCTGTGATTGCTCCTATTAAAC	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
Prunus avium	UCD-CH10	TCACGAGCAAAAAGTGTCTCTG CACTAACATCTCTCCCCTCCC	52 ℃	$C_L \times a$, $C_L \times c$
	UCD-CH11	TGCTATTAGCTTAATGCCTCCC ATGCTGATGTCATAAGGTGTGC	52 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	UCD-CH12	AGACAAAGGGATTGTGGGC TTTCTGCCACAAACCTAATGG	52 ℃	$C_L \times c$, $C_L \times d$
	UCD-CH13	ACCCGCTTACTCAGCTGAAC TTAGCACTAAGCCTTTGCTGC	52 ℃	$C_L \times d$
	UCD-CH14	GTACACGGACCCAATCCTG TCTAACATCATGTTAAACATCG	52 ℃	$C_L \times a$, $C_L \times d$
	UCD-CH15	TCACTTTCGTCCATTTTCCC TCATTTTGGTCTTTGAGCTCG	52 ℃	$C_L \times a$, $C_L \times d$

Table 2.2. Continued.

Source species	Primer name	Sequences 5' -3'	Annealing temperature	Polymorphism in three populations
Prunus avium	UCD-CH16	ATCACAAGGCAGACTGGTCC CTTAAACTTCAACAAGTTCAGG	52 ℃	$C_L \times a$, $C_L \times c$
	UCD-CH17	TGGACTTCACTCATTTCAGAGA ACTGCAGAGAATTTCCACAACCA	52 ℃	$C_L \times a$, $C_L \times c$
	UCD-CH19	GTACAACCGTGTTAACAGCCTG ACCTGCACTACATAAGCATTGG	52 ℃	$C_L \times a$, $C_L \times c$,
	UCD-CH21	TTGTTGACCATCGAATATGAAG GAAGGTACATGGCGTGCC	52 ℃	$C_L \times c$,
	UCD-CH31	TCCGCTTCTCTGTGAGTGTG CGATAGTTTCCTTCCCAGACC	52 ℃	$C_L \times a$, $C_L \times c$,
	UCD-CH39	CACTGTCTCCCAGGTTAAACTC CCTGAGCTTTTGACACATGC	52 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	PS7a2	CAGGGAAATAGATAAGATG TCTAATGGTGGTGTTCATT	57 ℃	$C_L \times a$, $C_L \times c$
	PS08E08	CCCAATGAACAACTGCAT CATATCAATCACTGGGATG	57 ℃	$C_L \times a$,
	PS12A02	GCCACCAATGGTTCTTCC AGCACCAGATGCACCTGA	57 ℃	$C_L \times a$, $C_L \times c$
	PMS67	AGTCTCTCACAGTCAGTTTCT TTAACTTAACCCCTCTCCCTCC	57 ℃	$C_L \times a$, $C_L \times c$
Prunus cerasus	PceGA34	GAACATGTGGTGTGCTGGTT TCCACTAGGAGGTGCAAATG	57 ℃	$C_L \times c$,
	PceGA59	AGAACCAAAAGAACGCTAAAATC CCTAAAATGAACCCCTCTACAAAT	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
Prunus salicina	CPSCT002	CATGTGCCTCAATGCATCTT CGGCCCACAAAATTGAACTA	62 ℃	$C_L \times a$, $C_L \times c$

Table 2.2. Continued.

Source species	Primer name	Sequences 5' -3'	Annealing temperature	Polymorphism in three populations
Prunus salicina	CPSCT004	GCTCTGAAGCTCTGCATTGA TTTGAAATGGCTATGGAGTACG	62 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	CPSCT006	ACAAAACCAAGCACCGTCTC GGGCAAATGCTTACCTGTTC	62 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	CPSCT007	GTGGCCGGACGAGAGAAC CGATCGAATGAAGCTCAGTG	62 ℃	$C_L \times c$,
	CPSCT010	TTGGGTAAATACTTTATCATTTCC TCCCTGAATAAGGGTTGTGC	62 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	CPSCT011	ATTTGGGTTTGCGACTCAAG ACTCATCCCTTGCCCTTTCT	62 ℃	$C_L \times c$, $C_L \times d$
	CPSCT012	ACGGGAGACTTTCCCAGAAG CTTCTCGTTTCCTCCCTCCT	62 ℃	$C_L \times a$, $C_L \times c$
	CPSCT018	AGGACATGTGGTCCAACCTC GGGTTCCCCGTTACTTTCAT	62 ℃	$C_L \times c$, $C_L \times d$
	CPSCT021	GCCACTTCGGCTAAAAGAGA TCCATATCTCCTCCTGCTTGA	62 ℃	$C_L \times a$
	CPSCT022	TGTCTGCCTCTCATCTTAACCA TTCTTGAGCAGCCCATCTTCT	62 ℃	$C_L \times c$,
Prunus armeniaca	PacA10	TGAGCATAATTGGGGCAG GCCAGAGAAGCCATTTCAGT	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	PacB35	ATTGCGATTTCGGTCTGTT CCATCCCAAATTGCTTACTT	57 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	PacC13	GCTTGCTGCTCATCATTTAC AATAACAACCATATTGGAGTATTTAC	57 ℃	$C_L \times a$, $C_L \times c$
	aprigms18	TCTGAGTTCAGTGGGTAGCA ACAGAATGTGCGTTGCTTTA	58 ℃	$C_L \times d$

Table 2.2. Continued.

Source species	Primer name	Sequences 5' -3'	Annealing temperature	Polymorphism in three populations
Prunus armeniaca	CPDCT006	GTCCTGCTGCCAGCTTCTCT GGTTTAGCGCAAAAGCTTCA	62 ℃	$C_L \times c$
	CPDCT008	GAAGCAGCCATTCCTAGTGC TGTTTATGGACCTTAGTAGTCTGG	62 ℃	$C_L \times a$, $C_L \times c$, $C_L \times d$
	CPDCT012	CAGACCGTCGTGTTGAAGTC GACCCGAATCGGACTTGTAA	62 ℃	$C_L \times a$
	UDA002	AAACGTGAGGTCTCACTCTCTC GCCATTTAAGGGTCTGGTCA	57 ℃	$C_L \times a$, $C_L \times c$
	UDA005	CATCACACACAAACACAAATGC GCATTGTGCTCTTCATGGAC	57 ℃	$C_L \times a$, $C_L \times c$,
	UDA006	ATTCTCCAAGGCGATAAGCA TTAGGCACCTGTCCCCTACA	57 ℃	$C_L \times a$
	UDA008	AGACGCTTTGCATACATACAAGT TGCAGGAACTGGGATTAGAGA	57 ℃	$C_L \times a$
	UDA009	AAAACATCTCTCTCCTCCATGC AGTTCTCTGGCAGCACAAGC	57 ℃	$C_L \times c$, $C_L \times d$

Table 2.3. Information for chokecherry SSR primers that produced polymorphic bands in chokecherry.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')	Polymorphism in three populations
C162	ga	6	226	GGTTGTGGTGGTGAGAGGTA TCAGCTGGATTAATGGCTCT	$C_L \times a$
C324	tta	5	293	TTTATGGTTCCCAGGCAGTA TAATAGCCGTTGTCGAGGTC	$C_L \times c$
C525	ct	5	205	CACCGTGTCACTGTACCAAG CGGAGGATCTGAGTGAGAGA	$C_L \times c$
C629	ctt	5	262	CCTCTTTCTCTGCCTCAAAA GTTTCTGGTGCTTGTCTGCT	$C_L \times c$, $C_L \times d$
C1114	at	5	313	TTCCCCTACTGAAGGTCCTC ACATTTGGACGTTGTTGGAC	$C_L \times a$
C1181	tg	5	355	CTAGGCATGATTTGGGATTG TAGGCAGGAAGCTAGCTGAA	$C_L \times d$
C1231	ttg	5	311	TTCGATCTTTGGGTTTAGGA CATTGAGGTGGAGGATTCTG	$C_L \times c$
C1322	at	5	203	ATCAATCGACAGCGAGAGAG GCAATGATTAGTCCTTGAGCTT	$C_L \times a$
C1476	ag	6	256	TTTCCAGGGAAAAGTGATGA CGGTCAGGTGCTTCTCAGTA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C1585	at	6	202	CAAACACGGACGAGAGAAGT TAAATCCGGATGTCCAGAAA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C1795	atc	5	260	TAACGCCATGTGAAGGTTCT TGAAGAGTCCATGCATGTTG	$C_L \times a$
C1882	ag	5	318	CCAAAGCTTCACCTCTTTAGG TGCTAGATGGAGGTGGACAT	$C_L \times a$
C1933	atg	5	186	CCAACAATACGGAAACCAAA GATTGGTGGAGTGAATGAGG	$C_L \times c$, $C_L \times d$

Table 2.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')	Polymorphism in three populations
C2103	tc	6	133	CATTGCATGGTCTCTCAGTG AATCCTCTTTCCCATTCGAC	$C_L \times c$, $C_L \times d$
C2109	ct	6	201	AAGGGCATTTTGGGTATTTC ACGAGCCGGTTCTTTAGG	$C_L \times a$, $C_L \times c$, $C_L \times d$
C2175	ta	6	202	GTTTTGTGGTATGGCAGGAC TGCCGTAAATTTTGTGTGTG	$C_L \times a$
C2556	ta	5	339	ATTGGATGCATGGGGTTAT CCATCAACTCTGGCTCCTAA	$C_L \times c$
C2762	tc	6	246	TGCTTGATTCGAACTTCCAT CGCTATATATACTCACATCCAGGTC	$C_L \times a$, $C_L \times d$
C2997	ga	8	306	CCAAAAACAAAAGCTGGTTC CATGGCCTAAAGGCTACTGA	$C_L \times c$
C3205	atc	6	146	CCTCATGGATTCACCAACTC TGCAAGTGCAAGACACTGTT	$C_L \times a$, $C_L \times c$, $C_L \times d$
C3280-1	ag	6	308	GAATCGACTCCAACCAACC CCTCTCTCTAACCGGCTCTC	$C_L \times a$, $C_L \times c$
C3292	ga	8	175	CCTCTGATGGACCTGAAGAA CACCCTGCTAGAATGGAAAA	$C_L \times c$
C3332	at	5	184	AAGTGCTAGCCCCTGGTAAC TGCCATCGACATTGACTCTA	$C_L \times c$
C3635	ttg	5	206	GGAAATTGAATTCACCCAACT GGCCAATTTCTTGATTACCA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C3637	ta	5	170	CCCTATTATTTAAAAACCGTCGT TGAGTTGAAGAAAGATAGCGAAA	$C_L \times a$
C3722	ct	5	220	AGCACAAAAATCCCCTTGAT TGGTATCAAGAGCCAAGGTC	$C_L \times a$

Table 2.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')	Polymorphism in three populations
C3977	ga	9	360	CGAATCGTTCAACACCTACC CAGTTTTAGTAACTGATTCTCTCTCTC	$C_L \times a$
C4056	ta	6	161	TTGGGTTTCCGAATTTACTG GAAAACCCAAGCTTCCAAAC	$C_L \times a$, $C_L \times c$
C4136	gt	6	332	GAACCTATGGGCTTATTTCCA CCATTGCCATTTCATCTTTT	$C_L \times a$, $C_L \times d$
C4230	tc	5	244	TCGTTTTGAAAGCTAAATCCTC ACCGTTTGTTTTTCGCTAGG	$C_L \times a$, $C_L \times c$, $C_L \times d$
C4274-3	ag	6	274	ATCCCCTTTGTGATCACCTT TTGAGTTGCCATGTTAGCTG	$C_L \times c$
C4375	ggt	6	232	GGAGGTTAAGGAGGAGTTGG TAAAAGCAGTGGCCTTTTCA	$C_L \times a$, $C_L \times d$
C4399	ct	5	221	CTTTTAAAAACGCGGTCCTT GCATGTGAGATTAGGGCTTG	$C_L \times a$, $C_L \times c$, $C_L \times d$
C4402	tc	5	204	CAACACACACCATTCCAGAG GAGCTGAGCTTTTCACAGGT	$C_L \times a$
C4407	ta	6	392	ATCAAAGGATACGCACCTCA CAACGTCGTCCAAAATAACC	$C_L \times a$, $C_L \times c$, $C_L \times d$
C4441	gt	6	378	GGAAACGCTGAGACAGTCAT AGGCAACGAAGAACTCCAA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C4846	ca	6	334	GGACAATGGAGCAATCTGAC GGGTTTGTGTTCTTGTTGGA	$C_L \times a$, $C_L \times d$
C4882	ag	5	350	AGCCCTACTTATCAGAGCAATG TCCAGTTTTCGTGTAATGTTTTT	$C_L \times a$

Table 2.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')	Polymorphism in three populations
C4940	ag	9	320	GGAGGAAGAGTCATCGCATA TTAACCCGTTAAGCTCATGG	$C_L \times a$, $C_L \times d$
C5120	acaa	5	162	AAGAATTCCTGCAAAGAGCA CAAAGTGTGGGCTTTTGTTC	$C_L \times c$
C5595-1	ct	5	269	CAACCCTAAACCCAAATCCT GAGATCGAGGTCGTTTTGAG	$C_L \times a$, $C_L \times d$
C5595-2	tc	5	178	CAACCCTAAACCCAAATCCT CGACACAGAGAGGGAGAGAG	$C_L \times a$, $C_L \times d$
C5678	ta	5	274	TTATGAGTGGGAGGGTCGTA CCCAAACACTTTTCAATGCT	$C_L \times a$, $C_L \times c$
C5753	at	5	241	CTTCCTCCTCATGCACAATC GGCGTAAAGCAAGGGTTAAT	$C_L \times c$, $C_L \times d$
C5900	ga	7	216	TTGGAATTTTGGATATGGTTTC GATGGATGGCTGAGATTCAC	$C_L \times a$, $C_L \times c$, $C_L \times d$
C5948-1	ct	5	297	AAACCGGACTTtCTCTCTCC CAGAAACAGCAAGAGCATGA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C6099	tg	5	259	ACGCTTCTGATCCACACTTC CCCAGAATCAATTCCAGATG	$C_L \times a$, $C_L \times c$
C6100	ta	7	291	CTGCGTGAGAAAAGAGGAAG ATTCGTACATCACGCAACAG	$C_L \times a$, $C_L \times c$, $C_L \times d$
C6156	tc	5	397	GGTTGCTCTAGGCACATGAC CCAATCTTTGATGCCATAGG	$C_L \times a$, $C_L \times c$, $C_L \times d$
C6188	ta	5	362	CGCACAACCTCTCAACACTT AAATCTTCCTCCCGTAGTTTAGA	$C_L \times c$
C6255	tg	5	236	TGAAATGCATGCACCTAATC TGAAATCATTGGTGTTGGTCT	$C_L \times a$, $C_L \times d$

Table 2.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')	Polymorphism in three populations
C6256-1	ag	5	265	AGATTGTTCAGTGGGATTGC CCCAGATCAAAACACACACA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C6256-2	tg	6	341	ATTTGGAGGGAGAAATTTGG CTAAATGGGGGCCTTCTCT	$C_L \times a$, $C_L \times c$, $C_L \times d$
C6292	caact	5	286	ACAGACTGCAAGGGGAAAGA AAGCCTTGGATTGCTTGTGT	$C_L \times c$, $C_L \times d$
C6293	gt	5	248	CTGCCATTCCTGTAGCCATA TCCAGGTTTTGTTTTGTTGTCA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C6363	aga	5	208	TCAGCCATAATTGTACAGAATAGTTT T TTGTTGGCTGCATTCTCATC	$C_L \times a$, $C_L \times c$
C6387	ga	5	297	CCATGATAGAGAAACCATCAGGA CAGCCTAGTGCCTCTTCCAC	$C_L \times a$, $C_L \times c$, $C_L \times d$
C6434	tca	6	322	CATGGACTCCACCAAGAGGT CCACTGAATTGGGAGACCCTA	$C_L \times a$, $C_L \times d$
C6669	ct	6	201	CCTGCGACAAAATACCCAAA GCGACTTAGGTGGGTCTGAA	$C_L \times a$
C6740	ta	5	299	CAGTGCAGTGGCGATATAGG AGGGGGATATGATGGTGATG	$C_L \times c$, $C_L \times d$
C6797	ca	5	356	GATCTGCATCATCTGAAACTGC GCCACAGGAGCAAAAGTCTC	$C_L \times c$
C6957	ga	5	193	AAATCTGGCCAAGAGCACAG TGACCATCGAGTTGGCATAA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C7106	tggtt	5	151	ATGCCTAAACAAGCCGAACC CGATTTGACCCTCAAACCAC	$C_L \times c$, $C_L \times d$
C7247	tc	9	278	GCGTCTTTTTATTGGGGTCA GCCTCGAGCAATTGTCTTCT	$C_L \times a$, $C_L \times c$, $C_L \times d$

Table 2.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')	Polymorphism in three populations
C7319	ta	5	308	CATTTGGAAGAACAAGCATTATAC AACTCACAAGGGGGTGGTTT	$C_L \times a$, $C_L \times c$, $C_L \times d$
C7533	ct	5	198	ACGATGATTCCATCGAAAGC TCAGCGATGAGAAAGGGAGT	$C_L \times a$, $C_L \times d$
C7642	ga	5	178	TCCTCATCACAATCGAACCA GCCCTTGGCTCTCTCTACCT	$C_L \times c$, $C_L \times d$
C7670	tca	8	173	GTAACGCATCATGGGCAAG GCCAGACATGAAAGGCAAAT	$C_L \times a$, $C_L \times c$, $C_L \times d$
C8064	tg	6	231	TGTTGCCTAGCTCACACCAG GAATGTGGGGTATTGCTCGT	$C_L \times a$, $C_L \times c$, $C_L \times d$
C8086	tca	7	279	ACCCCTAGTGCTTGGTCCTT CCTTGAAGTGAAGGCTGAGG	$C_L \times a$, $C_L \times d$
C8107	ct	5	175	TTTAGGCGAATCCAATGAGG CACCTGCAATTTCCTTGGTT	$C_L \times a$, $C_L \times d$
C8169	gtt	6	206	CTTCAAGGGGTTGTCGGTTA TGCGAGCGTTGAAGAGATTA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C8386	gt	6	336	CTTCCAGATCCAGCCATGAT GATCCAGCTGCTGTGCATAA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C8477	ct	5	166	TCTTGGCTCCGTCTCTCTCT TGAGCTTCGATGAACACACA	$C_L \times c$
C8537	ct	7	159	TCCCTGTGTATTGAGCACCA GCCGATGGAGAAGTTGTGAG	$C_L \times c$, $C_L \times d$
C8627	tc	5	185	GCTAGGAGCAATGGCTAGGA ATTTTGGGAGCACAGAGGAA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C8761	ca	5	151	CCGTGTTGACTACTGTTACCC TTCTTTAGATTGCTCTGATATTGCTC	$C_L \times c$, $C_L \times d$

Table 2.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')	Polymorphism in three populations
C8812	tc	12	297	TGCCAACTACTGGAATCTTGC AAGATGTTGGCGGTCTTGAG	$C_L \times d$
C9108	tg	5	182	GGGATCACCAAGAGGACGTA AAGGGAAGAAGCGTGTGTTG	$C_L \times d$
C9267	ga	16	209	GGGGTGGGATAAGTCTTGGA CCTCACCCACCTCTCTCTCA	$C_L \times d$
C9480	at	6	283	GCAACTATTCTCAATCCATTCCA CCCGGTTGACCAATATGACT	$C_L \times a$
C9500	aag	6	395	ACCAATCACAGCATCAACGA CTTGCTCGAGAGGCTTCTGT	$C_L \times a$, $C_L \times d$
C9559	ag	5	150	AAATGGTTTGCGAGGTCAAC AAGAAGCGCGCATTTTGAA	$C_L \times a$, $C_L \times c$
C9600	tg	5	233	CTGGAGATGAGGGAGCACAT TGGGTGAAGTTGATGGTTCA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C9657	ga	5	287	CGGGTATTTTGGGAAGATGA GGGCTTCTTAGGGTTCGAGA	$C_L \times a$, $C_L \times c$,
C9736	ga	5	214	GGTCACGTAAACTGGGGAGA TCTCTCTGTCTCTCTCAACACGA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C9746	ac	5	285	TCGTTGTAATGGCAAGTGGA GGACGTCCTGCTCTGAGAAT	$C_L \times d$
C9824	ag	6	293	AATGGATAGGGCACGTCAAG CTCTCTCTTCCCTCCGGTTT	$C_L \times a$, $C_L \times c$, $C_L \times d$
C9912	at	5	185	ATGTGTTGGCAATTGGGTTT CCACAACCCACTCACTTTCC	$C_L \times c$
C9976	gaa	5	205	AAAATGCCAAAAGTCCGATG TGACATGCTCATCGCTTACC	$C_L \times c$

Table 2.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')	Polymorphism in three populations
C10367	ga	5	166	GCGGTACAAGCTTCTTCTTCTT TGGGTTGATGTCATGTCAGG	$C_L \times a$, $C_L \times d$
C10404	cca	5	158	TCGGTGCAACATAGACCTCA GTAACTGGCGGGTTGTGTTT	$C_L \times d$
C10447-2	ga	9	281	TGGGGTTACCTTGAGATTGC ACCCCAAAATCTCAGCCTCT	$C_L \times a$
C10542	tct	6	208	TCATCATGGGCTTCAGATCA CCTCCTCTCACGAGCCTGTA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C10685	gag	5	285	TCCGATATCCCAACATCCAT GTGGCTTCCACAGTTGGAGT	$C_L \times a$, $C_L \times c$, $C_L \times d$
C10694	ggt	5	220	CTCAAAATTGTTGGCTGCAA CGTGTATGCAACGTTCTCGT	$C_L \times a$
C11107	ga	5	215	ATCTGGCCAAGAGCACAAAG CATCTTGAGCTCTCCCACAA	$C_L \times c$
C11197	tga	5	347	CAGAACCGTTGGAGTTGGTT ACAACTGGGCACATTTGACA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C11377	cac	6	210	ACCACGTCATCAAAACCAC TCAGATGAGAGGCAATCACG	$C_L \times a$, $C_L \times c$, $C_L \times d$
C11508	tc	7	201	TGCACACTTCTTGAGTCTTCG CTATGCGGGGCTCGAAAG	$C_L \times a$, $C_L \times c$, $C_L \times d$
C11610	ac	9	216	GGGACTTGCACACCTTCACT AGTGGTGCAGTGAGCAGCTA	$C_L \times a$, $C_L \times c$
C11662	ta	5	245	TTGAGAATTTGGTTTCGGTTG AAGCTCACACGCCAAAGAAT	$C_L \times c$, $C_L \times d$
C11992	ga	5	271	CTGACAACAGTGGTCCAAATTC CGGAAATGAAATGGCTTTGT	$C_L \times c$, $C_L \times d$

Table 2.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')	Polymorphism in three populations
C12352	ga	9	283	CACAGGGTTAAGTGGGCAGT AAACCTATCTTTTCACCCACCA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C12361	ag	5	255	GTGAGTTGGCAAGGGAGAGA GGCTTCAACGTAAGCTGCTC	$C_L \times a$, $C_L \times c$, $C_L \times d$
C12477	gt	5	245	CAGGTTGGTTTCGGTTGAGT TCCATTCACACGTTCATTGC	$C_L \times d$
C12735	tc	8	268	CCCGTAAGTTTCCCAAAATTC AGTGATAGATGAAGATGGGTTTTT	$C_L \times c$, $C_L \times d$
C12916	at	5	171	TCCAATCTCAATTTCCGAAC TTGGATTGTTTTCCCTTGGT	$C_L \times d$
C13401	ag	6	206	TGTGAGGTGATTAGATTGCTTGA CCGTCAAGACCTCCGTTAAA	$C_L \times a$, $C_L \times c$, $C_L \times d$
C13993-1	ta	5	239	CAGAACAGGGGGAGGTACAA GGTCTGTTATGCGGTCGTTT	$C_L \times a$
C14145	cct	5	215	GGCTCCGACTAGCTCCAC ATCAGAGCAACCCCAGGAG	$C_L \times a$, $C_L \times d$
C14193	tc	5	208	ACACGAACCAACCCGTTAAG GGTTGTTGAGAGAGGTTTTTGAA	$C_L \times d$
C14227	ac	5	241	TGTGGTAAGCCTAGCATTTTCC GAGCAGGTGTGTGTGGAGTG	$C_L \times a$
C14231	ag	7	206	GGCCGGTGATGTTCTATGAT TCTTTTCCTCCCTTAACCTTCC	$C_L \times a$, $C_L \times c$, $C_L \times d$
C14600	aga	5	203	TGAAGTTGTAGGCGTGTCT TGCTCACATCCAAATTCGTC	$C_L \times c$
C16053	cac	5	150	GATAGAGAGGCTATGGCTCATCA CATTGGCACCGTCTGATACTT	$C_L \times c$

4.3 Construction of genetic linkage maps

4.3.1 The $C_L \times$a linkage map

A total of 134 SSR primers were selected for mapping the population $C_L \times$a. As a result, 232 markers were identified in which 169 markers were considered as informative markers that were single dose restriction fragments (SDRFs) (Cali, 2004). Using TetraploidMap, 110 markers were mapped as maternal loci (C_L), 88 markers were paternal loci (a), and 34 markers were common loci that were represented in both parents. Another five markers were unlinked (3.0%) and not mapped into any linkage groups. A total of 164 markers (97.0%) were ordered onto the 16 linkage groups (Fig. 2.2 and 2.3). The total length of the genetic distance for C_L and a were 1379.3 and 1105.8 cM, respectively. The average distance between two markers was 12.5 cM in LGs (Linkage groups) of C_L and 12.6 cM in LGs of a. The number of markers on each linkage group ranged from 3 to 27 and the linkage distance spanned by individual linkage groups from 29.4 cM (LG13) to 294.4 (LG11) in the C_L map. In contrast, the map of parent a had a range of marker number from 3 to 27 and the distance of linkage groups were ranged from 77.9 cM (LG12) to 222.4 cM (LG11). In the map of C_L, 12 linkage groups were clustered by markers and four linkage groups were missing: LG4, 6, 7, and 15, in which markers on LG4 and 6 were mapped on the parental map of a. In the map of a, nine linkage groups (LG2, 4, 6, 9, 10, 11, 12, 13, and 14) were clustered in the map and no marker was mapped on LG1, 3, 5, 7, 8, 15, and 16, in which LG1, 3, 5, 8, and 16 belonged to C_L parental LG groups. Linkage group 7 and 15 were missing in both maps (Fig. 2.2 and 2.3) because the two markers in each group belonged to the different parental type. Marker BPPCT018-1 was the single marker clustered on the group 4 of the map C_L. There were 32 and 23 gaps that were larger than 15 cM for the parental maps and

the largest gap was found in LG10 (70.5 cM) and LG13 (105.5 cM) on the map of C_L and a, respectively.

By using JoinMap, the genotype data was separated on the basis of their parental genotypes (lm and ll or nn and np), two sets of genetic linkage maps were generated (Fig. 2.2 and 2.3). Eighteen groups were clustered and a total of 93 markers were mapped in C_L parental map. The total length of the linkage maps were 935.3 cM and the average distance between two markers was 10.1cM. The number of markers for each group ranged from 2 to 16, while the length for each group ranged from 16.2 to 97.3 cM. The distance of five linkage groups that contained 2 markers were found to be larger than 32.3 cM. For the map of a, 15 linkage groups were generated. In total, 71 markers were mapped in the linkage groups and the total length was 838.8 cM with an average distance of 11.8 cM between two markers. The number of markers for each group ranged from 2 to 10 and the length for each group ranged from 20.9 to 98.5 cM. Four linkage groups had only 2 markers and the largest distance between the markers was 38 cM. Comparing these two parental maps, 14 markers were shared by both maps. In this case, 153 (90.5% out of 169 SDRFs) markers were included in this map constructed by JoinMap.

4.3.2 The $C_L \times c$ linkage map

Two hundred and forty markers were identified in the population $C_L \times c$. Of which, 150 markers were SDRFs. By using TetraploidMap, 75 and 99 markers were mapped on the maternal (C_L) and paternal (c) maps, respectively and 25 markers were identified to be shared by two parental maps and 2 markers remained unlinked (1.3% in total) (Fig. 2.4 and 2.5). In total, 148 markers (98.7%) were mapped onto both parental maps. The total length was 1237.2 cM for C_L and 1165.3 cM for c and the average distance between two markers were 16.5cM and 11.8cM in the map of C_L and c, respectively. In the map C_L, 12 linkage groups were obtained and four

linkage groups had no marker: LG7, 8, 14, and 16. For the map c, 14 linkage groups were clustered and only one marker was positioned on LG1 and LG9. The number of markers on each linkage group for the map of C_L ranged from 3 to 12, while for the map of c, the number was ranged from 3 to 14. The length of each linkage group for both parental maps (C_L and c) ranged from 24.1 (LG15) to 188.7 cM (LG6) and 23.5 (LG4) to 149.6 cM (LG3). Thirty and 26 gaps larger than 15 cM were found in the map of C_L and c, respectively and the largest gap was found in LG10 of the map C_L (70 cM) and LG7 of the map c (60.5 cM).

Fifteen and 16 linkage groups were clustered with the software JoinMap in parental C_L and c map, respectively (Fig. 2.4 and 2.5). In total, 63 markers were mapped in the parental C_L map with a length of 929.6 cM. The average distance between two markers was 14.8 cM. The number of markers for each linkage group was ranged from 2 to 9 and the distance of each linkage group was ranged from 13.3 to 96.7 cM. In contrast, 94 markers were included in the parental c map. The total length of the linkage groups was 1028.3 cM with an average distance of 10.9 cM between two markers. The number of markers for each linkage group was ranged from 2 to 20 and the distance of each linkage group was ranged from 2.8 to 120 cM. In total, 148 marker loci (98.7%) were mapped in this population with 9 common markers shared by two sets of maps.

4.3.3 The $C_L \times d$ linkage map

Within 195 marker loci identified for the population $C_L \times d$, 149 loci were informative SDRFs. By using software TetraploidMap, 86 markers were mapped to 13 linkage groups in the C_L map and 94 markers were mapped to 12 linkage groups in the d map (Fig. 2.6 and 2.7). In total, 148 markers (99.3%) were included in the two maps of this population. There were 32 common markers that were shared by both parental maps. For the map C_L, the total length was

1079.7 cM with an average of 12.6 cM between two markers and no marker was positioned on LGs 11 and 14, while in the map d, the total length was 1135.6 cM with an average of 12.1 cM between two markers and no marker was located on the LGs 3, 5, and 12. Linkage group 16 was missing in both maps because only one marker C12352-3 was assigned to this group although the marker fit 3:1 ratio and was considered as a common marker. The length of each linkage group in the maps of C_L and d were ranged from 42.7 (LG10) to 151.7 cM (LG2) and 31.8 (LG15) to 143.8 cM (LG4), respectively. The marker number on each linkage group were from 4 (LG6, 9, and 12) to 12 (LG8) in the map C_L and from 3 (LG15) to 12 (LG4 and LG13) in the map d. In total, 25 and 27 gaps that were larger than 15 cM existed on the map of C_L and d, respectively. The largest gap was found on the LG2 of the map C_L (51.6 cM) and LG6 (59.7 cM) of the map d.

In contrast, 16 linkage groups were generated for both parental maps of the population $C_L \times d$ using JoinMap and 77 and 79 markers were assigned to the parental C_L and d maps, respectively (Fig. 2.6 and 2.7). In total, 24 common markers were found in this population, so that 132 marker loci were mapped in the linkage maps. In the C_L map, the total length was 921.4 cM and the average distance between two markers was 12 cM. The number of markers and the length of each linkage group were ranged from 2 to 10 and 20.1 to 117 cM, respectively. In the d map, the total length was 821.2 cM and the average distance between two markers was 10.4 cM. The number of marker and the length of each linkage group were ranged from 2 to 14 and 2.4 to 153.4 cM, respectively.

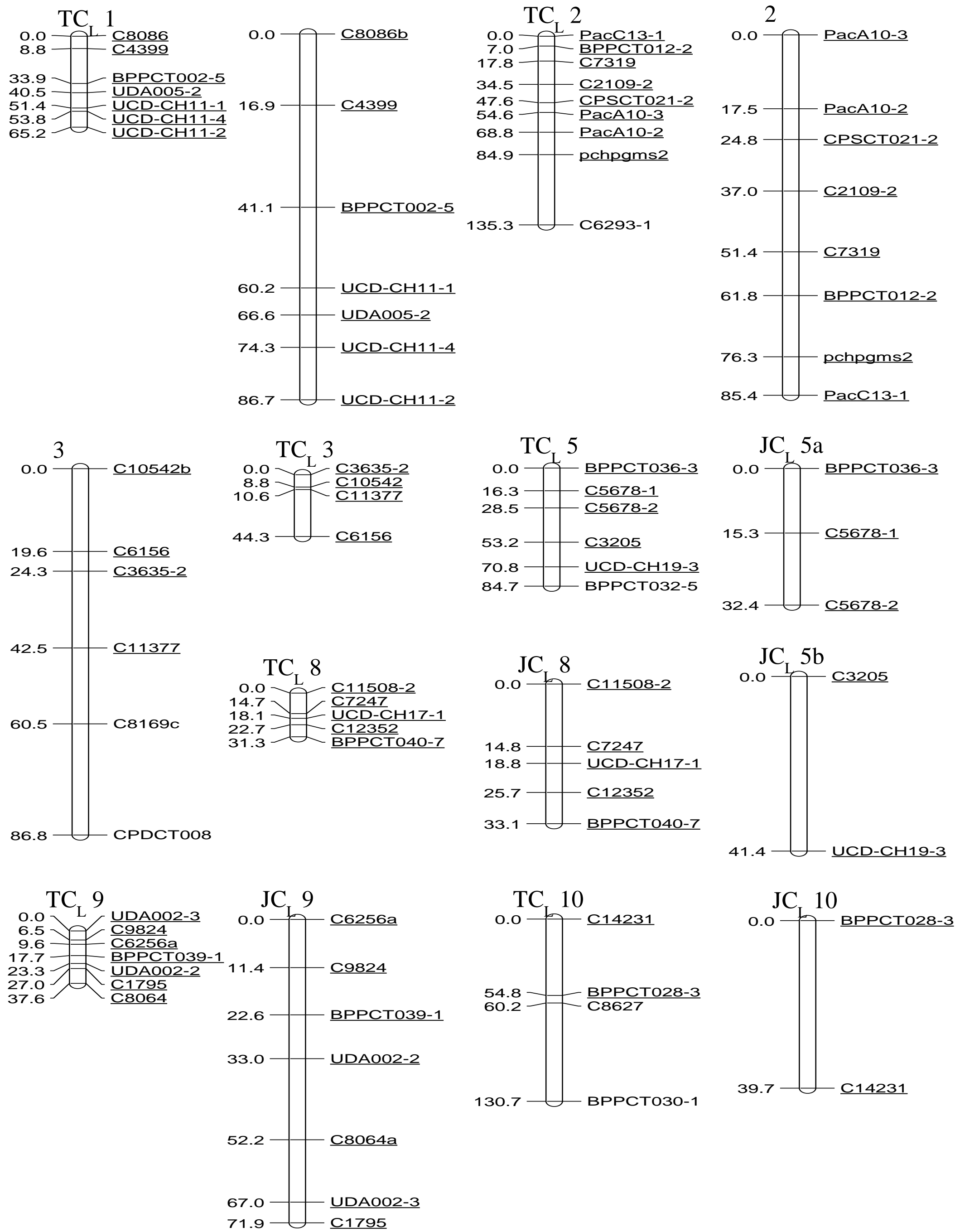

Fig. 2.2. Genetic linkage maps were constructed using TetraploidMap and JoinMap for the parent C_L in the population $C_L \times$ a. “T” refers to the map constructed by TetraploidMap; “J” refers to the map constructed by JoinMap. Markers underlined are homologous between two sets of maps.

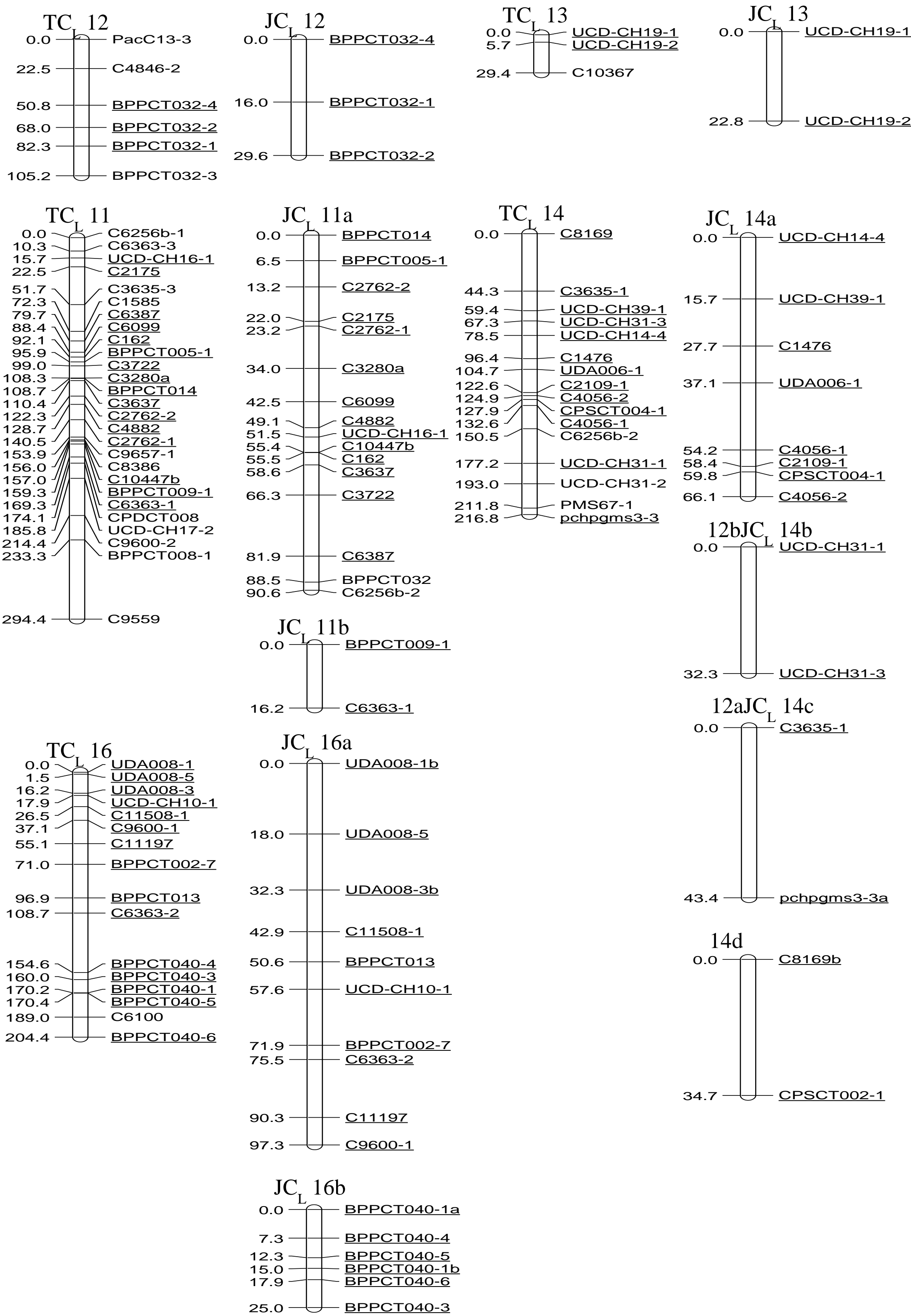

Fig. 2.2. Continued.

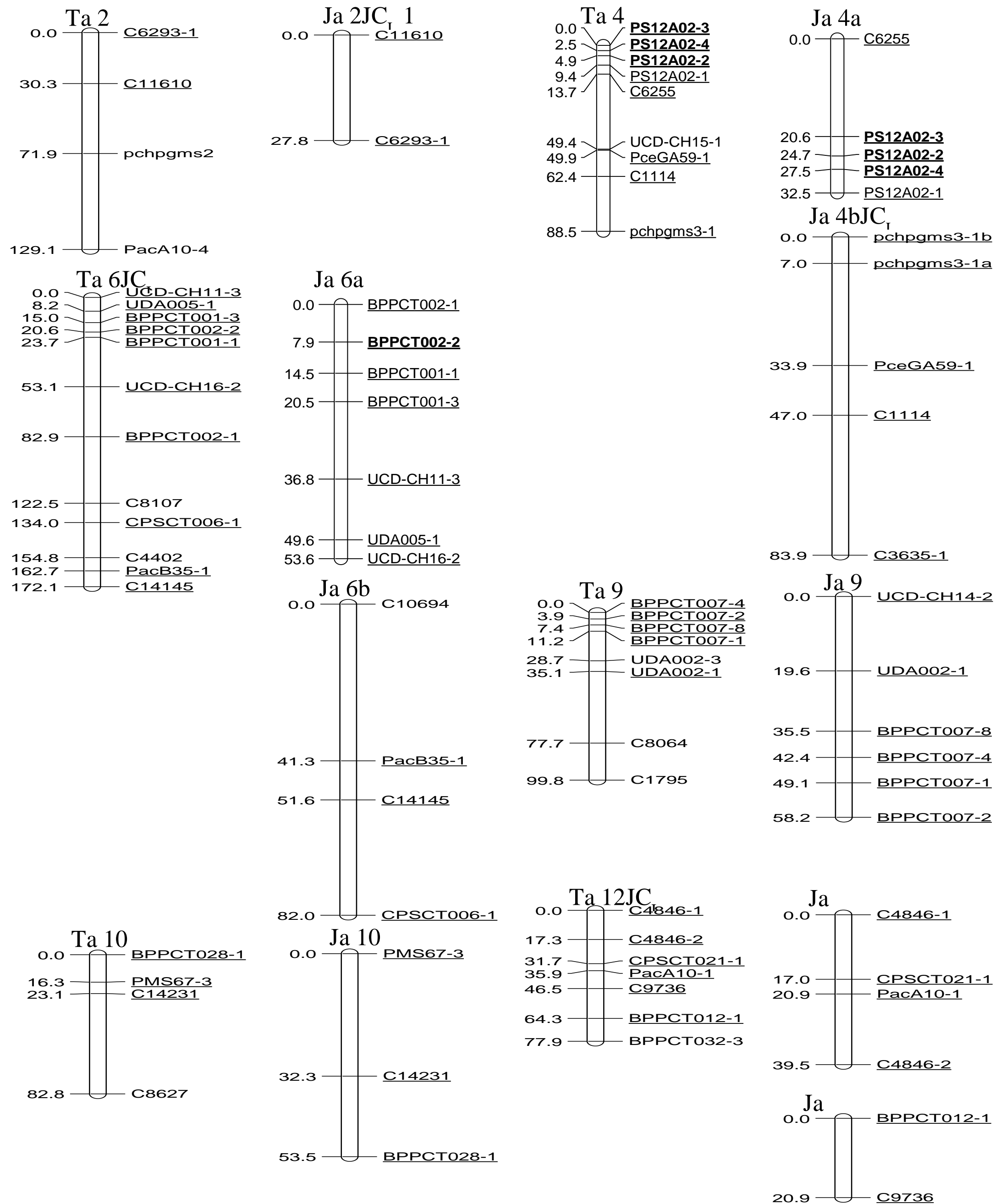

Fig. 2.3. Genetic linkage maps were constructed using TetraploidMap and JoinMap for the parent a in the population C_L × a. “T” refers to the map constructed by TetraploidMap; “J” refers to the map constructed by JoinMap. “?” refers to no corresponding linkage group number was assigned. Markers underlined are homologous between two sets of maps. Markers bolded are identified X-disease linked markers.

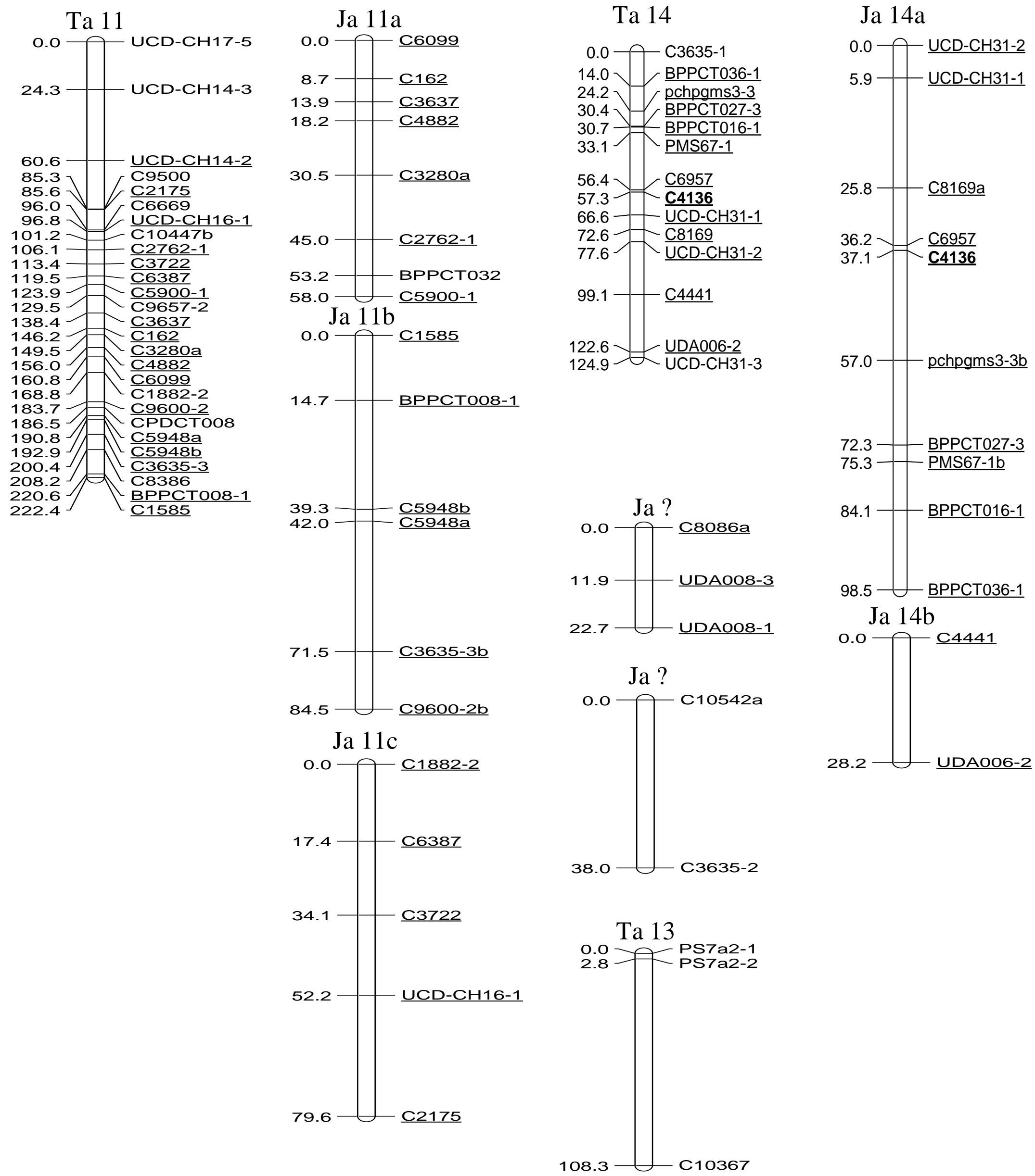

Fig. 2.3. Continued.

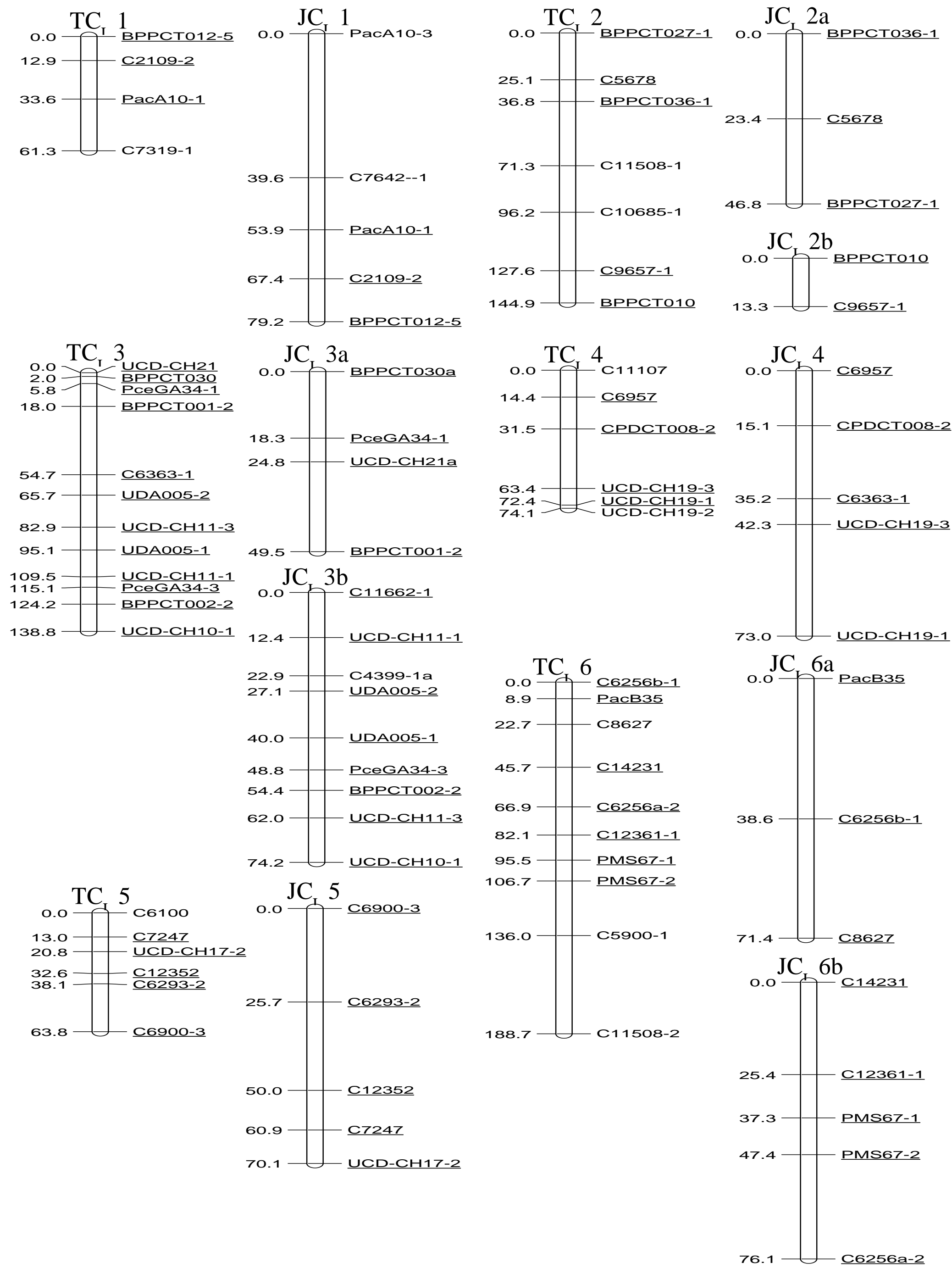

Fig. 2.4. Genetic linkage maps were constructed using TetraploidMap and JoinMap for the parent C_L in the population $C_L \times c$. "T" refers to the map constructed by TetraploidMap; "J" refers to the map constructed by JoinMap. Markers underlined are homologous between two maps.

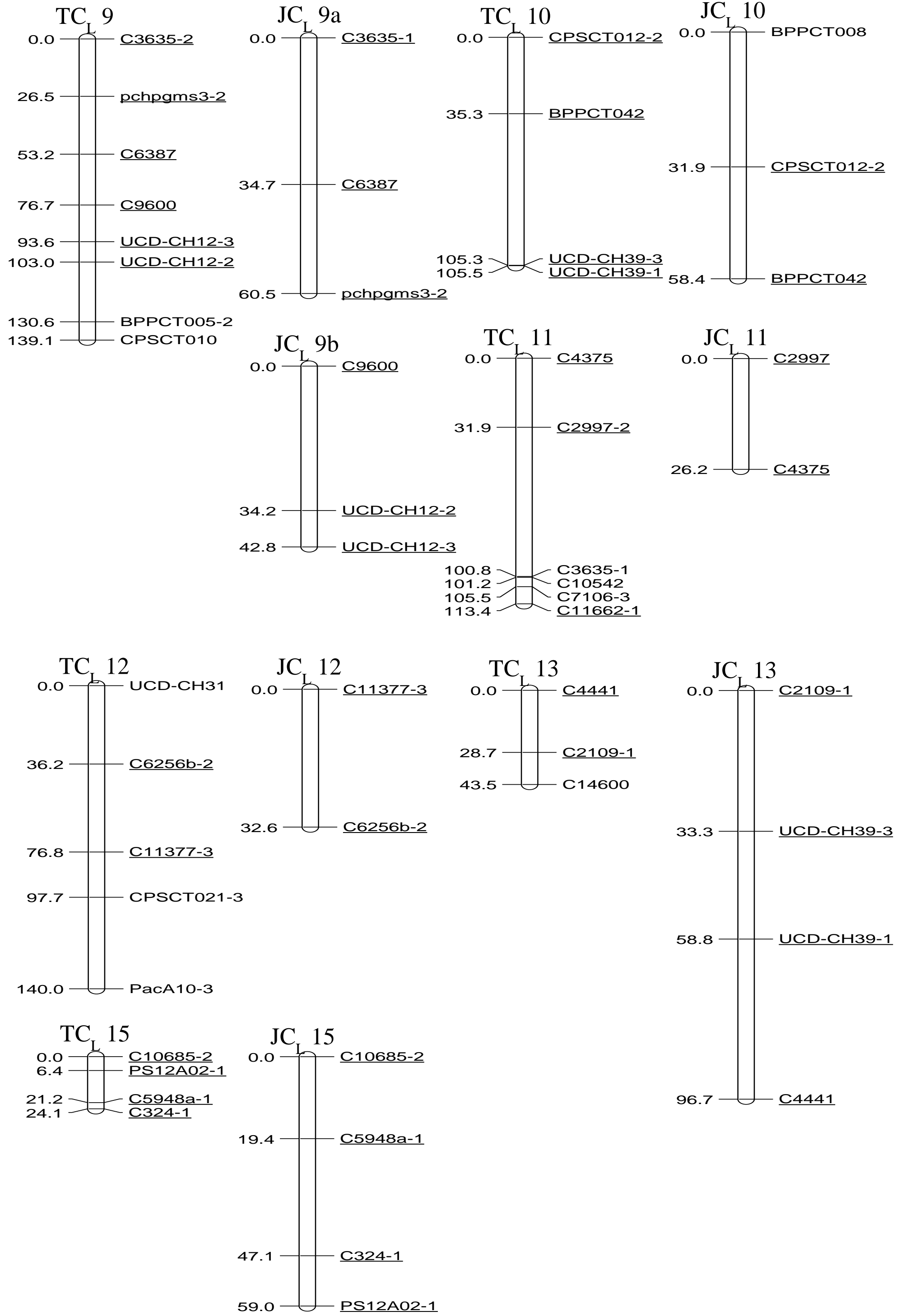

Fig. 2.4. Continued.

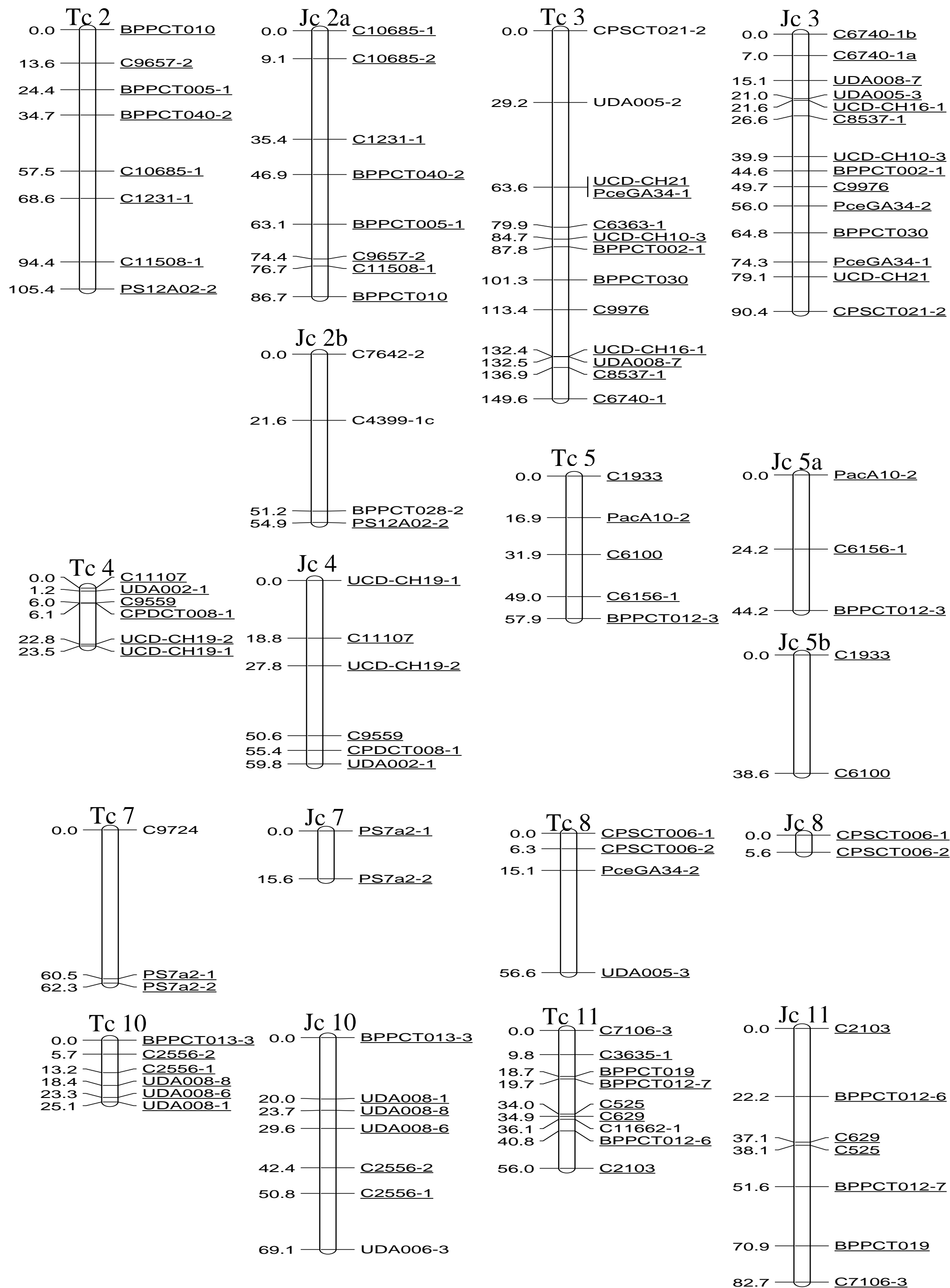

Fig. 2.5. Genetic linkage maps were constructed using TetraploidMap and JoinMap for the parent c in the population C_L × c. “T” refers to the map constructed by TetraploidMap; “J” refers to the map constructed by JoinMap. “?” refers to no corresponding linkage group number was assigned. Markers underlined are homologous between two sets of maps.

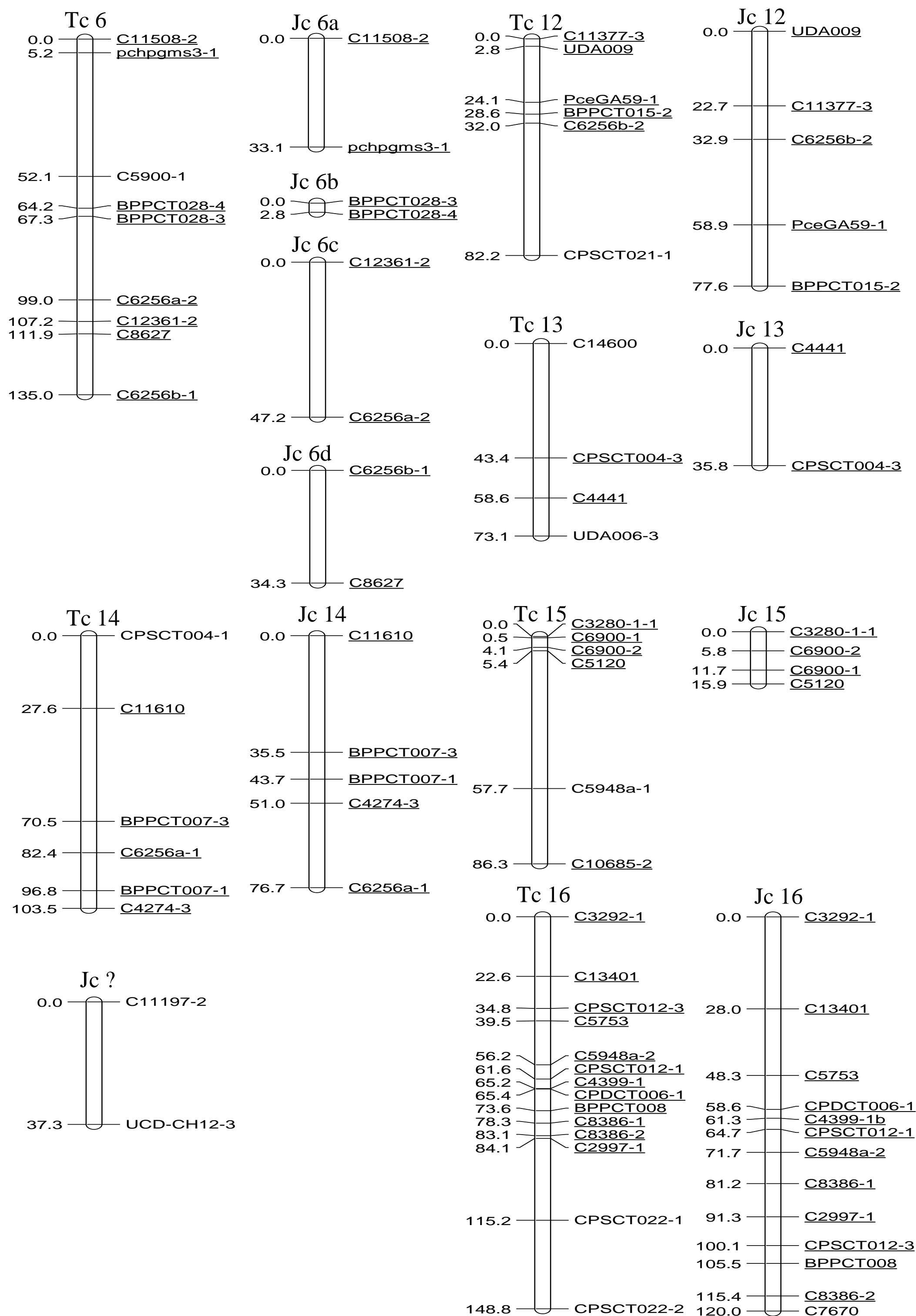

Fig. 2.5. Continued.

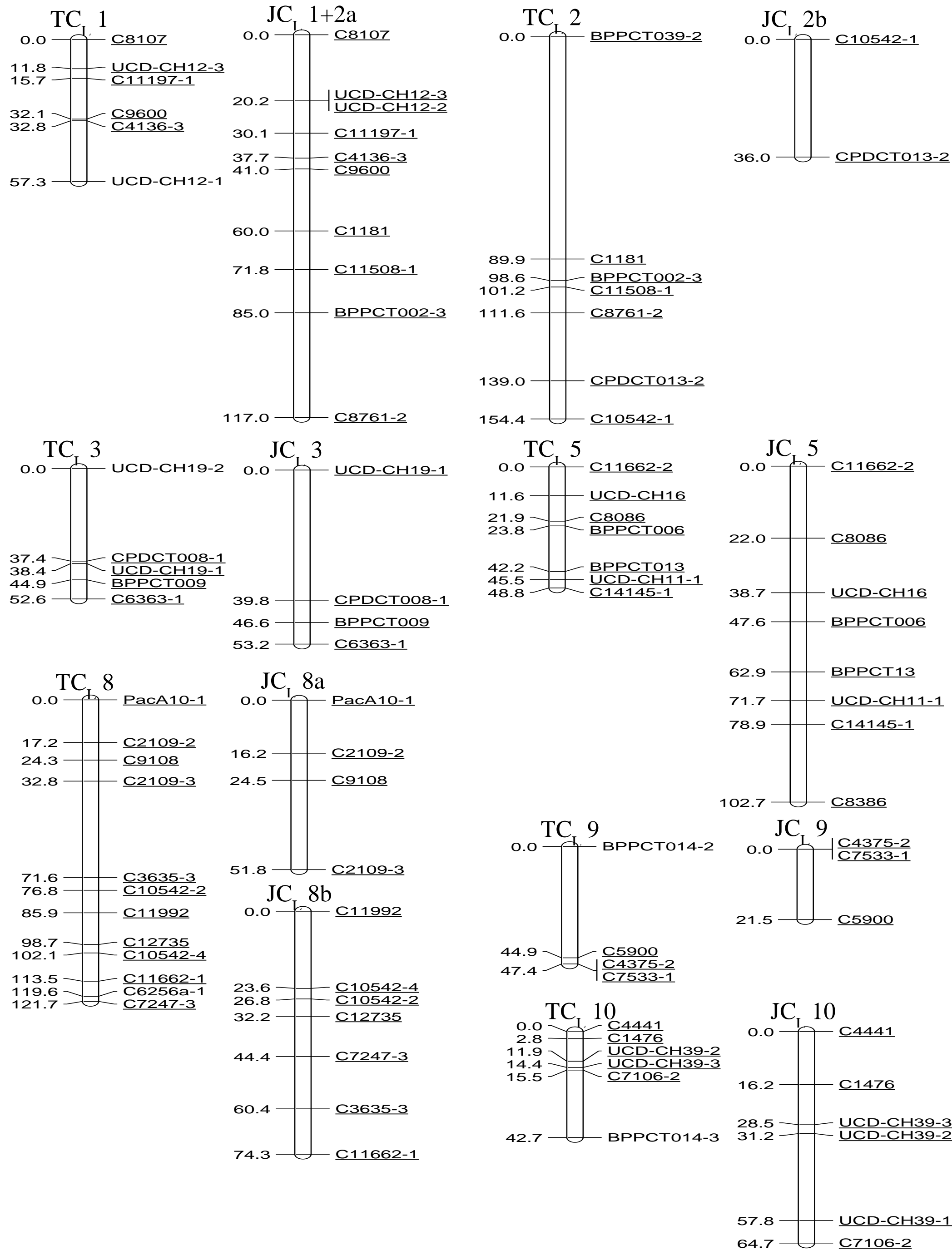

Fig. 2.6. Genetic linkage maps were constructed using TetraploidMap and JoinMap for the parent C_L in the population $C_L \times$ d. “T” refers to the map constructed by TetraploidMap; “J” refers to the map constructed by JoinMap. Markers underlined are homologous between two sets of maps

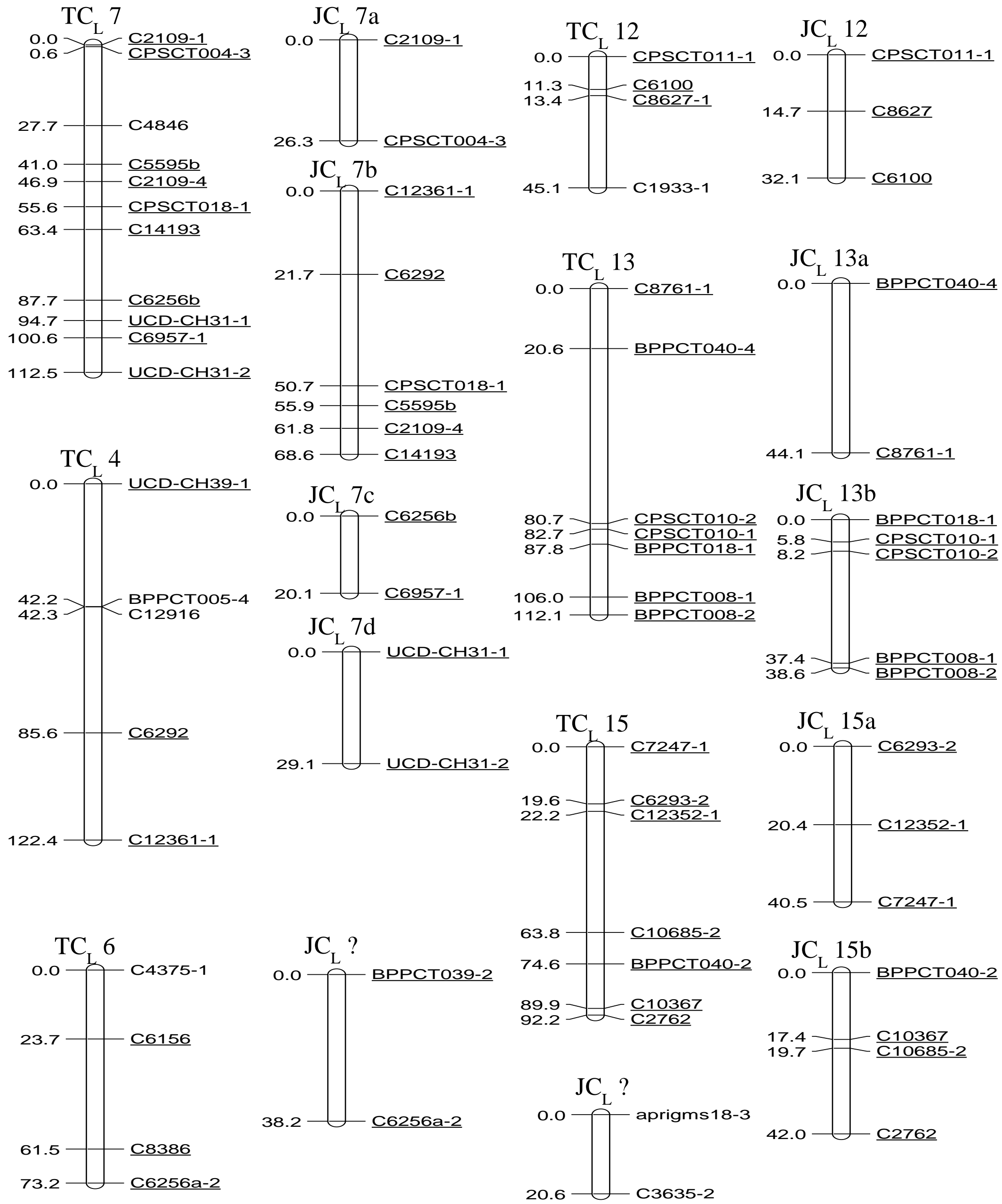

Fig. 2.6. Continued.

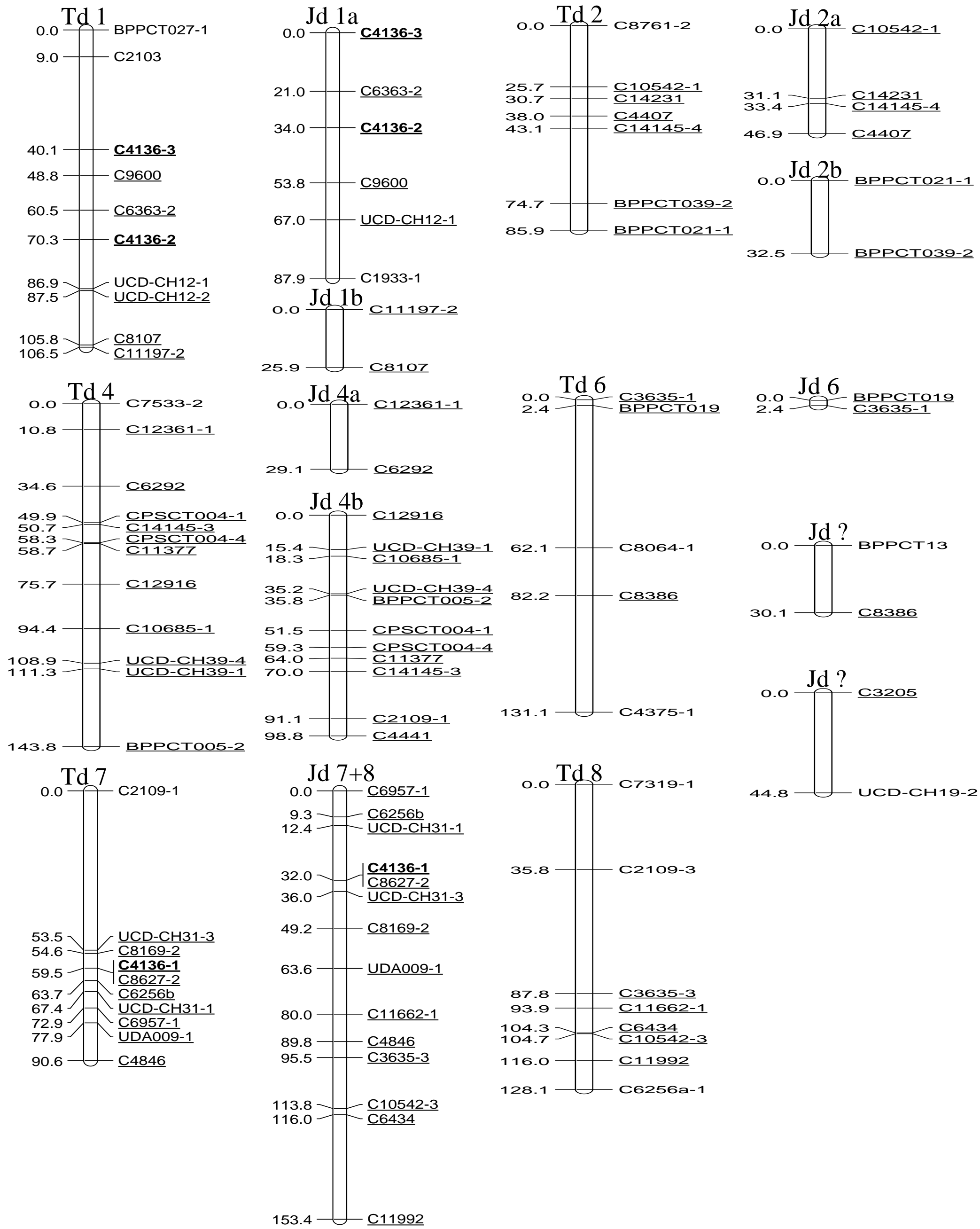

Fig. 2.7. Genetic linkage maps were constructed using TetraploidMap and JoinMap for the parent d in the population C_L × d. “T” refers to the map constructed by TetraploidMap; “J” refers to the map constructed by JoinMap. “?” refers to no corresponding linkage group number was assigned. Markers underlined are homologous between two sets of maps. Markers bolded are identified X-disease linked markers.

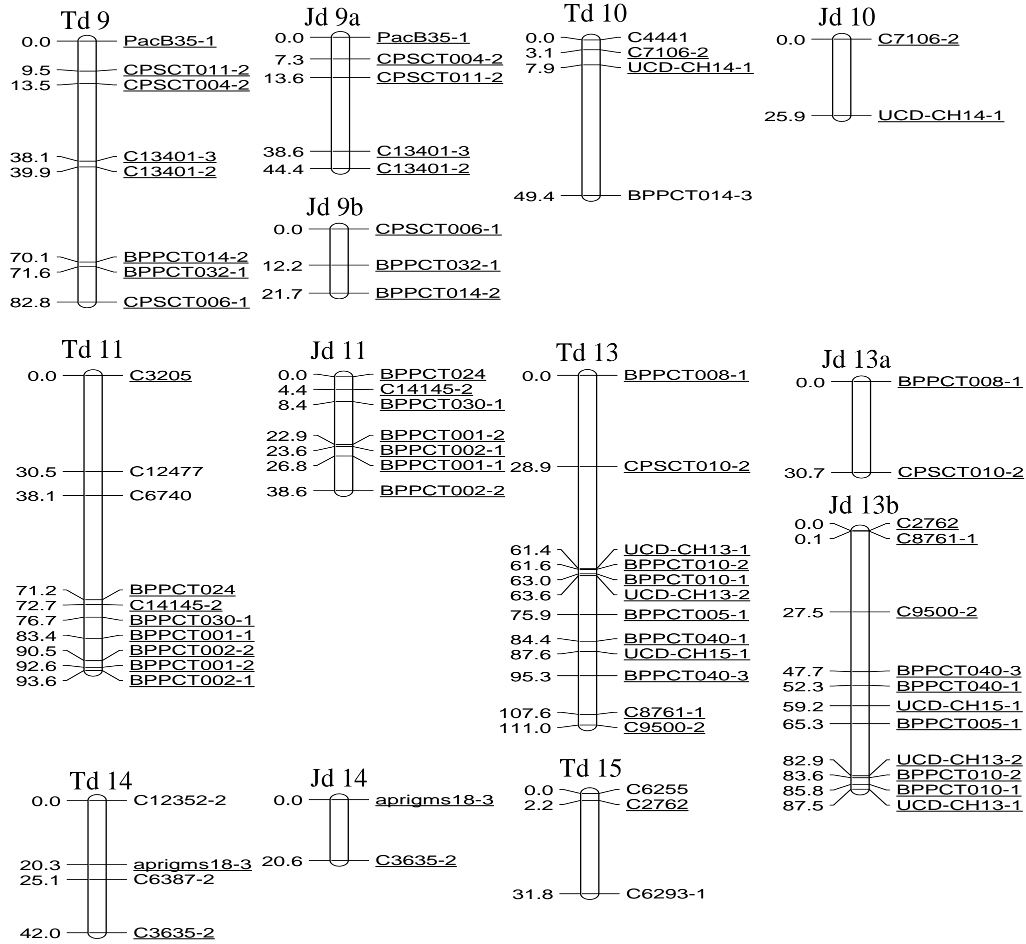

Fig. 2.7. Continued.

4.3.4 Joint linkage groups

In this study, all three populations had the same female parent C_L. A few linkage groups from the different populations shared two or more markers; therefore these groups were joined together using the software JoinMap (Fig. 2.8). A total of six joint-groups were generated and all of them were reassigned. The first linkage group was merged with the LG1 of the population $C_L \times a$ and the LG3b of the population $C_L \times c$. The length of this group was 125.6 cM and a total of 14 markers were assigned in it, which was different from the original linkage groups (86.7cM with 7 markers and 74.2 cM with 9 markers for LG1 and LG7b, respectively). The average distance between two markers also changed from 12.4 and 8.2 to 9.0 cM. The second joined linkage group was joined by the linkage groups from the three populations and the original groups were LG14a in the population $C_L \times a$, LG13 in the population $C_L \times c$, and LG10 in the population $C_L \times d$. Ten markers were reassigned to this linkage group with the total length of 100.6 cM and the average distance of 10.6 cM between two markers. All markers were included from three linkage groups except markers UCD-CH39-2 and C7106-2, which were missing from the LG10 in the population $C_L \times d$. The third linkage group was joined with the linkage groups originally from the three populations as well: LG8 in the population $C_L \times a$, LG5 in the population $C_L \times c$, and LG15a in the population $C_L \times d$. The total length of the new linkage group was 75.9 cM with eight markers and the average distance between two markers was 9.5 cM. The rest of three linkage groups were all reassigned with the groups from the population $C_L \times c$ and $C_L \times d$. The forth linkage group was reassigned with ten markers from the LG9b in the population $C_L \times c$ and LG(1+2a) in the population $C_L \times d$. The length was 81.4 cM and the average distance between two markers was 8.1 cM. The fifth linkage group was joined by the LG4 and LG3 from the population $C_L \times c$ and $C_L \times d$, respectively. The total length was 82.3 cM

with seven markers and the average distance between two markers was 11.8 cM. The last linkage group was joined with the LG1 and LG8a from the population $C_L \times c$ and $C_L \times d$, respectively. Seven markers were assigned and the total length was 104 cM with an average distance of 14.9 cM between two markers.

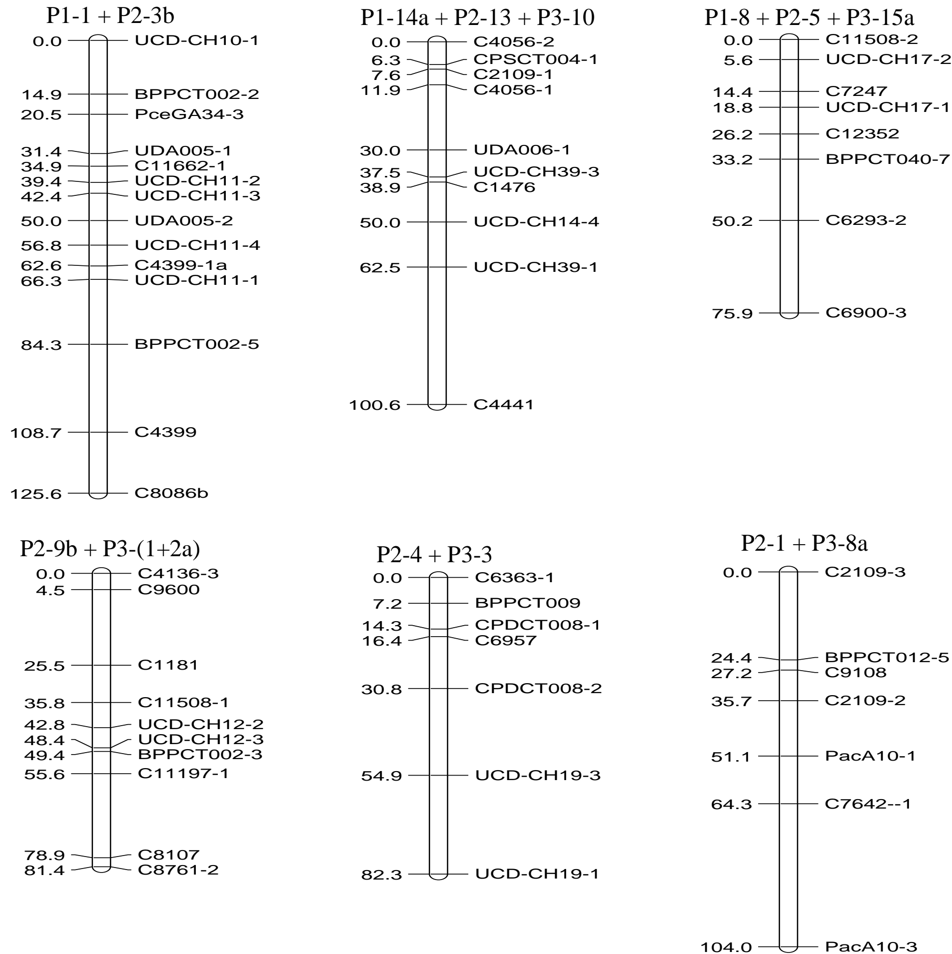

Fig. 2.8. The consensus map of three populations was joined based on two or more common markers. P1: Population $C_L \times a$; P2: Population $C_L \times c$; and P3: Population $C_L \times d$. The number after "-" is the linkage group number.

4.4 X-disease resistance in mapping populations

4.4.1 X-disease inoculation and verification

X-disease phytoplasma was inoculated using the grafting method (Fig. 2.9). Based on the previous experiment, phytoplasmas can be successfully transmitted from the infected scion to the chokecherry within one week (personal communication with Dr. Walla) although the symptom may not be seen for several months or until the next growing season after the grafting.

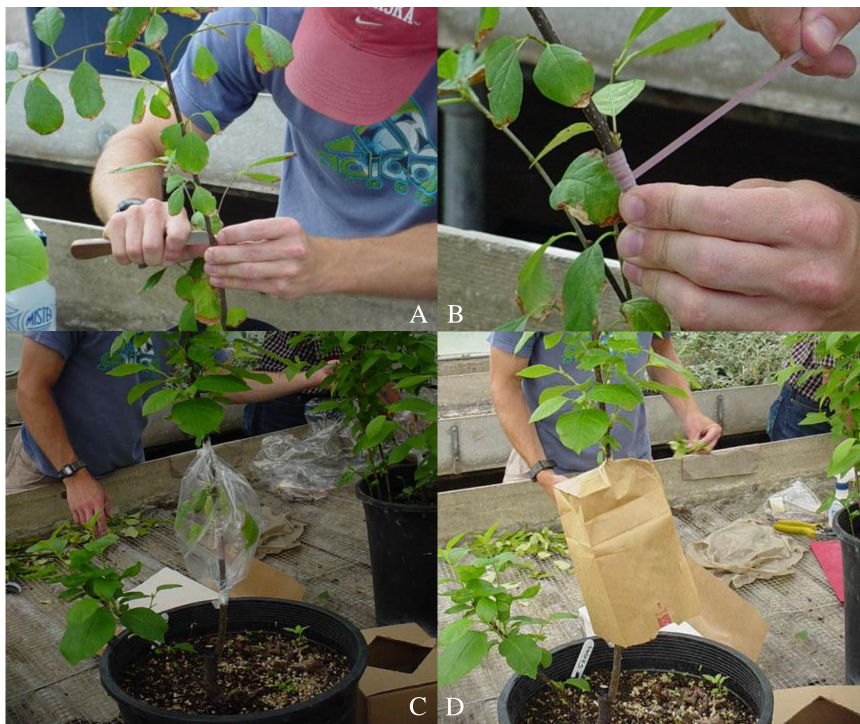

Fig. 2.9. Graft-inoculation of X-disease pathogen to chokecherry seedlings. A: Making cut on the stem of the seedling; B: Inserting the X-disease-infected scion to the cut and tightening with a plastic tape; C: Covering the grafted area with a plastic bag; and D: Covering with a paper bag.

Nested PCR analysis was used to verify the inoculation of X-disease. X-disease was detected on four individuals one week after inoculation. In the 6thweek, 12 chokecherry seedlings were detected infected. In the 8th week, DNA samples were extracted from both leaf veins and roots and a total of 20 plants were detected infected with X-disease phytoplasma (Fig. 2.10). It

appeared that DNA samples extracted from roots showed more amplification than the ones extracted from main vein. DNAs were re-extracted from all survived individuals in the next spring and nested-PCR confirmed that all samples were successfully inoculated with X-disease phytoplasma (Fig. 2.11).

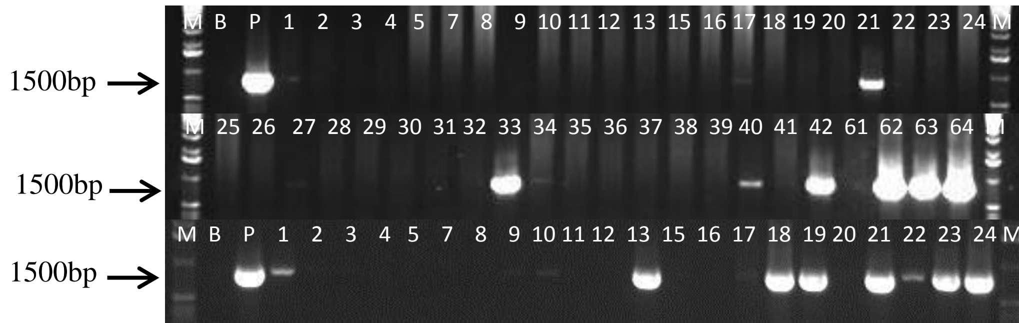

Fig. 2.10. Nested PCR amplified products from chokecherry lines inoculated with X-disease phytoplasma in the 8th week. A. DNA extracted from leaf tissues; B. DNA extracted from roots. Lanes: M = 1 kb DNA ladder; B = Blank; P = positive control.

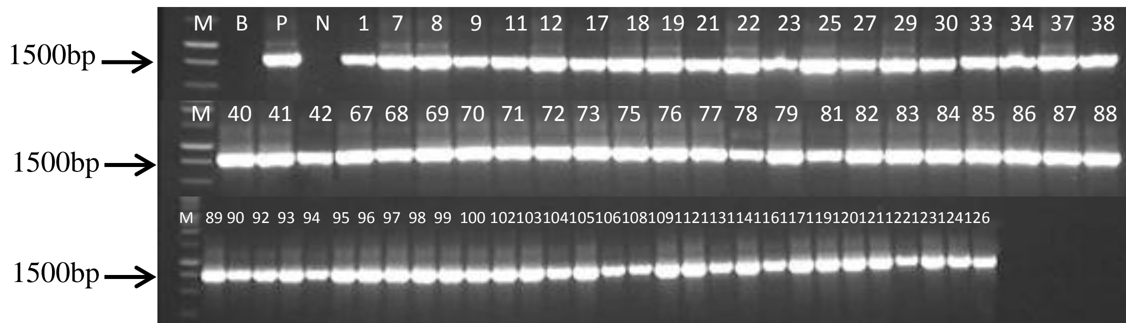

Fig. 2.11. Nested PCR amplified products from chokecherry lines in the next growing season (2011). Lanes: M= 1 kb ladder; B = Blank; P = positive control; N = negative control.

4.4.2 Evaluation of X-disease resistance in the population $C_L \times a$

Evaluation of X-disease resistance has been only done for the population $C_L \times a$. Forty individuals in the population $C_L \times a$ were evaluated for four years (2008, 2009, 2010, and 2011) and the rest of the lines in this population were evaluated for two years (2010 and 2011) (Table

2.4). In 2008, the numbers of lines rated as 5, 4, 3, and 2 were 19, 9, 9, 3, respectively; no line was rated as 1 and 0. In 2009, 21, 7, 8, and 4 lines were rated as 5, 4, 3, and 2, respectively, and still no line was rated as 1 and 0. However, more lines were rated as 3, 2, and 1 as plants were transplanted in the field. In 2010, 20, 7, 39, 12, 8, and 1 lines were rated as 5, 4, 3, 2, 1, and 0, respectively, while in 2011, the numbers were changed to 23, 6, 18, 19, 7, and 13 for each rating classes. If the lines rated as 5 were considered as resistant, the ratio between resistant and susceptible lines fitted to 1:3 (20R: 67S and 23R:63S in 2010 and 2011, respectively) by chi-square analysis (χ^2 = 0.19 and 0.14, respectively; $< \chi^2_{0.05,\,1}$=3.84). If the lines rated as 5 and 4 were both considered as resistant, the ratio between resistant and susceptible lines was also acceptable to be 1:3 by chi-square analysis (χ^2 = 1.68 and 3.49, respectively; $< \chi^2_{0.05,\,1}$=3.84). However, in this case, the ratio also fitted to be 1:2 (χ^2 = 1.07 and 0.005, respectively; $< \chi^2_{0.05,\,1}$=3.84).

X-disease symptoms appeared to be changeable year by year due to changes of environmental conditions every year. The phenotyping data of the population $C_L \times a$ became stable since the third year evaluation (2010). The total number of resistant lines was 27 in 2010 and 29 in 2011. However, the rating of the lines that were previously rated 3 and above tended to be lower to 2 or 1 and some were dead in 2011 (Fig. 2.12).

Table 2.4. Evaluation of X-disease resistance in the population $C_L \times a$.

Plant no.	X-disease severity[a]			
	8/6/2008	8/6/2009	8/11/2010	8/3/2011
1	5	5	5	5
2	4	4	-[b]	-
3	5	4	-	-
4	4	5	2	0
5	5	5	-	-
7	5	5	2	3
8	3	3	2	3
9	5	5	5	5
10	5	5	-	-

Table 2.4. Continued.

Plant no.	X-disease severity[a]			
	8/6/2008	8/6/2009	8/11/2010	8/3/2011
11	5	5	5	5
12	4	4	3	3
13	5	5	2	0
14	-	-	-	-
15	5	5	2	0
16	4	5	2	0
17	3	3	2	3
18	2	2	1	4
19	4	4	1	4
20	5	5	-	-
21	5	5	5	5
22	2	3	2	3
23	5	5	2	4
24	3	2	0	0
25	2	2	1	2
26	3	4	2	0
27	5	5	5	5
28	5	5	-	-
29	3	3	2	3
30	5	5	5	5
31	4	4	-	-
32	4	5	3	0
33	3	2	1	2
34	5	5	5	5
35	4	4	-	-
36	4	3	1	0
37	5	5	1	2
38	3	3	2	2
39	5	5	1	3
40	3	3	3	3
41	5	5	5	5
42	3	3	1	1
67	-	-	5	5
68	-	-	3	2
69	-	-	3	3
70	-	-	3	1
71	-	-	3	4
72	-	-	3	1
73	-	-	3	1
74	-	-	3	0
75	-	-	3	3
76	-	-	3	5
77	-	-	5	5

Table 2.4. Continued.

Plant no.	X-disease severity[a]			
	8/6/2008	8/6/2009	8/11/2010	8/3/2011
78	-	-	4	2
79	-	-	5	5
80	-	-	3	5
81	-	-	4	3
82	-	-	3	2
83	-	-	3	3
84	-	-	3	2
85	-	-	3	2
86	-	-	3	2
87	-	-	4	2
88	-	-	3	5
89	-	-	5	0
90	-	-	3	2
91	-	-	-	-
92	-	-	5	5
93	-	-	3	5
94	-	-	4	4
95	-	-	3	2
96	-	-	3	2
97	-	-	4	1
98	-	-	3	3
99	-	-	4	3
100	-	-	3	5
101	-	-	3	0
102	-	-	5	5
103	-	-	3	3
104	-	-	5	5
105	-	-	5	5
106	-	-	3	4
107	-	-	-	-
108	-	-	5	5
109	-	-	3	0
110	-	-	3	0
111	-	-	-	-
112	-	-	3	2
113	-	-	5	5
114	-	-	3	3
115	-	-	5	-
116	-	-	3	3
117	-	-	3	2
118	-	-	-	-
119	-	-	3	1
120	-	-	4	2

Table 2.4. Continued.

Plant no.	X-disease severity[a]			
	8/6/2008	8/6/2009	8/11/2010	8/3/2011
121	-	-	3	2
122	-	-	5	5
123	-	-	3	1
124	-	-	3	3
125	-	-	-	-
126	-	-	3	2

[a]: Rank of X-disease severity: 0 = whole plant died; 1 =leaves were discolored, most shoots and leaves were very stunted, and very low growth vigor; 2 =leaves were discolored, most shoots and leaves were stunted, and low growth vigor; 3 =leaves were discolored, most shoots and leaves were moderately stunted, moderate growth vigor; 4 = all or part of tree with slight symptoms, high growth vigor; and 5 = no symptoms, high growth vigor. [b]: Missing data.

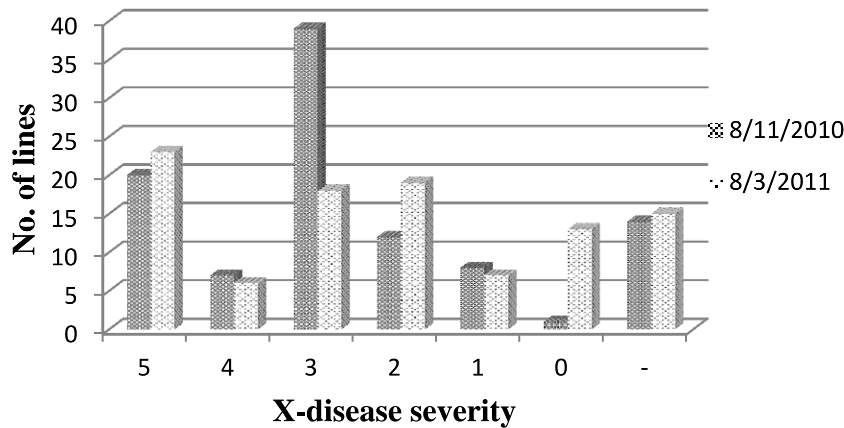

Fig. 2.12. Distribution of X-disease resistance in population $C_L \times a$ in 2010 and 2011.

4.4.3 X-disease resistance in the populations $C_L \times c$ and $C_L \times d$

A total of 181 and 84 individuals were obtained in $C_L \times c$ and $C_L \times d$, respectively. All inoculated individuals were evaluated for X-disease resistance in 2010. In the populations $C_L \times c$, the phenotyping data was collected only in 2010. Because of the severe environment (flooding), all lines of this population were dead in 2011. So the data for the population $C_L \times c$

was discarded. A similar situation occurred in the population $C_L \times d$; however, half of these lines were still surviving in 2011(Table 2.5). The number of lines rated as 5, 4, 3, and 2 was 21, 7, 42, and 1, respectively; no line was rated as 1 and 0 in the first year. In 2011, 9, 5, 15, and 11 lines were rated as 5, 2, 1, and 0, respectively. If only the lines rated as 5 were considered to be resistant, the number of resistant lines in 2010 changed to 21, and the ratio fitted into both 1:2 and 1:3 (χ^2 =0.45 and 0.8, respectively). If the lines rated as 5 and 4 were considered to be resistant, the ratio of resistant to susceptible lines was 28R:43S in 2010, which fitted the ratio of 1:2 (χ^2 =1.19, $<\chi^2_{0.05,\,1}$=3.84) by chi-square test at p = 0.05 level. In 2011, there were no lines rated as 4, the ratio between resistant and susceptible lines was 9R:31S, which fitted to 1:3 (χ^2 = 0.13, $<\chi^2_{0.05,\,1}$=3.84) by chi-square analysis.

Table 2.5. X-disease severity of population $C_L \times d$ in 2010 and 2011.

Plant no.	X-disease severity[a]		Plant no.	X-disease severity	
	8/11/2010	6/28/2011		8/11/2010	6/28/2011
1	2	0	43	3	-
2	3	0	44	3	-
3	5	-[b]	45	3	1
4	-	-	46	3	2
5	3	5	47	-	-
6	3	-	48	5	5
7	5	-	49	-	-
8	3	5	50	-	-
9	3	-	51	5	-
10	4	-	52	3	1
11	3	1	53	5	5
12	4	-	54	3	0
13	5	-	55	3	0
14	-	-	56	3	1
15	3	1	57	5	5
16	3	-	58	3	2
17	3	2	59	3	1
18	3	-	60	3	1
19	3	2	61	4	-
20	3	5	62	3	-

Table 2.5. Continued

Plant no.	X-disease severity[a]		Plant no.	X-disease severity	
	8/11/2010	6/28/2011		8/11/2010	6/28/2011
21	3	1	63	3	1
22	3	0	64	5	-
23	4	-	65	5	-
24	4	-	66	-	-
25	-	-	67	-	-
26	4	-	68	3	1
27	3	-	69	3	0
28	3	0	70	4	-
29	3	0	71	5	-
30	3	1	72	5	-
31	3	-	73	-	-
32	5	5	74	5	-
33	3	1	75	5	0
34	3	1	76	5	-
35	3	0	77	5	0
36	3	2	78	5	-
37	-	-	79	5	-
38	-	5	80	5	-
39	3	1	81	5	-
40	3	1	82	-	-
41	3	-	83	-	-
42	3	-	84	5	5

[a]: Rank of X-disease severity: 0 = whole plant died; 1 =leaves were discolored, most shoots and leaves were very stunted, and very low growth vigor; 2 =leaves were discolored, most shoots and leaves were stunted, and low growth vigor; 3 =leaves were discolored, most shoots and leaves were moderately stunted, moderate growth vigor; 4 = all or part of tree with slight symptoms, high growth vigor; and 5 = no symptoms, high growth vigor.

[b]: Missing data.

4.5 Identification of molecular markers linked to X-disease resistance

4.5.1 BSA (Bulked segregant analysis)

A total of 354 pairs of SSR primers, including 246 pairs developed from chokecherry and 108 pairs adopted from other *Prunus* species, were used to screen the parents of population $C_L \times$ a (C_L and a) and 6 bulks (2 resistant bulks and 4 susceptible bulks). One SSR marker named as C4136 was identified to co-segregate with the susceptible parent (a) and 4 susceptible bulks (Fig. 2.13). This marker was mapped into the linkage group LG 14 of the population $C_L \times$a and LG 1 and LG 7 of the population $C_L \times$d (Fig. 2.14 and Fig. 2.15).

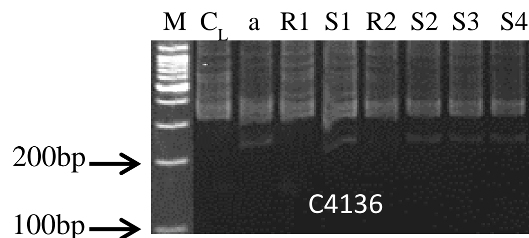

Fig. 2.13. BSA analysis of resistant and susceptible bulks developed from the population $C_L \times$a with C4136 SSR primer. C_L and a: parents; R1and R2: resistant bulks; S1, S2, S3, and S4: susceptible bulks.

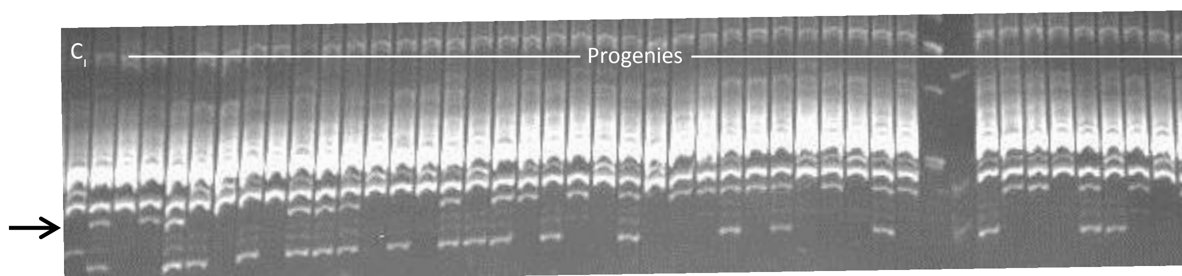

Fig. 2.14. Validation of C4136 marker in the population $C_L \times$a. M = 100 bp ladder; C_L: resistant parent; a: susceptible parent. Arrow pointed out the marker associated with X-disease resistance.

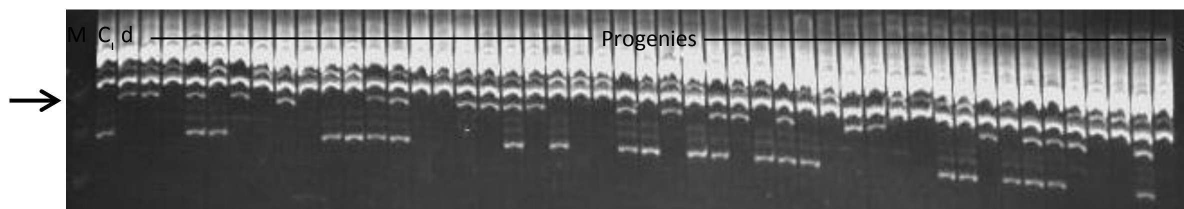

Fig. 2.15. Validation of C4136 marker in the population $C_L \times d$. M = 100 bp ladder; C_L: resistant parent; d: susceptible parent. Arrow pointed out the marker associated with X-disease resistance.

4.5.2 Mapping

The analysis of QTL was performed for the population $C_L \times a$ because its phenotyping data was stable. Marker and phenotype association was evaluated by TetraploidMap software with "markers" routine and "associate trait data" subroutine. As a result, 9 markers were identified to be associated with X-disease resistance with the data collected in 2010 by both Kruskal-Wallis test and ANOVA at 0.05 level, in which BPPCT002-2 and BPPCT017-1 were significantly associated with X-disease resistance (p= 0.01) (Table 2.6). Using the 2011 data, 11 markers were identified to be linked to X-disease resistance and PS12A02-2, PS12A02-3, and PS12A02-4 were significantly associated with X-disease resistance. The marker C4136, which was identified to be associated with X-disease resistance by BSA, appeared in both years.

Table 2.6. Markers associated with X-disease resistance identified using TetraploidMap.

Year	Marker	KWSig.[a]	AVSig.[b]
2010	BPPCT002-1	0.03036	0.0298
	BPPCT002-2	0.00789	0.00761
	BPPCT002-7	0.02874	0.02821
	BPPCT018-1	0.02579	0.02527
	BPPCT017-1	0.00194	0.00184
	BPPCT040-5	0.02471	0.02417
	CPSCT010-1	0.01649	0.01624
	C4136	0.0114	0.01106
	C6957	0.0114	0.01106
2011	BPPCT040-4	0.0483	0.04762

Table 2.6. Continued

Year	Marker	KWSig.[a]	AVSig.[b]
2011	BPPCT040-5	0.01365	0.01325
	BPPCT040-6	0.0483	0.04762
	PS12A02-1	0.03001	0.02944
	PS12A02-2	0.00407	0.0039
	PS12A02-3	0.0003	0.00028
	PS12A02-4	0.00277	0.00263
	C4136	0.02369	0.02318
	C6255	0.01582	0.01544
	C6434-2	0.01511	0.01493
	C6957	0.02369	0.02318

[a] Significance of the Kruskal-Wallis test;

[b]Significance of the analysis of variance.

5. Discussion

5.1 Structure of mapping population

A highly segregated and good size population is critical to construct an ideal genetic linkage map (Collard et al., 2005). Selection of parents that have contrasting characteristics in one or a few traits of interest will result in a high recombination of genes; therefore, these traits will be highly segregated. In this study, X-disease resistance was the trait of interest and the resistance of all four parental chokecherry lines was evaluated for more than ten years. These lines were selected from a large chokecherry germplasm collection collected from different regions. They possess a highly complex genetic background. All three segregating populations were developed from the cross between a resistant and a susceptible line from the collection aforementioned; thus they should provide a larger array of polymorphisms for X-disease resistance.

The size of a segregation population is another important factor for map construction because large population size benefits to estimating the gene recombination. Collard et al. (2005)

suggested that a population size of 50 to 250 would meet the basic requirement for the construction of a linkage map and gene mapping. The size of the optimum population may be affected by the population structure and crop species. For woody species, particularly *Prunus* species, the population size is often restricted by limited seed number (each fruit has only one seed) and a low seed germination rate (required for ideal stratification before germination). It is not surprising that some genetic maps were constructed using a relatively small population, such as sour cherry (84 progenies) (Canli, 2004), sweet cherry (94 progenies) (Clarke et al., 2009), dogwoods (94 progenies) (Wang et al., 2009). A peach linkage map was constructed only using 55 progenies (Lu et al., 1998). In this study, the size of three populations were 82 ($C_L \times d$), 101 ($C_L \times a$), and 177 ($C_L \times c$). The relatively small populations, particularly for the $C_L \times d$ progeny might have caused the segregation distortion detected in the study. For example, common markers were detected mostly between the map of population $C_L \times c$ and $C_L \times a$, or $C_L \times d$, but no consensus linkage groups were generated with the populations of small size ($C_L \times a$ and $C_L \times d$).

Selection of mapping populations depends on the species and the marker system employed (Semagn et al., 2006). For example, the second filial generation (F_2), backcross (BC), recombinant inbred lines (RILs), double haploids (DHs), and near isogenic lines (NILs) are usually used for mapping of self-pollination crop species. For open pollination species, two-way pseudo-testcross, half-sib, and full-sib families that derived from controlled crosses are often used for mapping studies. Most woody species, particularly trees, are open pollinated and they have a long juvenile time, complicated genetic make-up, and inbreeding depression; therefore, it is not applicable to develop true F_2 or backcross populations for mapping (Wedden et al., 1994). In open pollination, particularly in those that are self-incompatible woody species, seeds derived

from a single tree may represent similar haploid genotypes and they can be considered as pseudo-F_1 plants, thus, a pseudo-F_2 population can be developed by crossing two pseudo-F_1 trees (Weeden et al., 1994). This strategy was recently used for genetic linkage map construction of flowering dogwood (Wang et al., 2009). Canli (2004) used the pseudo-testcross strategy to construct a genetic linkage map for sour cherry. Chokecherry is a self-incompatible species. Three populations developed in this study are pseudo-F_2 segregating populations and two parental lines of each cross have a contrasting X-disease resistance. These populations should have a high degree of X-disease resistance segregation; therefore they are suitable for genetic linkage map construction and gene mapping.

5.2 Molecular mapping in polyploid plant species

Polyploid species have complexity of inheritance and uncertainty of genome constitution; therefore, construction of a genetic linkage map for a polyploid species is always a challenge (Wu et al., 1992). Single-dose restriction fragments (SDRFs) have to be calculated to meet the requirement of mapping software for diploid species. An SDRF is defined as the fragment present in one polyploid parent of the cross and segregated in a single-dose ratio (1:1) in the offspring. An SDRF marker is equivalent in both autopolyploid and allopolyploid species where it is considered as a simplex allele in autopolyploids and an allele at one heterozygous locus in allopolyploids (Wu et al., 1992). Hemmat et al. (1994) expanded the usage of SDRFs to loci that are heterozygous in both parents and segregate in a 3:1 ratio for dominant loci in the progenies. Thus, SDRFs combined with pseudo-testcross strategies have been used to construct genetic maps in many polyploid species, such as potato (Ghislain et al., 2004), sugarcane (Hoarau et al., 2001), and sour cherry (Canli, 2004).

5.3 Synteny analysis of molecular loci in two maps

MapMaker is the most common software for map construction of crop plants. MapMaker is usually used with the markers that are polymorphic between two parents. In this study, TetraploidMap and JoinMap were used for map construction and mapping of X-disease resistant genes in chokecherry. TetraploidMap, developed by Hackett and Luo (2003) is map-make software specifically for constructing linkage maps with co-dominant or dominant molecular markers under chromosomal segregation for tetraploid species. JoinMap is usually used to construct integrated genetic maps (Stam, 1993) and different mapping algorithm and genotype code should be used for polyploid species.

In this study, two sets of genetic linkage maps were constructed using TetraploidMap and JoinMap individually. JoinMap was also used to join three female parent maps (C_L) from three populations for comparison of the chokecherry maps with the *Prunus* reference maps (T $\times$ E). The SSR marker order and distance between the two map sets had been compared. Both sets of each population map used the same data sets calculated for SDRFs using TetraploidMap, but not all markers were mapped on the maps. Generally, most markers located on the maps constructed by JoinMap (J map) were also mapped on the ones constructed by TetraploidMap (T map) (Fig. 2 to 4). Linkage groups in the J map were numbered according to the homologous linkage groups in the T map. For instance, 110 and 93 markers were mapped on the parental (C_L) linkage groups by T and J, respectively. All markers but 5 (CPDCT008, C8169, BPPCT032, C6256b-2, and CPSCT002-1) on the J map were included in the T map. Similar situations were observed in other parental linkage maps (a, c, and d) in other populations. The high percentage of common SSR markers in J maps showed in T map indicated that the grouping calculation of software JoinMap was stricter than TetraploidMap. Some markers grouped in T map remained unlinked in

J map. Although a high number of common markers was detected in this research, the marker distance and order were different from each other, which is consistent with the result in alfalfa research (Robins et al., 2008) where a recent genetic map constructed by TetraploidMap was different from the original map constructed by JoinMap. Such difference may be caused by the different ordering algorithm: two-point linkage analysis and regression mapping algorithm, were used in these two software programs (Stam, 1993; Luo et al., 2001). Moreover, some linkage groups in the T map corresponded to two or more sub-linkage groups of the J map. For example, the linkage group 6 of the T map in the population $C_L \times c$ corresponded to four sub-linkage groups in the J map, which may also be caused by the different analysis methods used in the two software programs. In general, the T map here can be treated as a frame with more markers, while the J map was constructed with stricter conditions and is more reliable than theT map.

5.4 Synteny analysis with the *Prunus* reference maps

The number of SSRs in three chokecherry maps was compared to the $T \times E$ map, the *Prunus* bin reference map (Dirlewanger et al., 2004a) (Table 2.7). In the map of $C_L \times a$, 10 linkage groups were found to share two or more markers with the linkage groups in the reference map. In the map of $C_L \times c$, seven linkage groups shared common markers with six linkage groups in the reference map. In the map of $C_L \times d$, eight linkage groups detected common markers shared in the $T \times E$ linkage groups. The result indicated that these linkage groups in the chokecherry linkage maps might be homologous to the corresponding linkage groups in the $T \times E$ map. Comparing the joined J map (Fig. 5) to the reference map, four linkage groups were found to be homologous to the linkage groups in the $T \times E$ reference map: the linkage group P1-1 + P2-3b shared 3 common markers with the linkage group 2 in the $T \times E$ map; the linkage group P1-14a + P2-13 + P3-10 had 3 common markers compared to the linkage group 7; the

linkage group P1-8 + P2-5 + P3-15a and P2-9b + P3-(1+2a) were homologous to the linkage group 4 and 2 with 2 common markers each group, respectively. Comparing to the result of alignment between the sweet cherry map and the T × E map reported by Olmstead et al. (2008), more SSRs were found shared between the two parental maps and the *Prunus* reference map in all eight linkage groups. It can be explained with the difference between diploid and tetraploid species.

Compared to our chokecherry linkage maps, three markers (pchgms3, PS12a02, and PceGA34) tested in this research were anchored in the sour cherry map (Canli, 2004). Marker pchgms3 produced 4 alleles from sour cherry DNA, while it amplified 3 alleles from chokecherry DNA. One allele was mapped on the linkage group 1 in the map of sour cherry. In contrast, two alleles were mapped on two different linkage groups in the map of chokecherry (C_L × a) and both linkage groups were homologous to linkage group 1 in the T × E reference map.

Table 2.7. Linkage groups in chokecherry maps homologous to the T × E reference map.

Map	LG[a] in chokecherry map	LG in T × E map
C_L × a	LG1	G2
	LG4	G1
	LG6	G2
	LG8	G4
	LG9	G3
	LG10	G1
	LG11	G4
	LG14	G1
	LG15	G6
	LG16	G2
C_L × c	LG2	G4
	LG3	G2
	LG4	G3
	LG6	G1
	LG8	G2
	LG11	G8

Table 2.7. Continued

Map	LG[a] in chokecherry map	LG in T $\times$E map
	LG16	G6
$C_L \times d$	LG2	G3
	LG3	G3
	LG4	G7
	LG7	G1/7
	LG9	G5
	LG10	G7
	LG11	G2
	LG13	G4/6

[a]: LG: linkage group.

Three of four alleles of PS12a02 were mapped on the map of sour cherry and located on linkage groups 3 and 11. Two alleles produced from PS12a02 were positioned on the LG4 in the map of $C_L \times a$ and the LG2 and LG15 in the map of $C_L \times c$, in which the LG2 was homologous to the LG4 in the reference map. The third marker PceGA34 was located on the different linkage groups in the chokecherry map and sour cherry map, while it is positioned on the homologous linkage group of the reference map. The low number of SSRs used in the sour cherry map makes it hard to compare these two maps of tetraploid species in *Prunus* genus. More common markers will be present once more SSR primers that were used for the sour cherry map construction are tested in the future.

5.5 Transferability of SSR markers from other *Prunus* species to chokecherry

In this study, 108 SSR primers developed from other *Prunus* species were used to amplify SSRs in chokecherry. A total of 93 primers (86.1%) amplified bands in chokecherry and 73 primers (67.6%) were polymorphic and can be used for map construction. Dirlewanger et al. (2004b) reported that 264 out of 277 (95.3%) SSR markers developed from peach, cherry, plum, apricot, and other *Prunus* species can be amplified in their hybrid progenies and 204 primers within those primers (73.6%) had polymorphisms. Dondini et al. (2007) observed 70% of

primers developed from almond, apricot, peach, and sweet cherry can be transferable to apricot and 58.6% of SSR primers produced polymorphism in their pseudo-F_2 mapping population. The suitability of the transferable SSR primers for mapping in chokecherry depends on the species from which the primers were developed. For example, primers developed from sweet cherry amplified more bands in chokecherry than the ones developed from other species. Primers developed from apricot produced the lowest number of bands in chokecherry. This indicates that the performance of the transferrable primers is related to the genetic distance between species. However, the result might be biased because of the small number of primers tested. These transferable primers amplified more bands from chokecherry (an average of 3.3) than from other *Prunus* species (an average of 2.6) (Downey et al., 2000; Struss et al., 2003; Mnejja et al., 2004, 2005); however, a similar number of bands was amplified from sour cherry (Cantini et al., 2001). It may be explained by the tetraploid nature and the same genome complexity of chokecherry and sour cherry.

As mentioned in the sour cherry linkage map study, tetraploid *Prunus* species should have 16 linkage groups and cover twice the length of the linkage map of diploid *Prunus* species (Canli, 2004). The average length of the chokecherry linkage map constructed in this study is around 1050 cM, which was shorter than the expanded $T \times E$ reference map (1144 CM) constructed based on the cross between peach and almond (Bliss et al., 2002), but much longer than that of the sour cherry linkage map (442.4 cM). It is the first attempt to construct genetic linkage maps for chokecherry, a unique species for research on both polyploid and X-disease resistance in woody fruit species. The maps will be expanded when more markers are available and will be further used for gene mapping and other genetic research.

5.6 X-disease resistance in mapping populations

Comparing four years' phenotyping data for part of the population $C_L \times a$, the severity of X-disease rating was not consistent in the first two years, especially for those individuals rated to be resistant. The rating of 12 individuals dropped in the last two years. Aldaghi et al. (2007) reported that the typical symptoms of phytoplasma can be observed during the second month after grafting on periwinkles that were infected by apple proliferation (AP). However, the speed of symptom appearance is not so fast in trees. Aldaghi also reported that 75% of the apple rootstocks showed symptoms by the end of the 7th month. Peterson (1984) studied the spread and damage of X-disease for chokecherry in eastern Nebraska and found that the typical symptoms of X-disease appeared on 60% of the chokecherry trees within 3 years after the X-disease pathogen was introduced and, more trees had symptoms after another two years. It means that the appearance of X-disease symptoms cannot be stable until 5 years after grafting. In this study, the rate of X-disease resistance appeared to be stable since the third year after inoculation. It might be due to the small size of hybrid lines (rootstocks) or strong X-disease strain used as inoculum. Minor changes of the rate may be seen in the next few years.

According to the phenotyping data in the latest two years (2010 and 2011) for the population $C_L \times a$, the segregation ratio of the progenies fitted to a 1R:3S ratio at $p = 0.05$ level by chi-square analysis. It indicated that X-disease resistance might be governed by a single recessive gene. This conclusion was consistent with the research conducted by Singh et al. (2007), where they found a single recessive gene controlled phyllody (a phytoplasma disease) resistance in cultivated sesame varieties. In 2011, the ratio 1R:3S was also observed in the population $C_L \times d$ as well. Additionally, one dominant marker (C4136) was identified by BSA. This marker was associated with the susceptible parent and susceptible DNA pools and gave 1:1

ratio in progenies of population $C_L \times a$ and $C_L \times d$. This observation further confirmed that the resistance to X-disease in chokecherry might be controlled by a single recessive gene.

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CHAPTER 3. DEVELOPMENT AND CROSS-SPECIES/GENERA TRANSFERABILITY OF MICROSATELLITES DISCOVERED FROM 454 GENOME SEQUENCES IN CHOKECHERRY (*PRUNUS VIRGINIANA* L.)

1. Abstract

Chokecherry (*Prunus virginiana* L.) (2n = 4x = 32) is a unique *Prunus* species for both genetics and disease resistance research due to its tetraploid nature and X-disease resistance. A partial chokecherry genome was sequenced using Roche 454 sequencing technology. A total of 145,094 reads covering 4.5 Mb of chokecherry genome were generated and 15,113 contigs were assembled, in which 11,675 contigs were larger than 100 bp in size. A total of 481 SSR loci were identified from 234 (out of 11,675) contigs, in which 246 had flanking sequences suitable for polymerase chain reaction (PCR) primer design; thus, 246 primer pairs were designed. Of 246 primers, 212 (86.2%) effectively produced amplification from genomic DNA of chokecherries. All 212 amplifiable chokecherry primers were used to amplify genomic DNA from other 11 rosaceous species (sour cherry, sweet cherry, wild cherry, peach, apricot, plum, apple, crabapple, pear, june berry, and raspberry). As a result, chokecherry SSR primers can be transferable in *Prunus* species or other rosaceous species. An average of 63.2% and 58.7% of amplifiable chokecherry primers amplified DNA from cherry and other *Prunus* species, respectively, while 47.2% of amplifiable chokecherry primers can be transferable to other rosaceous species. Using random genome sequence data generated from the next-generation sequencing technology to identify microsatellite loci appears to be rapid and cost-efficient, particularly for species with no sequence information available. Sequence information and confirmed transferability of the identified chokecherry SSRs among species will be valuable for the genetic research in *Prunus* and other rosaceous species.

2. Introduction

Chokecherry (*Prunus virginiana* L.) is a small tree or large shrub widely distributed across the United States and Canada. Native to North America, chokecherry is one of the native species (pincherry, cranberry, blueberry, etc.) used in small fruit production for beverages, jellies, dried fruit products, and wine. It is also used as an ornamental plant because of the beautiful white flowers in spring and colorful leaves and fruits in fall. Chokecherry belongs to the genus *Prunus* in the Rosaceae family. The genus *Prunus* consists of more than 200 species of trees and shrubs including many important stone fruit species, such as peach, sweet and sour cherry, nectarine, apricot, plum, almond, etc. Chokecherry has the same base chromosome number as other *Prunus* species ($x = 8$); however, it is a tetraploid, having 32 chromosomes ($2n = 4x = 32$). Among diseases in *Prunus* species, X-disease, caused by X-disease phytoplasmas, is a severe disease of stone fruit trees in North America. Phytoplasmas are known to cause diseases in more than 300 higher plant species including many economically important food, fiber, forage, fruit and ornamental plants (Lee et al., 1992). X-disease is one of the limiting factors for production of many major *Prunus* species (Rosenberger, 1982) and is particularly devastating to peaches, cherries, nectarines, and chokecherries (Gilmer et al., 1954; Rosenberger and Jones, 1977; Peterson, 1984). So far, no effective and practical methods are available to control X-disease. X-disease resistance in chokecherry is the only reported case within single stone fruit species (Walla et al., 1996). Such resistance is unique and currently not available for other stone fruit species due to the genetic incompatibility among species in *Prunus* (Moore and Janick, 1983); therefore chokecherry is considered as a model for phytoplasma disease research in woody species, particularly in stone fruits.

In the last two decades, advances in plant genetics and genomics facilitate the development of molecular makers that can largely improve plant breeding efficiency through marker assisted selection (MAS), gene mapping, and genetic transformation. Microsatellites or simple sequence repeats (SSRs) are repeating sequences of 1-6 base pairs of DNA. The DNA sequences flanking SSRs are conserved and can be used to design PCR primers that are used to amplify the intervening SSR. SSR markers are known to be co-dominant, reproducible, relatively abundant, and multi-allelic and widely used for genotype fingerprinting, genetic map construction, gene identification, and marker-assisted selection (Agarwal et al., 2008; Parida et al., 2009). SSRs can be discovered from SSR-enriched genomic libraries or random genomic sequences. A large number of SSR markers have been developed from EST (expressed sequence tags) database because EST-SSRs are targeting to the transcribed region of the genome and these SSRs appear to be more closely related to the important agronomical traits (Qi et al., 2010). However, EST-SSR markers are less polymorphic and their development is restricted by the availability of sequence database (Gupta et al., 2003). Recently, the application of next-generation sequencing (NGS) technology to genome sequencing allows us to discover a large amount of genome-wide and gene-based microsatellites in a much more efficient way (Mardis, 2008; Jun et al., 2011; Zalapa et al., 2012). The method of 454 sequencing is one of the two NGS technologies (the other one is Illumina sequencing) used for the discovery of SSR in plants. The 454 sequencing method not only has one major advantage of NGS: avoiding the SSR enrichment step because a great number of SSR can be detected from a large DNA sequence data, but also produces longer reads (350-600 bp per read) that increases the likelihood to design SSR primers. To date, SSR markers have been recently developed using 454 genome sequences for more than 20 plants and this number is increasing rapidly (Zalapa et al., 2012).

It has been well recognized that some molecular markers including SSRs can be transferable from different genotypes within or between species or even between genera (Kalia et al., 2011). Such an interspecifc or intergeneric transferability makes SSRs a widely useful tool for genetic studies, such as fingerprinting, genetic map construction, and molecular marker identification. The transferability rate of SSRs depends on the genetic distance between the individual genotypes. The closer the genetic relationship between genotypes, the more transferable the SSR is (Luro et al., 2008). For instance, Singh et al. (2011) found that SSR markers derived from sugarcane had a high transferability rate within *Saccharum* complex (98.0%) and cereal genomes (88.3%).

Transferability of SSRs has been well applied to many aspects of the genetic research in *Prunus* and other rosaceous species since the first SSR marker was developed in peach (Cipriani, et al., 1999; Mnejja et al., 2010). Microsatellites have been discovered from many species in the family of Rosaceae, particularly from most of the commercial species, such as peach (Aranzana et al., 2002), almond (Mnejja et al., 2005), apricot (Hagen et al., 2004), sweet cherry (Olmstead et al., 2008), sour cherry (Canli, 2004), apple (Gasic et al., 2009), pear (Yamamoto et al., 2002), and strawberry (Lewers et al., 2005). Transferability of these SSRs in or between rosaceous genera has been evaluated. Decroocq et al. (2003) tested 10 apricot EST-SSR markers in a few *Prunus* and other rosaceous species (apple and pear) and found that only one marker was transferable across all tested species. Moreover, transferability of SSRs depends on the relationship between species tested and the one from which the SSRs were identified. Mnejja et al. (2010) reported that SSR markers developed from peach and almond had a higher transferability rate in *Prunus* species than in three other non-*Prunus* rosaceous genera (apple, pear, and strawberry). Among *Prunus* species, a higher transferability rate was found from peach

to plum than to cherry because the genetic distance between peach and plum is closer than the one between peach and cherry. Wünsch (2009) reported that 13 out of 18 SSR markers developed from peach and cherry were transferable in 27 varieties of 10 *Prunus* species, but only two loci were polymorphic in all species.

The objectives of this study were to develop SSR markers from chokecherry genome sequences generated using Roche 454 sequencing technology and to evaluate transferability and polymorphism of the SSRs in other *Prunus* and rosaceous species. The SSR primer resource developed from this study will provide useful information and tools for the genetic research in *Prunus* and other species in the Rosaceae family.

3. Materials and methods

3.1 Plant material and genomic DNA extraction

A total of 17 genotypes from seven *Prunus* and five other rosaceous species were used. Seven *Prunus* species were chokecherry (*Prunus virginiana*, three lines), sour cherry cultivars (*P. cerasus* 'Rheinische Schattenmorelle', 'Balaton', and 'North Star'), sweet cherry (*P. avium* 'Emperor Francis' and 'Schneider'), wild cherry (*P. serotina*), peach (*P. persica*), apricot (*P. armeniaca*), and plum (*P. nigra* × *P. salicina* 'Pembina'). Five other rosaceous species were apple (*Malus* × *domestica* 'Haralson'), crabapple (*Malus domestica* 'Dolgo'), pear (*Pyrus communis*), juneberry (*Amelanchier alnifolia*), and red raspberry (*Rubus idaeus* 'Boyne').

Genomic DNA was extracted followed the method described in Chapter 2 (3.4.1).

3.2 NGS 454 sequencing and sequence assemblies

Approximately 20 μg of genomic DNA extracted from the root tissues of a X-disease resistant chokecherry was sent to the Center for Genetic, Proteomic, and Bioinformatic Research at University of Hawaii-Manoa for library preparation and sequencing using the 454 Genome

Sequencer FLX Titanium (Roche Applied Science) following emulsion polymerase chain reaction (emPCR). Newbler was used to analyze the sequencer-generated SFF data and assemble the reads into contigs in FASTA format files via a command line (runAssembly), which was kindly provided by Dr. Zheng Jin Tu at the Supercomputing Institute for Advanced Computational Research at the University of Minnesota.

3.3 Identification of SSRs and design of SSR primers

All contigs or sequences longer than 100 bp were searched for microsatellites using the software SSRIT (Simple Sequence Repeat Identification Tool) available at www.gramene.org/db/markers/ssrtool. The minimum number of repeat motifs to be considered as a microsatellite was 12 repeats for a mononucleotide motif and more than five repeats for 2-5 bp-nucleotide motifs. Primers were then designed using the online software Primer3 (http://frodo.wi.mit.edu/primer3/).

3.4 Amplification of SSRs in chokecherry and other species

To validate the newly-designed SSR primers and their transferability within the genus *Prunus* and among the species in other genera of Rosaceae, PCR amplification was conducted. Amplification reactions were carried out in 18 μl volumes containing 60 ng of template DNA, 2.0 mM $MgCl_2$, 10× buffer, 200 μM dNTP, 0.2 pmol of each primer, and 0.125 U *Taq* DNA polymerase. The amplification was performed under the condition of denaturing for 30 seconds at 94 ℃ (5 min for the first cycle), annealing for 30 seconds at 57 ℃, and extension for 30 seconds at 72 ℃ (7 min for the final cycle) for 35 cycles. The PCRs were performed on Programmable Thermal Controller PTC-100™ or Applied Biosystems 2720 Thermal Cycler.

PCR products were separated in a non-denaturing 6% polyacrylamide gel (29 : 1 acrylamide : bis, J.T. Baker, Mallinckrodt Baker, Inc. NJ). The gel was prepared followed the method described in Chapter 2 (3.4.2.3).

3.5 Scoring of amplification

Gel images were scored based on the presence and absence of each band in the image. The number of bands amplified by each primer was recorded when the number is equal or less than 5 and "M" is used when the number of amplified bands was more than 5.

4. Results

4.1 Analysis of the 454 sequences of chokecherry

A total of 145,094 reads were generated from a half run of 454 sequencing of the chokecherry genome with an average length of 32.85 bp per read and a total length of 4,766,864 bp nucleotides. After assembling all reads, 15,113 contigs were obtained with an average contig length of 315.4 bp nucleotides in which 3,438 of the contigs were smaller than 100 bp. Of 11,675 contigs that were larger than 100 bp, 9,651 (82.7 %) were in the range of 100 to 600 nucleotides in size. The other 17.3 % of contigs were larger than 601 bp including 49 scaffolds (0.42 %) with the length of nucleotide greater than 2,000 bp (Fig. 3.1).

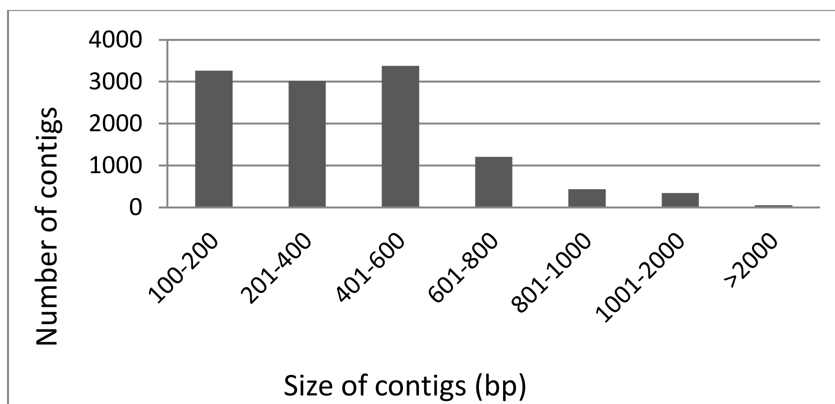

Fig. 3.1. Distribution of contigs which are greater than 100 nucleotides after assembly of sequence reads from a half run of 454 sequencing of chokecherry.

4.2 Discovery and analysis of chokecherry microsatellites

Of all contigs (11,675) that were larger than 100 nucleotides, 405 contigs contained one or more microsatellites and a total of 481 SSR loci were identified (Table 3.1). Among these SSR loci, 413 were dinucleotides (85.86%), followed by 62 trinucleotides (12.89%), 3 tetranucleotides (0.62%), and 3 pentanucleotides (0.62%). The CT/GA repeat (24.32%) was the most frequent repeat motif, followed by AT/TA (22.45%) and AG/TC (21.83%). Using the online software Primer3, 234 contigs that contain SSRs were chosen to design SSR primers and a total of 246 primers were designed. Within the 234 contigs, 11 contigs contained more than one SSR locus. The primer C3280 and C4274 contained three SSR loci and C5595, C5948, C5956, C8681, C10447, C10749, C13713, C13993, and C15115 contained two SSR loci.

4.3 Amplification of the new SSR primers in chokecherry and cross-species/genera

To verify the effectiveness of newly-designed SSR primers, they were used to amplify genomic DNA of three chokecherry lines, six other *Prunus* species, and four other rosaceous species (Fig. 3.2 and Fig. 3.3). Of all 246 new primers, 212 (86.2%) effectively amplified DNA from chokecherries (Table 3.3). Tested in other cherry species, 176 (71.5%), 160 (65.0%), and 147 (59.8%) of chokecherry primers produced amplification of three sour cherry cultivars, ‘RS’, ‘Balaton’, and ‘North Star’, respectively, followed by wild cherry (155 primers, 63.0%), two sweet cherry cultivars ‘EF’ (149, 60.6%) and ‘Schneider’ (152, 61.8%). For other *Prunus* species, 152 (61.8%) of primers amplified DNA of peach, followed by plum (142, 57.7%) and apricot (139, 56.5%). Amplification rate of these new chokecherry SSR primers in other non-*Prunus* species in Rosaceae family was decreased, ranging from 58.9% in apple to 53.7% in crabapple, 49.2% in juneberry, 46.3% in raspberry, and 28% in pear.

Table 3.1. Characterization of SSR loci discovered in 405 contigs of chokecherry genome.

Motif type	Repeat motif	No. of a given motif type	Frequency (%)
Dinucleotide	CT/GA	117	24.32
	AT/TA	108	22.45
	AG/TC	105	21.83
	AC/TG	41	8.52
	CA/GT	28	5.82
	CG/GC	14	2.91
Subtotal		413	85.86
Trinucleotide	CTC/GAG	8	1.66
	CCA/GGT	7	1.46
	AAC/TTG	6	1.25
	AAG/TTC	6	1.25
	CTT/GAA	6	1.25
	AGA/TCT	4	0.83
	AGT/TCA	3	0.62
	ATC/TAG	3	0.62
	AAT/TTA	3	0.62
	CCT/GGA	3	0.62
	ACT/TGA	2	0.42
	CAA/GTT	2	0.42
	CAC/GTG	2	0.42
	CTG/GAC	2	0.42
	ACA/TGT	1	0.21
	ACC/TGG	1	0.21
	ATA/TAT	1	0.21
	ATG/TAC	1	0.21
	CTA/GAT	1	0.21
Subtotal		62	12.89
Tetranucleotide	ACAA/TGTT	1	0.21
	GTGC/CACG	1	0.21
	TAAA/ATTT	1	0.21
	TTTG/AAAC	1	0.21
Subtotal		3	0.84
Pentanucleotide	CAACT/GTTGA	1	0.21
	TGGTT/ACCAA	1	0.21
Subtotal		3	0.42
Total		481	100

A total of 76 (30.9%) chokecherry primers produced amplicons in all twelve *Prunus* genotypes, in which, 26 (10.6%) primers amplified DNA from both *Prunus* and other rosaceous species. Nineteen (7.7%) primers were considered as chokecherry specific as they produced no amplicons in any other species tested. Two primers, C837 and C6387, can amplify DNA from all species but Cho1 and Cho2, respectively.

In this research, only sour cherry and sweet cherry had more than one cultivar. Most of the chokecherry SSR primers produced polymorphism within three genotypes of sour cherry species (172, 69.9%), while only 47 primers produced polymorphism between two sweet cherry genotypes.

4.4 Number of bands amplified in chokecherry and other species

The number of bands that individual SSRs amplified varied in species and genotypes (Table 3.2 and 3.4). Most of primers produced more than two bands, especially in polyploid species (chokecherry and sour cherry). In average, each chokecherry SSR primer amplified 2.46 bands (excluding the SSRs that produced more than 5 bands). Chokecherry primers amplified an average of 3.33 and 2.52 bands from chokecherry DNA and sour cherry DNA, respectively. For other diploid *Prunus* species, the number of bands amplified was 2.29 from apricot DNA, followed by wild cherry (2.22), plum (2.21), sweet cherry (2.18), and peach (1.86). For non-*Prunus* rosaceous species, the highest band number amplified was observed in apple (2.75), followed by crabapple (2.69), juneberry (2.18), raspberry (2.26), and pear (1.55).

Table 3.2. Performance of chokecherry SSR primers in *Prunus* and other rosaceous species.

Alleles number	Cho1[a]	Cho2	Cho3	RS	BA	NS	EF	Sch	Wch	Apr	Pch	Plu	App	Cra	Pea	Ras	Jbe
1	18	16	12	51	45	41	55	59	58	49	42	50	33	29	43	36	37
2	33	42	34	29	31	29	36	42	31	32	43	38	28	28	17	34	31
3	32	37	39	31	34	28	19	17	23	18	21	14	28	27	6	23	19
4	43	37	42	23	17	18	15	11	15	14	21	18	21	14	3	10	6
5	46	38	39	24	18	10	10	10	12	13	13	9	20	18	0	4	11
M[b]	40	41	46	18	15	21	14	13	16	13	12	13	15	16	0	7	17
subtotal	212	211	212	176	160	147	149	152	155	139	152	142	145	132	69	114	121
0	34	35	34	70	86	99	97	94	91	107	94	104	101	114	177	132	125
Total	246	246	246	246	246	246	246	246	246	246	246	246	246	246	246	246	246

a. Cho1= chokecherry line (C_L); Cho2= chokecherry line (c); Cho3 = chokecherry (d); RS = sour cherry (‘RS’); BA = sour cherry (‘Balaton’); NS = sour cherry (‘North Star’); EF = sweet cherry (‘EF’); Sch = sweet cherry (‘Schneider’); Wch = wild cherry; Apr = apricot; Pch = peach; Plu = plum; App = apple; Cra = crabapple; Pea = pear; Ras = raspberry; Jbe = june berry.

b. M: the number of bands > 5.

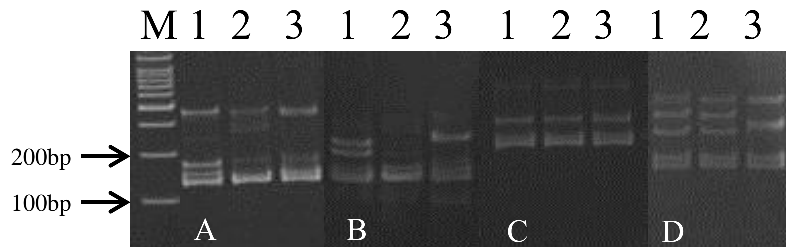

Fig. 3.2. A gel image of amplification patterns from three chokecherry lines using four primers. A: primer C1476; B: primer C1585; C: primer C1795; D: primer C7319. M=100bp DNA ladder; 1= C_L; 2 = c; and 3 = d.

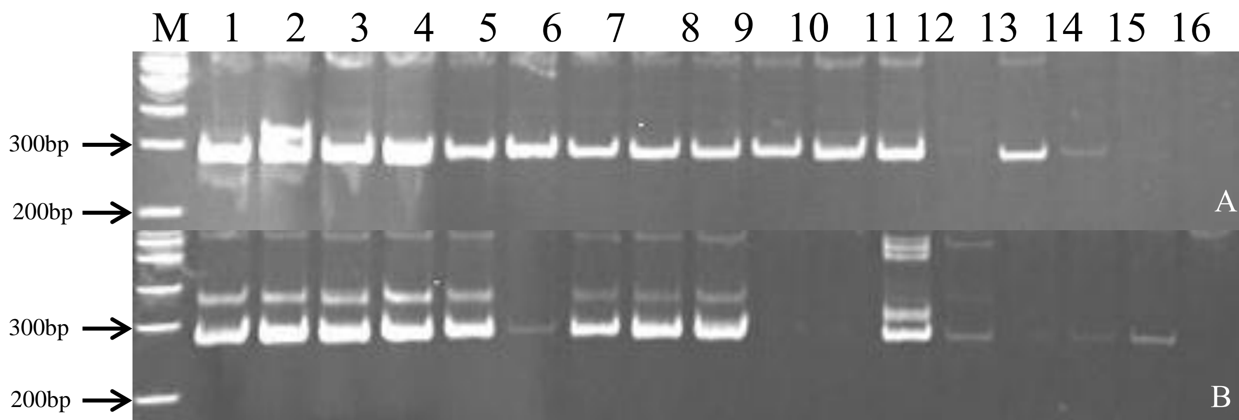

Fig. 3.3. A gel image of amplification patterns from different species using primers C162 (A) and C1795 (B). M = 100 bp DNA ladder; 1 = chokecherry (C_L); 2= chokecherry (c); 3 = chokecherry (d); 4 = sour cherry ('RS'); 5 = sour cherry ('Balaton'); 6 = sour cherry ('North Star'); 7 = sweet cherry ('EF'); 8 = sweet cherry ('Schneider'); 9 = wild cherry; 10 = apricot; 11 = peach; 12 = plum; 13 = apple; 14 = crabapple; 15 = pear; 16 = raspberry; and 17 = june berry.

Table 3.3. Characteristics of SSRs, primers, and products of all 212 amplifiable chokecherry SSRs.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')
C34	cg	5	280	CGAGTCGATTTGTTTCCAAT
				TATTGCCGATACCGATGAAT
C162	ga	6	226	GGTTGTGGTGGTGAGAGGTA
				TCAGCTGGATTAATGGCTCT
C205	gc	5	245	CGTGACACAGCCCCATAAT
				GGCCTTGTTCACTTTCTCCT
C324	tta	5	293	TTTATGGTTCCCAGGCAGTA
				TAATAGCCGTTGTCGAGGTC
C525	ct	5	205	CACCGTGTCACTGTACCAAG
				CGGAGGATCTGAGTGAGAGA
C629	ctt	5	262	CCTCTTTCTCTGCCTCAAAA
				GTTTCTGGTGCTTGTCTGCT
C837	ca	5	291	GAGCACGGTAGACATGGTGT
				ATTGACCATGGGGGTAGG
C1114	at	5	313	TTCCCCTACTGAAGGTCCTC
				ACATTTGGACGTTGTTGGAC
C1181	tg	5	355	CTAGGCATGATTTGGGATTG
				TAGGCAGGAAGCTAGCTGAA
C1231	ttg	5	311	TTCGATCTTTGGGTTTAGGA
				CATTGAGGTGGAGGATTCTG
C1322	at	5	203	ATCAATCGACAGCGAGAGAG
				GCAATGATTAGTCCTTGAGCtT
C1476	ag	6	256	TTTCCAGGGAAAAGTGATGA
				CGGTCAGGTGCTTCTCAGTA
C1585	at	6	202	CAAACACGGACGAGAGAAGT
				TAAATCCGGATGTCCAGAAA
C1795	atc	5	260	TAACGCCATGTGAAGGTTCT
				TGAAGAGTCCATGCATGTTG
C1882	ag	5	318	CCAAAGCTTCACcTCTTTAGG
				TGCTAGATGGAGGTGGACAT
C2103	tc	6	133	CATTGCATGGTCTCTCAGTG
				AATCCTCTTTCCCATTCGAC
C2109	ct	6	201	AAGGGCATTTTGGGTATTTC
				ACGAGCCGGTTCTTTAGG
C2175	ta	6	202	GTTTTGTGGTATGGCAGGAC
				TGCCGTAAATTTTGTGTGTG
C2194	ac	6	341	AGAGAGGAGGATGATTTCTCA
				CCGGTAAAAGTCAAACCTTG

Table 3.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')
C2250	gtg	5	359	CACACAGTTGAAGCCAGATG
				ATCAAGTCCAGGAGCTACCC
C2525	tg	5	338	TTCTCCTTCTCAGCAGCAAT
				GCTTTTGCTACAATCGGAAA
C2556	ta	5	339	ATTGGATGCATGGGGTTAT
				CCATCAACTCTGGCTCCTAA
C2743	ag	5	360	ATGAGAGGAATTTGCAGCAG
				TTCGCTTACGTTTGATTAGGA
C2762	tc	6	246	TGCTTGATTCGAACTTCCAT
				CGCTATATATACTCACATCCAGGTC
C2824	tg	5	397	TTGACCAAGTGGGAGAATGT
				GCCATTTGAATGATCGAAAG
C2838	gc	5	399	GACCTTTTTGCGCGTGTT
				CAATAGCAATTCGGGATCG
C2927	aac	5	311	TCCGGTAGAAAGACTTGTCG
				GAGGAAGAAGAATGGCTTCC
C2938	ga	5	344	TCAAACCCCAAGAGTGTTTC
				TCTCCCATTTCTTCATCTGG
C2997	ga	8	306	CCAAAAACAAAAGCTGGTTC
				CATGGCCTAAAGGCTACTGA
C3205	atc	6	146	CCTCATGGATTCACCAACTC
				TGCAAGTGCAAGACACTGTT
C3280-2	ga	6	301	ACCCCAGAAACATGATCAGA
				GGGATATTCCCTCTCTCTAACC
C3292	ga	8	175	CCTCTGATGGACCTGAAGAA
				CACCCTGCTAGAATGGAAAA
C3332	at	5	184	AAGTGCTAGCCCCTGGTAAC
				TGCCATCGACATTGACTCTA
C3522	at	5	232	TGGAGAGTTGGTGGAAAAGA
				TGGAAAGCCAAAAGATGAAG
C3603	gat	5	246	TGGTTCCATCTTTAGCTTGG
				TGACTTAAGCTTCGGAGTGC
C3635	ttg	5	206	GGAAATTGAATTCACCCAACT
				GGCCAATTTCTTGATTACCA
C3637	ta	5	170	CCCTATTATTTAAAAACCGTCGT
				TGAGTTGAAGAAAGATAGCGAAA
C3656	tc	5	400	GGCCCGTTTTAAGTTTCTTT
				ATCAACAATCAAAGCCCAAA

Table 3.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')
C3670	ga	6	305	CAGATCACACTTATGGCTTGG
				GAGCAAGCATCACCTCTCAT
C3722	ct	5	220	AGCACAAAAATCCCCTTGAT
				TGGTATCAAGAGCCAAGGTC
C3771	at	6	216	TCGAAACCAAGAACCGTATC
				ATATGTTTGGGACGGTGATG
C3869	at	5	276	TCAACAACCAAACGATAGCA
				CGTGAGTCATGTGCTACACC
C3874	ag	6	105	CATGACCGGGAGGAGGAG
				TCTCTCTCACCGACCCTCA
C3875	ct	5	373	CTCTCCTCTCCGGTCTCTCT
				CCGGAATAGAGGGAAATCAC
C3977	ga	9	360	CGAATCGTTCAACACCTACC
				CAGTTTTAGTAACTGATTCTCTCTCTC
C4056	ta	6	161	TTGGGTTTCCGAATTTACTG
				GAAAACCCAAGCTTCCAAAC
C4092	ta	6	355	ATGTTTGGAGGGCTTGACTA
				GTCGGTTGGGTAAGTGTTTG
C4121	ta	6	342	TGGACAGCTCATCCCTAGAC
				AAACCAATTCGAACCCTTTC
C4126	ga	5	233	TCTTGGGTTCTTTTCACCAA
				CTAGGCACCCCATCTCCTAT
C4136	gt	6	332	GAACCTATGGGCTTATTTCCA
				CCATTGCCATTTCATCTTTT
C4230	tc	5	244	TCGTTTTGAAAGCTAAATCCTC
				ACCGTTTGTTTTTCGCTAGG
C4273	ag	6	212	GAGGCTCAGACGAAGAACAA
				CAGCCAAATACTTTAATACTTCAAT
C4274-1	ac	5	386	GCAGGCTTCTTTTCTTTTCC
				ATGTAGGCAATTGCtGAACC
C4274-2	tc	5	400	CTACCACCTCCCTCGTTCTT
				AGCTAAGGCAAGCAAGAAAA
C4274-3	ag	6	274	ATCCCCTTTGTGATCACCTT
				TTGAGTTGCCATGTTAGCTG
C4285	at	5	162	GCCTTTGTGTCTTCATTTTTG
				ACGTTGATGAGACGTCATTG
C4399	ct	5	221	CTTTTAAAAACGCGGTCCTT
				GCATGTGAGATTAGGGCTTG

Table 3.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')
C4402	tc	5	204	CAACACACACCATTCCAGAG
				GAGCTGAGCTTTTCACAGGT
C4407	ta	6	392	ATCAAAGGATACGCACCTCA
				CAACGTCGTCCAAAATAACC
C4441	gt	6	378	GGAAACGCTGAGACAGTCAT
				AGGCAACGAAGAACTCCAA
C4551	ga	13	232	AGGGATGGAGTGAAGGAAAG
				CACAATTCAAGCAATTTGGA
C4580	ct	7	254	CCGAGATTTGAGAGGCATAA
				CAAGCTTCAACCGAGAACAT
C4581	ct	5	305	TTGGCCACTTTGTTTCTCTC
				AAGAACAAGCTGCAGTGGAC
C4846	ca	6	334	GGACAATGGAGCAATCTGAC
				GGGTTTGTGTTCTTGTTGGA
C4882	ag	5	350	AGCCCTACTTATCAGAGCAATG
				TCCAGTTTTCGTGTAATGTTTTT
C4940	ag	9	320	GGAGGAAGAGTCATCGCATA
				TTAACCCGTTAAGCTCATGG
C5068	tc	5	232	TGCATGCATGTCTTTCAATC
				TGAGCTTGGGTAAACCTTTG
C5120	acaa	5	162	AAGAATTCCTGCAAAGAGCA
				CAAAGTGTGGGCTTTTGTTC
C5226	ta	5	158	TTGGGAAGAGTGGTATTTTCA
				TTCATATGACAAGATTTGATGGA
C5269	ga	5	280	GTGACTGCCAAGCCTCTAAA
				TGGCTCAATGAGTGATGCT
C5515	ag	5	267	GCGGAGAGAACAAAGAAGAA
				TTCGAGAACCGTGAGGTTAG
C5595-1	ct	5	269	CAACCCTAAACCCAAATCCT
				GAGATCGAGGTCGTTTTGAG
C5595-2	tc	5	178	CAACCCTAAACCCAAATCCT
				CGACACAGAGAGGGAGAGAG
C5602	ga	5	258	GAGGGTTGGTTTCGTACCTT
				TCACCCGCGTCTCCTCTC
C5678	ta	5	274	TTATGAGTGGGAGGGTCGTA
				CCCAAACACTTTTCAATGCT
C5753	at	5	241	CTTCCTCCTCATGCACAATC
				GGCGTAAAGCAAGGGTTAAT

Table 3.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')
C5900	ga	7	216	TTGGAATTTTGGATATGGTTTC GATGGATGGCTGAGATTCAC
C5948-2	ct	5	295	CGCACTCTCTCTCTCCTCTG TCCAAAACGACCTCCAATAA
C6012	ta	5	265	GAAGTCTCGGCCCTATTTTC CACAGATGACGCTGAAGATG
C6099	tg	5	259	ACGCTTCTGATCCACACTTC CCCAGAATCAATTCCAGATG
C6100	ta	7	291	CTGCGTGAGAAAAGAGGAAG ATTCGTACATCACGCAACAG
C6293	gt	5	248	CTGCCATTCCTGTAGCCATA TCCAGGTTTTGTTTTGTTGTCA
C6350	ag	11	225	TCAGGTTTATCAACTTTCTTAGCAGA TCCCGACGTTTTAAATCACA
C6363	aga	5	208	TCAGCCATAATTGTACAGAATAGTTTT TTGTTGGCTGCATTCTCATC
C6387	ga	5	297	CCATGATAGAGAAACCAtCAGGA CAGCCTAGTGCCTCTTCCAC
C6394	ca	5	251	GCTTCATTGACACTCCACCA TGGTACAAAATAAGCAGATGAAGAA
C6434	tca	6	322	CATGGACTCCACCAAGAGGT CCACTGAATTGGGAGACCCTA
C6669	ct	6	201	CCTGCGACAAAATACCCAAA GCGACTTAGGTGGGTCTGAA
C6740	ta	5	299	CAGTGCAGTGGCGATATAGG AGGGGGATATGATGGTGATG
C6797	ca	5	356	GATCTGCATCATCTGAAACTGC GCCACAGGAGCAAAAGTCTC
C6918	cca	5	254	ATTTTGATGGTTGCGCTTGT TTCATTCCCACTCGGCTTAG
C6957	ga	5	193	AAATCTGGCCAAGAGCACAG TGACCATCGAGTTGGCATAA
C7106	tggtt	5	151	ATGCCTAAACAAGCCGAACC CGATTTGACCCTCAAACCAC
C7153	ag	5	204	TCACTGTTTGGGATGTTGGA CGCTTCGAGCtTCTGAGATT
C7215	tc	5	212	GTGGAGCCCACCAATACAAT GCTAAAGCCCAATGTGGAGA

Table 3.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')
C7247	tc	9	278	GCGTCTTTTTATTGGGGTCA
				GCCTCGAGCAATTGTCTTCT
C7319	ta	5	308	CATTTGGAAGAACAAGCATTATAC
				AACTCACAAGGGGGTGGTTT
C7430	aat	5	199	CTGCAGACCAAGTGACATCAG
				TCCAACACACCCTCAACATC
C7533	ct	5	198	ACGATGATTCCATCGAAAGC
				TCAGCGATGAGAAAGGGAGT
C7642	ga	5	178	TCCTCATCACAATCGAACCA
				GCCCTTGGCTCTCTCTACCT
C7670	tca	8	173	GTAACGCATCATGGGCAAG
				GCCAGACATGAAAGGCAAAT
C7944	ac	6	317	TTTTGGCTTCTTGCTGGATT
				TGCTCTACTAAGATGTGCTTACTGC
C8049	ta	5	238	TAGTCGCAAAGGCAAAACCT
				GTTCTCCATCCCAACGACTG
C8064	tg	6	231	TGTTGCCTAGCTCACACCAG
				GAATGTGGGGTATTGCTCGT
C8066	at	6	212	TGTCCCCTTTCTGAATTGGA
				TCATTGCTGAAAGCGTTAAGTT
C8086	tca	7	279	ACCCCTAGTGCTTGGTCCTT
				CCTTGAAGTGAAGGCTGAGG
C8107	ct	5	175	TTTAGGCGAATCCAATGAGG
				CACCTGCAATTTCCTTGGTT
C8169	gtt	6	206	CTTCAAGGGGTTGTCGGTTA
				TGCGAGCGTTGAAGAGATTA
C8243	ag	5	271	GTGGATTTGAGACCGGAGAA
				CTCAAAGCCCAGCTCTCCTA
C8244	ca	6	356	GCATGAGTTGTGTCTTCATGG
				TGCTCTCTTGCTCTTTTGACC
C8277	at	5	364	GAACTTCCCAACACCAAACC
				TCAACCCAACATAATTCAAGG
C8386	gt	6	336	CTTCCAGATCCAGCCATGAT
				GATCCAGCTGCTGTGCATAA
C8439	ta	5	185	GGTTTGGTTTTGGTTTGGAA
				CCCCACCTTTTTGAAACTAATG
C8477	ct	5	166	TCTTGGCTCCGTCTCTCTCT
				TGAGCTTCGATGAACACACA

Table 3.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')
C8537	ct	7	159	TCCCTGTGTATTGAGCACCA
				GCCGATGGAGAAGTTGTGAG
C8627	tc	5	185	GCTAGGAGCAATGGCTAGGA
				ATTTTGGGAGCACAGAGGAA
C8681-1	ga	5	157	TTCGATGTTTGGGTTTAGGG
				CTCCCCAACTCGTTCTCTTG
C8681-2	ag	5	186	CCCCAATTCCCAATTGATTT
				AACAACGAGCACTCCTCCTC
C8761	ca	5	151	CCGTGTTGACTACTGTTACCC
				TTCTTTAGATTGCTCTGATATTGCTC
C8812	tc	12	297	TGCCAACTACTGGAATCTTGC
				AAGATGTTGGCGGTCTTGAG
C9016	gc	5	261	CCTACGCCGATGTATCCCTA
				GCCCGTCTGTTTCAACTCTC
C9108	tg	5	182	GGGATCACCAAGAGGACGTA
				AAGGGAAGAAGCGTGTGTTG
C9267	ga	16	209	GGGGTGGGATAAGTCTTGGA
				CCTCACCCACCTCTCTCTCA
C9480	at	6	283	GCAACTATTCTCAATCCATTCCA
				CCCGGTTGACCAATATGACT
C9500	aag	6	395	ACCAATCACAGCATCAACGA
				CTTGCTCGAGAGGCTTCTGT
C9559	ag	5	150	AAATGGTTTGCGAGGTCAAC
				AAGAAGCGCGCATTTTGAA
C9582	ttg	5	215	CTTGGAACGTGGTTGGTTCT
				GGAGGAGGTGGAGTCTGACA
C9600	tg	5	233	CTGGAGATGAGGGAGCACAT
				TGGGTGAAGTTGATGGTTCA
C9657	ga	5	287	CGGGTATTTTGGGAAGATGA
				GGGCTTCTTAGGGTTCGAGA
C9736	ga	5	214	GGTCACGTAAACTGGGGAGA
				TCTCTCTGTCTCTCTCAACACGA
C9746	ac	5	285	TCGTTGTAATGGCAAGTGGA
				GGACGTCCTGCTCTGAGAAT
C9824	ag	6	293	AATGGATAGGGCACGTCAAG
				CTCTCTCTTCCCTCCGGTTT
C9912	at	5	185	ATGTGTTGGCAATTGGGTTT
				CCACAACCCACTCACTTTCC

Table 3.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')
C9976	gaa	5	205	AAAATGCCAAAAGTCCGATG
				TGACATGCTCATCGCTTACC
C10146	ct	5	272	CCACCTCGACCATATCCATC
				ACAAGATCTGCCCCTTTCCT
C10238	at	5	200	CATGCACAAAAAGAGTTTCACA
				ACAACGCTTTGTGCCATGTA
C10367	ga	5	166	GCGGTACAAGCTTCTTCTTCTT
				TGGGTTGATGTCATGTCAGG
C10393	ct	5	274	CTGACGTATGTGGTGCAAGG
				TGTCGGAGACTCTGATGCAC
C10404	cca	5	158	TCGGTGCAACATAGACCTCA
				GTAACTGGCGGGTTGTGTTT
C10447-1	ga	5	244	GCCCAGTGAGGTTCCATAAA
				GGTTCTATCCGACCCCAAAT
C10447-2	ga	9	281	TGGGGTTACCTTGAGATTGC
				ACCCCAAAATCTCAGCCTCT
C10495	ctc	5	249	GCCACGTCCCTATCAAACTC
				GCTGAACTCCAAGCCAACTC
C10531	tg	6	201	AGGAGCTTCTTGTGGCCTAA
				TGAGAGGCAACTTCCTGCTT
C10685	gag	5	285	TCCGATATCCCAACATCCAT
				GTGGCTTCCACAGTTGGAGT
C10694	ggt	5	220	CTCAAAATTGTTGGCTGCAA
				CGTGTATGCAACGTTCTCGT
C10749-1	ct	5	372	TGATGGTTTTAGCGTCGTGA
				CGCGCAGATCTGATGGAT
C10749-2	ct	5	311	ATTTTCAATTTTCGCGCTGT
				ACGCGCAGAGCTGTTAGA
C11107	ga	5	215	ATCTGGCCAAGAGCACAAAG
				CATCTTGAGCTCTCCCACAA
C11139	ag	11	400	GCCTGCCTATGTGGGAATTA
				GCAAAAACATGGTGAACACG
C11197	tga	5	347	CAGAACCGTTGGAGTTGGTT
				ACAACTGGGCACATTTGACA
C11252	ta	5	301	TCGCGATAGACGTACACGAG
				TACCCATCATGAGGCAGACA
C11334	ct	6	201	AAAGCACACATGGATCATTGAC
				TGGTGTAAAGACGGAACAATCA

Table 3.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')
C11353	aag	5	260	CGGGCTTTAAGGAGGAGAGA
				TTTTTGAATCCGTCACCAAG
C11374	tc	6	253	GCCTGGCCTATGGAAGAAA
				GGCAGTCAGCAAAAGAGGTC
C11377	cac	6	210	ACCACGTCATCAAAACCAC
				TCAGATGAGAGGCAATCACG
C11424	ct	5	315	ATTTTCGGGAGGAAAAGAGC
				AGGAGGCGGAGGCATATACT
C11508	tc	7	201	TGCACACTTCTTGAGTCTTCG
				CTATGCGGGGCTCGAAAG
C11580	ag	6	202	GTCAGTTGACCCCTGCAAAC
				TGACAAAATATAAACTCTTCTCACTCC
C11610	ac	9	216	GGGACTTGCACACCTTCACT
				AGTGGTGCAGTGAGCAGCTA
C11662	ta	5	245	TTGAGAATTTGGTTTCGGTTG
				AAGCTCACACGCCAAAGAAT
C11815	tc	7	215	AGCCAATCCGGTTCTCTCTC
				CCGAGATTTTCAGAGCTTGC
C11816	ga	5	100	GAAGATTCCACGGTGAGGAG
				CCCTTGAACCAATCACATTTC
C11819	tg	6	291	CAATGCGTCTTGAGCCACTA
				AAAACTCACGTTTTCAAACACAAA
C11864	ta	8	219	TGGGAGTTAGTCCCCAGTTG
				AGGCACAACAAGCAAGGAAG
C11961	tc	8	232	GTTACGGATGTTTCGGAGGA
				GAGAAGGGGTGGGTTAGGAC
C11992	ga	5	271	CTGACAACAGTGGTCCAAATTC
				CGGAAATGAAATGGCTTTGT
C12096	ct	6	288	TGGCCATATAACATGGTGACA
				AGGGTTTGGGTTGGAGAGAG
C12100	tc	8	210	TCCCGACGTTTTAAATCACA
				TCCACATGCTTAGCAGAAATACA
C12338	at	7	103	CCAAACCCCGAAATGGTTAT
				GATTTGCGAATGCTTGGACT
C12352	ga	9	283	CACAGGGTTAAGTGGGCAGT
				AAACCTATCTTTTCACCCACCA
C12361	ag	5	255	GTGAGTTGGCAAGGGAGAGA
				GGCTTCAACGTAAGCTGCTC

Table 3.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')
C12442	ta	5	201	AATACGGAGTGGGCCCTACAA
				GTGTACTCAGTTAATTTTAGGGTTCGT
C12477	gt	5	245	CAGGTTGGTTTCGGTTGAGT
				TCCATTCACACGTTCATTGC
C12519	ga	6	291	GTTGAAGTCGTCGGAGCTGT
				TTGGATAAAAGAACCCTAGCTGA
C12531	ga	5	162	CGGGAAAGCTAGTGGGTCTT
				CTCTCTCCTCTCTCTCTCTCCTC
C12724	ct	8	277	TTTGCCTACGTCCTCTTTGG
				TTTGAGGTAGGCTGGTTTGG
C12735	tc	8	268	CCCGTAAGTTTCCCAAAATTC
				AGTGATAGATGAAGATGGGTTTTT
C12757	tc	5	201	AGCCAAGTTCGAGCTTCTTG
				GGCTTCTCTTCGCTCCTTTT
C12914	gt	6	150	AGGTACGAGGCCGAAACTCT
				CTGTTGTGCAGAAACACTCCA
C12916	at	5	171	TCCAATCTCAATTTCCGAAC
				TTGGATTGTTTTCCCTTGGT
C12965	ta	10	186	CCCATTGGTCCCATTAATCC
				TATCGTTCGCGAATGTCGT
C13295	ga	5	231	TTGAGGGGGAGAAGACTGTG
				AGCAGAATGGGTGTTGAACC
C13401	ag	6	206	TGTGAGGTGATTAGATTGCTTGA
				CCGTCAAGACCTCCGTTAAA
C13479	at	5	283	AAGCTCCTGTACGTGCGAGT
				TCTGTTTGACAACCCCTTCC
C13519	ta	5	271	TTCCAAGGAATGAAGCCAGT
				TGTGAGGCCATCACTAAGTTT
C13624	at	5	225	TGGGTCTTGGATTTCACGTT
				CACCCAAAATATTCACAATAAGAA
C13713-1	ct	5	267	CCTTCTCCTAGCCGACCTTT
				GCATGTAAGGGAGCCAACAT
C13713-2		6	224	ATGTTGGCTCCCTTACATGC
				GAAAGGTGGAATGGATGTGG
C13939-1	ct	5	290	CCTTCCCCCTTTTCCTTAGA
				CGTTCCTGGCAGCTACAAAT
C13993-2	ta	5	239	CAGAACAGGGGGAGGTACAA
				GGTCTGTTATGCGGTCGTTT

Table 3.3. Continued.

Primer name	Motif	No. of repeat	Product size	Primer sequence (5'-3')
C13993-3	at	7	299	AAACGACCGCATAACAGACC
				TTGGGACGTCCGGATATTTA
C14145	cct	5	215	GGCTCCGACTAGCTCCAC
				ATCAGAGCAACCCCAGGAG
C14179	at	5	156	CATTCGTGGTCCTCAAGGTT
				AAATTTCGCAGGACCATTTG
C14193	tc	5	208	ACACGAACCAACCCGTTAAG
				GGTTGTTGAGAGAGGTTTTTGAA
C14227	ac	5	241	TGTGGTAAGCCTAGCATTTTCC
				GAGCAGGTGTGTGTGGAGTG
C14231	ag	7	206	GGCCGGTGATGTTCTATGAT
				TCTTTTCCTCCCTTAACCTTCC
C14345	ct	5	286	CTTGGTGATCCCCTTGAGAA
				GCCAAGGAATCAGAAATCCA
C14439	ga	14	163	TCGGTTTGGCTATTTTGGAA
				CTCCCTTTCTCCATCGCTTT
C14600	aga	5	203	TGAAGTTGTAGGCGTGTCT
				TGCTCACATCCAAATTCGTC
C14610	ag	8	225	CGTCTTACCCGTGAGGATGT
				GTCTCCTCAATCGGTGGTGT
C14764	tg	6	217	GAAAACAAGTGTGGGGGATG
				CAAATGGCAGATTCAAGCAA
C14956	ag	5	219	CCAGAAAATCAAGCCCTCAA
				TCTCACAGAAACTCCCTGGTG
C15229	ac	6	293	GCTTCAGCTGCTGTGAACTG
				TCAAGGGGATCACCAAAGAG
C15460	ta	5	176	GAAGCCTCCACAACCAGAAA
				CCATTTCAGGTCAGATATTCTTTTT
C15686	tga	7	189	CTCCACCCGAAGAACAAGAG
				CTCAAAGCCGATCTCAATCC
C16053	cac	5	150	GATAGAGAGGCTATGGCTCATCA
				CATTGGCACCGTCTGATACTT
C16326	gt	5	159	CAAGAGGACGTAGGCAGGAG
				TTGTGCGAAAACACTCCAAG

Table 3.4. Amplifications of chokecherry SSR primers in seventeen genotypes.

Primer	Cho1[a]	Cho2	Cho3	RS	BA	NS	EF	Sch	WC	Apr	Pch	Plu	App	Cra	Pea	Ras	Jbe
C34	1[b]	1	1	-	-	-	1	1	-	-	-	-	2	1	-	2	2
C162	2	2	2	1	1	3	1	1	1	M	4	1	2	2	-	1	M
C205	1	-	-	-	-	2	-	-	-	-	2	1	1	2	-	2	-
C324	4	4	4	4	4	-	4	4	4	-	-	1	2	1	-	2	3
C525	4	4	4	2	2	4	1	2	2	4	5	3	5	2	-	4	5
C629	2	2	1	2	2	1	2	2	2	1	1	1	1	3	-	2	2
C837	-	2	M	2	2	M	-	-	-	3	4	3	3	M	2	1	-
C1114	1	1	-	1	1	-	1	1	-	1	2	1	2	2	-	-	-
C1181	1	1	1	3	3	1	2	2	2	1	1	1	M	M	-	4	2
C1231	1	1	1	4	3	1	2	2	3	1	-	5	5	-	-	4	5
C1322	1	1	1	1	-	3	-	1	-	2	5	2	M	M	-	M	2
C1476	4	3	3	3	3	-	2	2	2	2	1	-	2	1	-	1	-
C1585	3	2	3	4	3	3	-	-	3	2	1	4	3	2	1	2	2
C1795	3	3	3	3	1	-	1	1	3	2	-	4	5	3	-	2	3
C1882	4	4	4	4	4	1	4	4	4	1	2	2	-	-	-	2	4
C1933	M	M	M	3	3	2	2	2	4	M	M	M	M	M	-	M	M
C2103	1	2	2	3	2	3	1	1	-	-	1	-	-	-	-	-	-
C2109	M	2	2	-	-	3	1	-	-	-	2	-	2	4	-	3	-
C2175	5	3	3	1	1	2	-	1	1	5	M	4	M	M	4	M	M
C2194	1	1	1	1	1	3	1	1	1	-	-	-	3	3	-	2	2
C2250	4	2	4	3	3	4	1	1	1	4	3	4	M	M	-	3	M
C2525	2	2	2	5	5	2	-	2	5	1	2	2	-	-	-	-	-
C2556	3	1	3	5	2	2	-	1	1	1	1	1	-	-	-	-	-
C2619	-	-	-	1	-	-	1	-	1	-	-	-	-	-	-	-	-
C2635	-	-	-	1	-	-	-	-	-	-	-	-	2	2	-	3	-
C2743	2	2	2	1	-	2	-	-	-	-	2	2	1	4	-	2	2
C2762	2	3	2	5	-	-	-	-	-	-	-	-	-	-	-	-	-
C2824	4	4	4	3	1	3	-	1	2	5	2	3	4	4	1	3	2

Table 3.4. Continued.

Primer	Cho1	Cho2	Cho3	RS	BA	NS	EF	Sch	WC	Apr	Pch	Plu	App	Cra	Pea	Ras	Jbe
C2838	1	1	1	1	1	1	1	1	1	1	1	1	1	2	-	-	2
C2927	4	4	4	3	3	4	4	4	4	4	4	4	2	2	1	1	2
C2938	3	3	3	3	2	-	-	2	2	-	2	-	2	3	-	2	1
C2997	3	2	2	4	3	1	1	1	-	-	1	-	1	1	-	2	-
C3205	1	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C3280-1	M	M	M	5	2	M	1	3	3	M	M	M	M	2	3	3	M
C3280-2	2	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C3292	2	-	2	2	3	-	1	1	1	-	-	-	-	-	-	-	-
C3332	2	2	2	4	4	-	2	2	2	-	4	2	3	2	-	2	1
C3522	5	5	5	5	5	-	5	5	3	2	2	2	3	3	-	3	2
C3603	3	3	3	3	3	-	3	3	3	2	-	-	-	-	-	-	-
C3635	3	3	3	2	2	3	1	1	1	3	2	3	2	2	2	2	-
C3637	2	2	2	-	-	-	1	1	-	-	-	-	-	-	-	-	-
C3656	4	4	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C3670	5	5	5	5	5	-	-	2	5	-	3	-	3	4	-	3	-
C3722	2	2	2	-	1	-	-	-	-	1	-	1	2	-	1	-	-
C3771	4	4	4	4	4	1	4	4	4	1	1	1	5	5	-	M	3
C3869	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C3874	3	3	3	4	3	3	3	3	1	5	3	2	4	5	1	3	M
C3875	5	1	5	-	-	4	1	1	-	3	2	1	3	M	-	3	M
C3977	1	2	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C4056	4	4	5	4	4	1	3	3	3	1	2	1	5	3	-	-	1
C4092	M	M	M	2	2	M	2	2	1	1	3	1	M	M	-	2	3
C4121	3	3	3	1	1	4	-	-	-	1	3	2	1	2	-	-	1
C4126	3	3	3	2	2	2	-	1	1	2	2	3	1	1	1	1	1
C4136	M	M	M	4	3	M	2	2	4	2	2	M	-	-	-	-	1
C4230	5	5	5	5	5	1	5	5	5	1	1	-	1	-	-	-	-
C4273	M	2	2	4	2	4	5	4	4	M	4	M	4	2	1	1	1
C4274-1	5	5	5	5	4	4	4	5	4	2	3	M	M	5	-	3	-

Table 3.4. Continued.

Primer	Cho1	Cho2	Cho3	RS	BA	NS	EF	Sch	WC	Apr	Pch	Plu	App	Cra	Pea	Ras	Jbe
C4274-2	M	M	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C4274-3	M	M	M	M	M	2	M	M	M	-	-	2	2	-	-	-	-
C4285	M	M	M	5	5	M	5	5	3	M	2	M	-	-	1	1	1
C4375	M	M	M	M	M	M	M	M	M	M	M	M	M	M	-	M	M
C4399	3	5	M	4	4	2	2	2	3	-	1	1	1	1	-	-	1
C4402	4	4	4	4	4	-	5	5	M	-	-	-	1	-	1	-	-
C4407	3	3	4	-	-	1	-	-	-	1	-	2	-	-	-	-	-
C4441	2	2	2	1	1	2	-	-	-	1	1	1	-	-	-	-	-
C4551	3	3	3	3	3	3	3	3	3	-	2	-	4	-	-	2	1
C4580	3	4	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C4581	5	5	5	3	-	-	-	-	-	-	3	2	4	5	1	4	3
C4846	4	3	4	3	3	2	1	1	1	-	-	-	2	2	-	2	3
C4882	2	2	3	2	2	2	-	-	1	3	3	1	-	1	1	-	1
C4940	M	M	M	1	1	2	1	-	-	3	1	1	2	2	1	2	-
C5068	2	4	2	1	-	-	-	-	-	1	1	-	-	-	-	-	-
C5120	1	3	2	-	-	2	-	-	-	2	2	2	-	-	-	-	-
C5226	3	3	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C5269	4	4	5	4	4	1	4	4	4	1	1	-	4	1	1	-	-
C5515	2	2	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C5595-1	2	3	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C5595-2	4	4	4	M	M	M	4	4	M	M	M	M	M	M	M	M	M
C5602	4	2	3	1	-	-	-	-	1	1	1	1	5	3	-	1	2
C5678	3	M	M	1	1	M	1	1	3	M	M	M	-	-	-	-	-
C5753	4	3	3	2	2	1	2	2	2	1	1	1	2	3	2	1	4
C5900	3	4	2	1	1	-	1	1	1	-	2	1	1	1	-	3	1
C5948-1	M	M	M	5	4	M	3	3	4	M	M	M	2	M	-	M	M
C5948-2	2	3	3	-	-	2	1	-	-	5	M	1	5	4	3	5	5
C6012	4	4	4	4	4	5	4	4	4	5	5	2	5	3	1	2	5
C6099	4	4	5	1	2	3	2	2	3	4	2	4	4	4	4	2	4

Table 3.4. Continued.

Primer	Cho1	Cho2	Cho3	RS	BA	NS	EF	Sch	WC	Apr	Pch	Plu	App	Cra	Pea	Ras	Jbe
C6100	3	3	5	-	-	5	-	-	-	4	-	1	5	4	2	4	M
C6293	4	2	3	3	3	3	2	2	1	5	4	M	4	3	1	2	2
C6350	4	4	4	2	2	1	2	2	2	1	2	1	1	1	1	-	1
C6363	5	2	2	4	-	3	-	1	3	1	1	1	-	-	-	2	-
C6387	M	-	M	5	3	4	3	3	M	2	4	2	3	3	1	2	2
C6394	1	2	4	3	5	M	4	2	2	M	M	M	M	M	2	M	M
C6434	5	5	5	5	5	2	5	5	5	2	4	2	1	2	1	2	1
C6669	3	4	4	1	1	2	1	1	1	2	4	2	5	4	1	3	2
C6740	2	3	2	1	1	3	1	1	1	5	1	3	M	M	2	4	5
C6797	4	4	4	-	-	M	-	-	1	4	3	4	M	M	3	3	5
C6918	2	3	2	3	5	5	3	3	5	M	2	5	3	1	-	2	1
C6957	2	3	2	1	1	2	1	1	1	2	3	2	4	5	-	2	3
C7106	3	2	4	1	2	1	1	1	1	1	1	5	M	-	2	5	M
C7153	5	5	5	1	1	1	1	1	1	1	1	2	3	-	2	1	1
C7215	4	3	2	-	-	1	-	-	-	2	3	3	-	-	-	-	-
C7247	M	M	M	M	M	M	M	M	M	M	2	5	3	1	-	3	2
C7319	5	5	4	2	1	3	2	3	3	3	5	3	2	2	-	-	-
C7430	4	3	3	1	1	2	1	1	1	-	2	2	4	5	-	-	5
C7533	2	2	3	3	3	-	1	1	-	-	-	-	-	-	-	-	-
C7642	5	4	4	2	2	4	2	-	-	3	4	2	1	2	1	3	3
C7670	4	5	5	5	5	1	2	2	2	1	2	1	-	-	1	-	1
C7944	5	4	4	1	-	-	-	1	-	-	-	-	-	-	-	-	-
C8049	2	1	2	1	1	-	1	1	1	-	1	-	3	3	-	-	2
C8064	4	4	3	2	2	2	2	2	2	2	2	3	2	2	1	1	-
C8066	4	4	4	1	-	-	-	-	-	-	1	-	-	-	-	-	-
C8086	4	5	3	M	3	4	2	2	4	2	3	4	4	5	2	2	5
C8107	M	M	M	2	2	2	2	2	2	3	4	5	2	2	1	1	3
C8169	M	2	2	3	3	4	2	2	2	-	-	1	1	1	1	-	1
C8243	M	M	M	M	M	M	M	M	M	1	M	2	3	4	-	1	1

Table 3.4. Continued.

Primer	Cho1	Cho2	Cho3	RS	BA	NS	EF	Sch	WC	Apr	Pch	Plu	App	Cra	Pea	Ras	Jbe
C8244	5	5	5	2	2	3	1	1	2	-	1	4	2	2	-	1	1
C8277	2	2	3	3	3	-	1	1	1	-	-	-	4	5	-	2	4
C8386	5	5	5	2	2	3	-	-	-	3	5	5	-	-	-	-	-
C8439	M	M	M	1	2	1	-	2	4	-	1	-	-	-	-	4	3
C8477	M	M	M	3	3	M	4	3	5	5	M	M	3	5	1	1	M
C8537	M	M	M	M	M	1	M	M	M	2	4	2	4	5	2	3	2
C8627	3	2	4	2	-	1	2	-	-	2	1	3	5	5	2	3	4
C8681-1	1	1	2	1	1	1	1	1	1	2	2	1	2	1	-	1	-
C8681-2	M	M	M	M	M	1	M	M	M	5	3	3	5	4	-	3	5
C8761	5	3	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C8812	M	M	M	4	1	M	3	3	5	M	M	M	3	3	3	5	1
C9016	-	-	-	-	-	-	-	-	-	-	-	-	2	3	-	-	2
C9108	M	M	M	1	1	-	-	2	M	M	M	M	M	M	-	2	M
C9267	M	M	M	-	-	1	-	1	1	2	5	5	M	M	3	M	M
C9480	M	M	M	1	-	-	-	-	1	-	-	-	-	-	-	-	-
C9500	2	3	5	2	3	3	-	-	-	3	1	2	1	1	2	1	2
C9559	5	M	4	5	5	-	2	2	3	-	-	-	-	-	-	-	-
C9582	M	M	M	M	M	M	M	M	M	M	M	M	2	3	1	3	1
C9600	5	2	5	4	4	-	3	3	1	1	5	2	1	1	-	1	3
C9657	4	5	4	3	3	1	4	1	1	3	1	-	2	5	-	-	2
C9736	5	3	5	3	3	1	2	2	1	3	2	1	1	-	1	1	1
C9746	M	M	M	M	M	M	M	M	M	1	3	4	1	1	-	1	-
C9824	M	M	M	3	2	-	2	-	-	-	-	-	4	1	-	-	1
C9912	4	5	5	5	5	3	2	2	3	1	5	1	5	3	-	4	2
C9976	M	M	M	4	-	-	3	2	1	-	-	-	-	-	-	-	-
C10146	2	3	2	-	-	-	2	-	2	-	1	1	3	4	-	3	2
C10238	5	4	5	2	1	1	-	-	1	3	4	2	3	3	-	-	1
C10367	5	5	4	1	1	5	1	1	1	4	M	4	4	3	-	2	2
C10393	4	4	4	5	4	5	2	2	M	5	3	5	1	1	1	1	-

Table 3.4. Continued.

Primer	Cho1	Cho2	Cho3	RS	BA	NS	EF	Sch	WC	Apr	Pch	Plu	App	Cra	Pea	Ras	Jbe
C10404	3	3	5	2	3	4	3	1	3	-	3	2	4	5	-	3	1
C10447-1	3	3	3	3	3	1	3	3	3	1	1	1	-	-	-	-	-
C10447-2	5	2	4	1	-	M	-	-	-	-	1	1	4	4	1	1	3
C10495	5	5	5	-	-	1	-	-	-	1	3	1	-	-	1	-	2
C10531	3	3	3	1	1	2	-	-	1	2	2	-	1	1	-	1	1
C10542	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
C10685	5	5	3	M	4	3	2	1	2	-	-	1	-	-	-	-	-
C10694	M	M	M	-	2	-	-	-	3	2	2	2	3	3	2	-	3
C10749-1	5	M	M	3	1	-	1	1	1	-	1	-	1	-	1	-	1
C10749-2	M	M	M	1	-	-	-	-	-	-	-	-	-	-	-	-	-
C11107	5	5	M	5	5	-	5	5	5	2	2	2	3	-	-	-	1
C11139	5	5	5	1	1	3	1	-	1	5	5	4	2	2	-	-	1
C11197	3	2	2	1	1	1	1	1	2	4	1	-	5	3	1	2	3
C11252	2	2	2	2	2	2	-	2	-	4	2	-	1	3	-	-	2
C11334	5	5	5	-	-	5	-	-	-	3	4	4	3	-	1	2	1
C11353	3	4	3	2	1	4	2	2	2	4	5	4	3	5	-	4	5
C11374	2	2	3	2	2	2	1	1	3	1	-	-	2	1	-	-	-
C11377	5	4	5	5	5	3	3	2	1	1	5	1	4	5	-	3	M
C11424	4	4	4	-	2	1	1	2	2	-	1	-	2	-	-	-	-
C11508	4	3	3	4	3	1	2	2	2	1	-	2	2	4	-	2	3
C11580	5	5	5	5	5	-	5	5	5	-	-	-	-	-	-	-	-
C11610	4	3	3	-	-	2	-	-	-	2	1	2	M	M	3	2	M
C11662	5	5	5	2	1	4	1	1	-	3	2	-	1	3	-	1	-
C11815	4	5	5	-	-	1	-	-	-	1	2	-	5	-	-	5	-
C11816	-	3	3	-	-	3	-	-	-	1	-	-	4	5	1	4	3
C11819	5	4	4	4	4	-	2	1	1	4	4	4	3	-	1	-	2
C11864	4	4	4	5	3	4	3	3	2	2	5	3	4	2	1	1	1
C11961	5	5	5	2	1	1	-	-	2	1	-	1	-	1	-	1	1
C11992	4	5	5	-	-	5	1	-	-	4	1	4	-	-	-	-	-

Table 3.4. Continued.

Primer	Cho1	Cho2	Cho3	RS	BA	NS	EF	Sch	WC	Apr	Pch	Plu	App	Cra	Pea	Ras	Jbe
C12096	5	5	5	3	3	-	1	1	2	M	4	1	3	3	2	M	M
C12100	4	4	4	M	1	-	M	M	4	-	-	-	-	-	-	-	-
C12338	4	5	4	-	-	-	2	-	-	-	-	-	-	-	-	-	-
C12352	5	5	M	-	-	-	-	-	-	-	-	-	5	5	-	1	1
C12361	5	5	5	5	5	2	5	5	2	5	5	2	5	5	1	M	4
C12442	5	5	5	5	5	4	5	5	5	3	4	4	-	-	-	-	-
C12477	M	M	M	1	3	-	3	1	2	-	-	-	-	-	-	-	-
C12519	M	M	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C12531	3	3	3	3	3	-	3	2	1	M	M	M	M	M	M	M	M
C12724	5	5	5	4	4	5	3	3	3	1	4	2	5	4	-	1	-
C12735	2	M	5	2	2	-	2	2	1	-	-	-	-	-	-	-	-
C12757	5	M	M	5	5	1	4	4	3	1	4	1	4	2	-	-	1
C12914	4	5	3	3	2	5	3	3	1	4	3	5	5	M	-	1	5
C12916	3	3	2	1	1	1	1	1	1	1	1	1	-	-	-	-	-
C12945	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
C12965	4	4	4	3	1	-	4	4	2	-	2	1	-	-	-	-	-
C13295	5	4	5	2	2	-	2	2	2	1	2	1	3	2	-	-	-
C13401	1	2	3	3	3	-	2	2	2	-	-	-	-	-	-	-	-
C13479	5	5	M	M	M	2	M	2	1	1	1	2	1	2	-	1	-
C13519	4	4	4	1	1	-	-	1	-	-	-	-	-	-	-	-	-
C13624	4	4	4	4	4	1	4	4	5	1	-	1	1	1	1	-	-
C13713-1	5	5	5	1	1	1	-	-	-	2	3	3	3	3	-	1	2
C13713-2	M	M	M	1	-	3	-	-	-	1	2	1	5	2	2	-	3
C13939	3	2	4	1	3	3	1	1	5	2	2	3	-	-	-	-	-
C13993-1	5	5	5	-	-	1	-	-	1	2	2	1	1	1	1	-	-
C13993-3	5	5	M	M	M	-	4	2	2	-	-	-	-	-	-	-	-
C14145	2	1	3	1	-	-	-	1	1	-	-	-	-	-	-	-	-
C14179	4	4	4	1	1	3	1	1	1	3	4	4	-	-	2	-	2
C14193	5	5	5	M	M	4	M	M	2	3	1	-	M	M	4	2	M

Table 3.4. Continued.

Primer	Cho1	Cho2	Cho3	RS	BA	NS	EF	Sch	WC	Apr	Pch	Plu	App	Cra	Pea	Ras	Jbe
C14227	3	2	2	-	1	3	-	-	-	1	2	2	3	2	-	1	3
C14231	5	2	4	5	5	1	1	1	1	1	-	-	-	-	-	-	-
C14345	2	2	2	2	2	3	1	1	1	1	2	1	1	1	-	-	-
C14439	2	2	3	M	M	2	M	M	M	5	4	2	-	-	-	-	-
C14600	1	-	1	-	-	-	-	-	-	-	-	2	1	3	-	-	-
C14610	3	4	3	1	1	1	1	1	4	1	2	1	1	1	2	1	2
C14764	2	2	2	-	-	-	1	-	1	2	2	1	3	3	1	3	3
C14956	2	2	4	1	-	2	1	1	1	4	2	2	-	-	-	-	-
C15115-1	M	M	M	1	-	-	1	-	1	-	1	-	-	-	-	-	-
C15115-5	M	M	M	1	1	M	1	1	1	M	M	M	M	M	M	-	M
C15229	M	M	M	3	3	-	3	3	3	1	1	2	3	3	1	1	1
C15460	1	1	1	1	1	-	1	1	1	-	-	-	-	-	-	-	-
C15686	M	M	M	1	1	4	1	1	1	2	3	1	1	1	-	-	2
C16053	3	3	3	1	1	1	1	1	1	-	1	1	1	1	1	1	-
C16326	2	2	2	2	1	5	2	2	1	2	3	1	1	1	1	1	1

a. Cho1= chokecherry line (C_L); Cho2= chokecherry line (c); Cho3 = chokecherry (d); RS = sour cherry (‘RS’); BA = sour cherry (‘Balaton’); NS = sour cherry (‘North Star’); EF = sweet cherry (‘EF’); Sch = sweet cherry (‘Schneider’); Wch = wild cherry; Apr = apricot; Pch = peach; Plu = plum; App = apple; Cra = crabapple; Pea = pear; Ras = raspberry; Jbe = june berry.

b. The numbers indicated the number of bands; M stands for the number of bands > 5.

5. Discussion

With the recent advances in DNA sequencing technology, particularly the application of the next generation sequencing (NGS) technology, a large amount of sequence data of plant species are being rapidly generated, providing a valuable resource for genetic research including molecular marker identification. Microsatellites (SSRs) have been discovered from the next generation sequence data for many plant species (Tangphatsornruang et al., 2009; Cavagnaro et al., 2010; Csencsics et al., 2010; Zhu et al., 2012). In this study, Roche 454 sequencing technology was applied to sequence the chokecherry (*Prunus virginiana*) genome. With a half sequencing run, a total of 145,094 reads covering 4.5 Mbp of the chokecherry genome were sequenced. Based on the published genome size of sour cherry (*Prunus cerasus*, 2n = 4x = 32) (599 Mb) and sweet cherry (*Prunus avium*, 2n = 2x = 16) (338 Mb) (http://icgr.caas.net.cn/973/%BB%F9%D2%F2%D7%E9%B4%F3%D0%A1.htm), chokecherry (2n = 4x = 32) genome size can be estimated around 600 Mbp; therefore, the obtained sequences in this study only covered 0.75% of the chokecherry genome. With such a small coverage of the chokecherry genome, a total of 481 SSR loci were identified with a frequency of one SSR in every 10 kb (481 SSRs in 4.5 Mb). The SSR frequency in chokecherry genome is similar to the one in sweet potato (1/7.1 kb) (Wang et al., 2011), but lower than the ones in cucumber (1/1.8 kb) (Cavagnaro et al., 2010) and cranberry (1/2.5 kb) (Zhu et al., 2012) and much higher than the one in mungbean (1/67 kb) (Tangphatsornruang et al., 2009). After assembling all 454 sequencing reads, the majority of contigs (80%) are in the range of 100 to 600 nucleotides in size, which is optimal for SSR identification and primer design because the PCR products used for genotyping are usually 100-400 bp (Hayden and Sharp, 2001). In this study, 234 of 481 contigs had satisfactory primer design sites and 246 primers were designed and validated in chokecherry, in

which 212 (86.2%) effectively amplified DNA from chokecherry, yielding more than 1000 alleles that have potential to be used for genotyping populations. The efficiency of SSR identification (percentage of amplifiable SSR markers in all obtained contigs) from 454 sequences of the chokecherry genome is 1.82%, which appears to be similar to the ones in other species that are ranged from 0.1% in blue duck (Abdelkrim et al., 2009) to 5% in insect (Malausa et al., 2011).

It is not surprised that di-nucleotide repeats were the most frequent motif type (85.9%) followed by tri-nucleotide, tetra-nucleotide, and penta-nucleotide repeat types in chokecherry genome SSRs (Table 1), which is often observed in other plant species (Tangphatsornruang et al., 2009; Yonemaru et al., 2009; Cavagnaro et al., 2010; Zhu et al., 2012). There is a significant difference in the relative abundance of a specific repeat motif between chokecherry and other species. In chokecherry genome, CT/GA (24.3%) was the most abundant SSRs followed by AT/TA (22.5%) and AG/TC (21.8%), while CG/GC was the least frequent in di-nucleotide (Table 1). The other motif repeats appeared to be evenly distributed with a frequency lower than 1% except CTC/GAG, CCA/GGT , AAC/TTG, AAG/TTC, and CTT/GAA in tri-nucleotides (Table 1). The motif repeat AG was the most frequent SSR motif in cranberry 35% (Zhu et al., 2012), while in sorghum and mungbean sequences, AT-rich motifs accounted for the largest proportions at 26.1% and 89.3%, respectively (Tangphatsornruang et al., 2009; Yonemaru et al., 2009). However, the proportion of GC-rich motifs in this study was the smallest (2.91%), which is in agreement with the results of genomic-SSR from rubber tree (Yu et al., 2011), cranberry (Zhu et al., 2012), and mungbean (Tangphatsornruang et al., 2009).

Interspecific and intergeneric transferability of SSRs make them useful for genetic research, such as fingerprinting and genetic map construction. Transferability of SSRs from one

species to other species or genera has been reported in many plant species, including cereals (Tang et al., 2006; Sim et al., 2009; Castillo et al., 2010), vegetables (Ince et al., 2010), and woody species (Gasic et al., 2009; Park et al., 2010; Yu et al., 2011). Recent research showed that *Prunus* SSRs were transferable within *Prunus* or across species in the Rosaceae family (Mnejja et al., 2010). The rate of SSR transferability in different species is related to the genetic distance between the species from which the SSRs developed and other species. In this study, six *Prunus* species that belong to three subgenera: *Cerasus* (sour cherry, sweet cherry, and wild cherry), *Amygdalus* (peach), and *Prunophora* (apricot and Japanese plum) were compared for the transferability of chokecherry SSRs in these species. Similar transferability rate was observed within the same group. For example, an average of 65.4, 63.0, and 62.4% of chokecherry SSR primers amplified bands from sour cherry, wild cherry, and sweet cherry, respectively, while 56.5 and 57.7% of chokecherry primers amplified bands from apricot and plum. Such a correlation between the genetic distance and SSR transferability rate was also proved in other research studies. Mnejja et al. (2010) reported 100% amplification rate was observed between peach and almond genomic SSRs, apricot and Japanese plum or almond EST SSRs, and European plum and Japanese plum genomic SSRs. They also found that peach DNA showed the highest amplification rate (91.6%) and cherry DNA had the lowest (76.6%) when the *Prunus* SSRs used, which is evident in this study that 61.8% of chokecherry primers amplified in peach DNA, higher than in apricot (56.5%) and plum (57.7%). A relatively high percentage of amplification and polymorphism of SSRs were also observed in this dissertation in Chapter 2, where 93 out of 108 SSR primers (86.1%) adopted from other *Prunus* species were transferable to chokecherry and 73 primers (67.6%) showed polymorphism in our populations. Further research confirmed that 70 out of 234 SSR chokecherry SSR sequences are homologous to the

peach sequences in the NCBI database (Altschul et al., 1990). The result also showed that chokecherry SSR primers produced a high amplification rate (58.9%) of apple DNA, which is supported by the previous research that a high degree of sequence similarity exists between *Prunus* and *Malus* (Gasic et al., 2009).

In this study, transferable chokecherry SSR primers were also tested for their ability to produce polymorphism in both sour and sweet cherry species. The result showed that 172 of 212 amplifiable chokecherry SSRs (81.1%) produced polymorphism in sour cherry species, while only 22.2% (47 of 212 SSRs) were polymorphic in sweet cherry. The low polymorphism in sweet cherry may be caused by the difference in ploidy level between chokecherry (4×) and sweet cherry (2×) and/or the low number of genotypes included in this research.

Although polymorphism of the chokecherry SSRs within species except cherries was not determined, variations of the amplification pattern were observed between chokecherry and other species. When using chokecherry primers that produced bands in chokecherry to amplify DNA of three other *Prunus* species, 66.5, 60.4, and 59.0% of amplification patterns in peach, plum, and apricot, respectively, were different from the ones in chokecherry. A higher variation in amplification pattern was also determined in other rosaceous species (63.7% in apple, 60.8% in crabapple, 54.7% in june berry, 51.9% in raspberry, and 31.6% in pear). Our result appears to not be consistent with the research of Gasic et al. (2009) in which only a few primer pairs of apple EST-SSRs amplified additional bands in other rosaceous species including pear, strawberry, rose, apricot, plum, almond, peach, sweet cherry, and sour cherry.

The sequence information of chokecherry generated from Roche 454 sequencing provides a powerful resource for genetic research of not only chokecherry, but also other species in *Prunus* and other member of the Rosaceae family. The high transferability rate of chokecherry

SSRs to other rosaceous species will be particularly useful for the species from which the genetic information is not available.

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CHAPTER 4. GENERAL CONCLUSION

Chokecherry (*Prunus virginiana* L.) is a small tree or large shrub widely distributed across the United States and Canada. As a member of *Prunus* genus, chokecherry has the same base chromosome number as other *Prunus* species ($x = 8$); however, it is a tetraploid, having 32 chromosomes ($2n = 4x = 32$). Among diseases in *Prunus* species, X-disease, caused by X-disease phytoplasmas, is a severe disease of stone fruit trees in North America. X-disease is one of the limiting factors for production of a few major *Prunus* species, particularly to peach, sweet and sour cherry, nectarine, and chokecherry. So far, no effective and practical methods are available to control X-disease. Development of resistant genotypes offers an excellent disease management option.

Advances in plant molecular genetics and genomics facilitate the development of molecular markers that can be used for genotype fingerprinting, map construction, and gene mapping. Integration of molecular marker technology, such as marker assisted selection (MAS), into a conventional plant breeding program will largely speed up the entire breeding process. Simple sequence repeat (SSR) markers have become the most favorable molecular markers due to their multi-allelic nature, reproducibility, co-dominant inheritance, high abundance, and extensive genome coverage. Many genetic linkage maps have been constructed using SSR marker systems. In this study, two sets of genetic linkage maps of chokecherry were constructed using TetraploidMap and JoinMap individually. A total of five SSR markers were identified to be associated with X-disease resistance in chokecherry. A total of 246 primer pairs were designed using Roche 454 chokecherry genome sequences. These newly-designed primers were validated in both chokecherry and other *Prunus* and rosaceous species.

This dissertation consisted of two main parts. Part 1 was a Literature Review, which provided the general background of chokecherry, the genus *Prunus*, commonly used molecular marker systems, X-disease and its management, mapping in polyploid species, and SSR marker development, etc. Part 2 was composed of two papers that were formatted as a formal manuscript. Paper 1 reported the construction of genetic linkage maps and identification of molecular markers associated with X-disease resistance in chokecherry. Paper 2 described the development and cross-species/genera transferability of microsatellites discovered from 454 genome sequences in chokecherry.

Three segregating populations of chokecherry were developed by crossing one X-disease resistant line (C_L) with three susceptible chokecherry lines (a, c, and d), of which the progenies were 101, 177, and 82, respectively. A total of 354 pairs of SSR primers including 246 pairs developed in chokecherry and 108 pairs adopted from other *Prunus* species were used to screen individuals of all three populations. Using TetraploidMap, 169, 150, and 87 loci were mapped on the map of $C_L \times a$, $C_L \times c$, and $C_L \times d$, respectively. Maps of each population contained two sets of linkage groups, one for parent (C_L) and the other for parent (a, c, or d). Maps constructed using JoinMap were composed of 153, 148, and 132 loci on the three maps, respectively. Five markers were identified to be associated with X-disease resistance. One SSR marker identified BSA was also mapped on the linkage group 6 of the map of $C_L \times a$ and on the linkage group 9 of the map of $C_L \times d$.

A partial chokecherry genome was sequenced using Roche 454 sequencing technology. A total of 145,094 reads covering 4.5 Mb of the chokecherry genome were generated and 15,113 contigs were assembled, in which 11,675 contigs were larger than

100 bp in size. A total of 481 SSR loci were identified from 234 (out of 11,675) contigs, in which 246 had flanking sequences suitable for polymerase chain reaction (PCR) primer design; thus, 246 primer pairs were designed. Of 246 primers, 212 (86.2%) effectively produced amplification from genomic DNA of chokecherries. All 212 amplifiable chokecherry primers were used to amplify genomic DNA from other 11 rosaceous species (sour cherry, sweet cherry, wild cherry, peach, apricot, plum, apple, crabapple, pear, june berry, and raspberry). As a result, chokecherry SSR primers can be transferable in *Prunus* species or other rosaceous species. An average of 63.2% and 58.7% of amplifiable chokecherry primers amplified DNA from cherry and other *Prunus* species, respectively, while 47.2% of amplifiable chokecherry primers can be transferable to other rosaceous species. Using random genome sequence data generated from the next-generation sequencing technology to identify microsatellite loci appears to be rapid and cost-efficient, particularly for species with no sequence information available.

In conclusion, the genetic maps, identified molecular markers, and information on chokecherry genome sequences and SSRs obtained in this research are valuable resources for the molecular genetics and X-disease research on *Prunus* woody species. Thus, this research will eventually facilitate the development of X-disease resistance in chokecherry and other *Prunus* species.