Scientia Horticulturae 127 (2011) 220–227

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Genetic diversity and phylogenetic relationships of *Prunus microcarpa* C.A. Mey. *subsp. tortusa* analyzed by simple sequence repeats (SSRs)

Mehmet Nuri Nas[a,*], Yuksel Bolek[b], Adem Bardak[c]

[a] *Kahramanmaras Sutcu Imam University, Faculty of Agriculture, Department of Horticulture, 46100 Kahramanmaras, Turkey*
[b] *Kahramanmaras Sutcu Imam University, Faculty of Agriculture, Department of Field Crops, 46100 Kahramanmaras, Turkey*
[c] *Bingol University, Faculty of Agriculture, Department of Field Crops, Bingol, Turkey*

ARTICLE INFO

Article history:
Received 28 August 2009
Received in revised form
18 September 2010
Accepted 21 September 2010

Keywords:
Microsatellite
Genetic diversity
Geographic origin
Prunus microcarpa

ABSTRACT

Prunus microcarpa C.A. Mey. *subsp. tortusa* is a deciduous shrub well adapted to severe winter and dry-hot summer conditions. As the first step to explore the genetic and horticultural potential of *P. microcarpa* C.A. Mey. *subsp. tortusa*, we used SSRs to elucidate the genetic variation within its populations dispersed in upper Mesopotamia. We also investigated its phylogenetic relationship with economically important *Prunus* species; almond, apricot, sweet cherry, peach and plums. Using 47 amplifying SSR primer pairs, 63 *P. microcarpa* C.A. Mey. *subsp. tortusa* genotypes sampled from five locations and 15 cultivars belonging to other *Prunus* species were assayed. The cross-species transportability of SSRs was 96% indicating a high degree of homology between *P. microcarpa* C.A. Mey. *subsp. tortusa* and the other *Prunus* species. The genetic distance between *P. microcarpa* C.A. Mey. *subsp. tortusa* genotypes belonging to a particular geographic site was lower than that between genotypes of different geographic origins. Cluster analysis differentiated *P. microcarpa* C.A. Mey. *subsp. tortusa* genotypes according to their geographic sites and separated them from the other *Prunus* species. *P. microcarpa* C.A. Mey. *subsp. tortusa* and sweet cherry, the subgenus *Cerasus*, were located in the same major cluster, the other *Prunus* species, belonging to the subgenera *Amygdalus* and *Prunus*, were located in another one. The analysis of molecular variance (AMOVA) revealed that genetic variation among individuals within populations (59.10%) was much higher than among *Prunus* groups (29.28%) and among *P. microcarpa* C.A. Mey. *subsp. tortusa* populations of different geographic sites (11.61%). The results indicate a substantial genetic diversity in *P. microcarpa* C.A. Mey. *subsp. tortusa* and the need of exploring a wider area to increase the chance of finding a particular genotype.

1. Introduction

Among Rosaceae, an economically important angiosperm family, the genus *Prunus* covers some of the most important temperate stone fruit species. In 2007, world annual production of stone fruit (almond, apricot, cherry, nectarine, peach and plums) exceeded 35.4 million metric tons (FAO, 2007). Because of its ecological, morphological and genetic diversity, and its economic importance, the genus *Prunus* has become the object of numerous horticultural and molecular studies (Zhebentyayeva et al., 2008).

The genetic diversity within *Prunus* species is highly variable and related to whether the species is self-fertile or self-sterile, and whether the species is wild or cultivated. The genetic variation within cultivated germplasm is generally low and it restricts the *Prunus* production to specific areas and conditions (Scorza et al., 1985).

In *Prunus*, various factors such as limited genetic variation within commercial cultivars (Scorza et al., 1985), susceptibility to plum pox virus (sharka) and self-incompatibility hinder development of new cultivars (Vilanova et al., 2003). Thanks to the high degree of homology among *Prunus* species (Cipriani et al., 1999; Sosinski et al., 2000; Testolin et al., 2000; Aranzana et al., 2003) interspecific hybridization of closely related species is possible. In several breeding programs, mainly aiming to develop better-adapted rootstocks, the introduction of genes from related *Prunus* species through interspesific hybridization has been utilized (Webster et al., 2000; Martínez-Gómez et al., 2005). Nevertheless, some commonly used rootstocks have undesired traits. For instance, dwarfing cherry rootstocks that provide precocious bearing, i.e. Gisela 5 and Maxma 14, are susceptible to crown rot *Phytophthora* spp. (Exadaktylou and Thomidis, 2005), and some peach-almond hybrid rootstocks are difficult to root if not micropropagated (Felipe, 1995). Thus, there is still a need to develop new rootstocks and/or cultivars.

Since the genetic variation within cultivated *Prunus* germplasm is generally low, to achieve breeding goals, utilization of wild

* Corresponding author. Tel.: +90 344 2191565.
E-mail address: mnurinas@ksu.edu.tr (M.N. Nas).

0304-4238/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.scienta.2010.09.018

germplasm and employment of molecular techniques, along with the currently available gene pool have been suggested (Webster et al., 2000). One of wild stone fruit noteworthy of exploration is *Prunus microcarpa* C.A. Mey. *subsp. tortusa* (syn. *Cerasus microcarpa*). *P. microcarpa* C.A. Mey. *subsp. tortusa* is usually found on dry calcareous and rocky mountain slopes at 400–1800 m elevations (http://www.tubitak.gov.tr/tubives). It is well adapted to severe winter and dry-hot summer conditions. In East and South East Anatolia, including upper Mesopotamia, it used to be an important part of the forest along side of other prominent species such as oak. Nowadays, as the majority of natural forest is destroyed, its population has been decreasing dramatically. Although it is highly resilient, continuous human and animal pressure holds back its restoration and reproduction, and may cause the loss of genetic variation.

P. microcarpa C.A. Mey. *subsp. tortusa* is a wild tart fruit-bearing deciduous shrub, occasionally used as an ornamental and living-fence plant, yet it may have the potential of being a dwarfing rootstock that provides precocious bearing as well (unpublished data). Whilst all members of cultivated and some of wild *Prunus* species have been studied, *P. microcarpa* C.A. Mey. *subsp. tortusa* has been neglected. Excluding taxonomic reports, studies dealing with *P. microcarpa* C.A. Mey. *subsp. tortusa*, if there is any, are scarce or not readily accessible. To realize the potential uses of *P. microcarpa* C.A. Mey. *subsp. tortusa*, it is necessary to know its genetic diversity, its relationship with other *Prunus* species, and develop effective conservation strategies.

In the present study, as the first step to explore the genetic and horticultural potential of *P. microcarpa* C.A. Mey. *subsp. tortusa*, we used SSRs to elucidate the genetic variation within its populations dispersed in upper Mesopotamia, Sirnak and Mardin province of Turkey (Fig. 1). The aim of the work was to evaluate the genetic variation in relation to geographic regions. We also investigated its phylogenetic relationships with other economically important *Prunus* species; namely almond (*P. amygdalus*), apricot (*P. armeniaca*), sweet cherry (*P. avium*), peach (*P. persica*) and plums (*P. cerasifera*, *P. domestica* and *P. salicina*). To the best of our knowledge, this is the first study investigating genetic variation and phylogenetic positioning of *P. microcarpa* C.A. Mey. *subsp. tortusa*.

In *Prunus* genetic studies, different types of molecular markers including isoenzymes (Martínez-Gómez and Gradziel, 2003), RFLPs (Dettori et al., 2001), RAPDs (Baránek et al., 2006), AFLPs (Aradhya et al., 2004) and simple sequence repeats (SSRs, microsatellites) have been employed. In the present work, we used SSRs as the choice of molecular markers to study genetic diversity in *P. microcarpa* C.A. Mey. *subsp. tortusa*: SSRs are co-dominant single-locus, highly polymorphic and dispersed relatively evenly throughout the genome. In related species, they often have well conserved flanking regions and high cross-species transportability. A considerable number of SSRs has been developed and effectively been applied in *Prunus* species (Cipriani et al., 1999; Downey and Iezzoni, 2000; Sosinski et al., 2000; Testolin et al., 2000; Dirlewanger et al., 2002; Aranzana et al., 2003; Sánchez-Pérez et al., 2005; Baránek et al., 2006). An integrated web-based Genome Database for Rosaceae (GDR) containing maps, markers and all available Rosaceae sequences is also available at http://www.bioinfo.wsu.ede/gdr.

2. Materials and methods

2.1. Plant materials

A total of 78 plants were assayed in this study. They included 63 genotypes of *P. microcarpa* C.A. Mey. *subsp. tortusa* dispersed in five sites of upper Mesopotamia (Sirnak and Mardin province of Turkey) and 15 cultivars belonging to other *Prunus* species [five almond (*P. amygdalus*), two apricot (*P. armeniaca*), two sweet cherry (*P. avium*), three plum (one *P. cerasifera*, one *P. domestica* and one *P. salicina*), two peach (*P. persica*) cultivars and one peach × almond hybrid rootstock (GF677)]. The chosen locations (K, M, Z, O, and B) were 5, 7, 30, 40 and 73 km away from each other (Fig. 1). *P. microcarpa* C.A. Mey. *subsp. tortusa* plants included 10 genotypes from site K (37°21′ N, 41°47′ E), 11 genotypes from site M (37°19′ N, 41°45 E), 13 genotypes from site Z (37°16′ N, 41°46′ E), 16 genotypes from site O (37°25′ N, 40°58′ E) and 13 genotypes from site B (37°29′ N, 41°29′ E).

2.2. Total genomic DNA extraction

Total DNA was first extracted from young leaves of wild grown plants. The leaves and extracted DNA were rich in phenolics and were inconvenient for PCR work. Therefore, cuttings taken from field grown plants were used for DNA extraction. Cuttings were forced to grow out in a growth room at 22 ± 2 °C and 16/8 h (light/dark) photoperiod under cool white fluorescent light. Basal ends of cuttings were placed in a forcing solution containing 10 mg l^{-1} Giberallic Acid 3 (GA_3) + 200 mg l^{-1} hydoxyquinioline citrate + 20 g l^{-1} sucrose (Read and Yang, 1987), and the forcing solution was changed every third day. In 2–3 weeks leaves of newly developed shoots were harvested and stored at −80 °C until used.

Total DNA was extracted from approximately 0.5 g of young leaves according to a slight modification of the procedure described by Zhang and Stewart (2000). The leaves were ground in liquid nitrogen to fine powder, put into 2-ml eppendorf tubes and extracted with 0.5 ml extraction buffer (0.1 M Tris–HCl (pH:8), 1 M NaCl, 0.02 M EDTA (pH:8), 2% (w/v) CTAB, 2% polyvinylpyrrolidone-40, 1 mM 1,10-phenanthroline monohydrate, 0.2% P-mercaptoethanol). The mixture was incubated at 65 °C for 1 h, then 0.5 ml chloroform:isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was transferred to a new 1.5-ml eppendorf tube; 0.5 ml of isopropanol was added, mixed and incubated at −20 °C for 1 h, centrifuged at 12,000 rpm (4 °C) for 10 min. The supernatant was removed, the pellet was washed with 0.5 ml of 70% ethanol centrifuged at 13,000 rpm (4 °C) for 2 min, and a second wash with 0.5 ml of 100% ethanol, centrifuged at 13,000 rpm for 2 min, the supernatant was removed and the pellet was dried at room temperature. The pellet was dissolved in TE buffer; DNA was quantified spectrophotometrically and by metaphor agarose gel electrophoresis.

2.3. PCR amplification and product electrophoresis

Extracted genomic DNA was PCR-amplified using 56 SSR primer pairs developed for other *Prunus* species (Table 1). The primers were synthesized by Iontek, Turkey. PCR reactions were performed in a 20 μl volume. The reaction mixture contained 0.25 mM of each dNTP, 2 μl of 10× PCR buffer (Favorgen), 5 μM of each primer, 1 unit of Taq DNA polymerase (Fermentas) and 60 ng of genomic DNA. The PCR-amplification program consisted of one cycle at 95 °C for 3 min, then 35 cycles of [94 °C for 1 min, 55–60 °C (depending on primer annealing temperatures) for 1 min, 72 °C for 1 min], a final cycle at 72 °C for 5 min. The PCR reactions were carried out in a 96-well block Eppendorf Mastercycler. Amplified PCR products were separated by electrophoresis using 2.5% (w/w) Metaphor agarose (Lonza, USA) + 1.5% (w/w) low melting agarose (Sigma, A5093) gel, stained with ethidium bromide, visualized and photographed under UV light using an AlphaImager Gel Documentation and Analysis System, and fragment lengths were calculated by molecular weight comparison with 20 bp DNA step ladder mobility (Promega).