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Evaluation of the genetic structure present in natural populations of four subspecies of black cherry (*Prunus serotina* Ehrh.) from North America using SSR markers

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ABSTRACT

Black cherry (*Prunus serotina*) is a fruit tree native to North America, and almost all parts of this plant have some use. This species is a complex of five subspecies with morphological differences and distinctive habitats. The genetic structure of 18 natural populations of black cherry was evaluated with 16 microsatellite markers. One hundred sixty-four individuals were collected across seven states of Mexico, and another 14 in Texas. These individuals represented subspecies *capuli* (38), *eximia* (14), *serotina* (53), and *virens* (73). A total of 246 alleles was detected for the 16 markers. A Neighbor-Joining tree and a principal coordinate analysis (PCoA) revealed two major groups of individuals while Bayesian clustering analysis detected six main clusters that do not clearly correspond to the four putative subspecies studied. At the $p \geq 0.900$ cut off values, the composition of these clusters indicates geographical structuring of the samples; their genetic differentiation suggests gene flow among populations with geographical proximity. This is the first time that molecular markers have been used to assess the genetic structure present in natural populations of four subspecies of black cherry with the goal of evaluating their taxonomic classification and providing guidelines for *in situ* conservation of genetic resources of the species.

1. Introduction

Black cherry (*Prunus serotina* Ehrh.) is a fruit tree native to North America, almost all parts of which have some use. In Mexico, where the species is commonly known as capulín, its fruits are eaten fresh, dried, or as ingredients in other preparations (e.g., jellies, tamales, and liqueurs), and the seed is consumed after toasting the stony endocarp (Raya-Pérez et al., 2012). Its leaves and fruits are believed to have expectorant, sedative, and antispasmodic properties; additionally, inflorescences and leaves are considered to be an excellent source of antioxidants (Olszewska, 2007). Its wood has been used since pre-Columbian times as firewood (Adriano-Morán and McClung de Tapia, 2008). In the United States of America, the wood of *P. serotina* is appreciated because of its hardness and durability (Maynard et al., 1991; Rohrer, 2014), and owing to the thickness of the trunks and reddish color of the wood, it has a commercial value similar to sweet cherry (*Prunus avium* L.) (Barnd and Ginzel, 2008; Rohrer, 2014; Wang and Pijut, 2014). Historical accounts show that black cherry was introduced to South America and Europe in the 17th century by colonizers; in Ecuador it reached some importance for commercial trading, and in several European countries it became an invasive species (Popenoe and Pachano, 1922; Starfinger et al., 2003).

McVaugh (1951) described black cherry as a botanical complex of five native subspecies of North America (*capuli*, *eximia*, *hirsuta*, *serotina* and *virens*), and stated that instant visual field recognition of the different subspecies would be unfeasible if they were not geographically segregated in nature due to continuous variation and some degree of overlap among subspecies in nearly all distinguishing characters.

In Mexico, there are some selections of ssp. *capuli* that are promising for horticultural research (Segura-Ledesma et al., 2009). This Mexican subspecies is considered the cultivated form of ssp. *serotina* (McVaugh, 1951). Cultivated individuals are grown in the states of Tlaxcala, México and Veracruz for their large fruit and large round seed with thick endocarp (which facilitates the extraction of the edible embryo), and characteristically have larger broader leaves with long petioles and longer flower peduncles (Avendaño-Gómez et al., 2015). People commonly consume these fruits either fresh or prepared, while the seeds are roasted, salted and eaten as snacks.

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It has been established that black cherry is an allotetraploid ($2n = 4x = 32$) for which there is no certainty about its progenitor species (Pairon and Jacquemart, 2005). Diploid individuals had also been reported by Forbes (1969), and pentaploid and hexaploid individuals were referenced by Dickson et al. (1992). Nonetheless, these potential variations in chromosome number have not been clarified, or ascribed to differences among intraspecific groups.

The first published study of molecular variability in this species focused on demonstrating the usefulness of DNA markers developed from peach (*Prunus persica* (L.) Batsch), sweet cherry (*Prunus avium* (L.) L.), and sour cherry (*Prunus cerasus* L.) in the evaluation of black cherry diversity (Downey and Iezzoni, 2000). Subsequently, some molecular genetic analyses have been conducted with various objectives, including: to improve the understanding of its invasive behavior in European forests and to establish efficient control strategies (Pairon et al., 2006; Petitpierre et al., 2009; Pairon et al., 2010); to evaluate genetic variability of the species in Ecuador (Guadalupe et al., 2015); and to test the Abundant Center Model in central North America using black cherry populations (Beck et al., 2014). In the context of timber use, in the USA it has been included in genetic breeding projects that sought to obtain transgenic black cherries more resistant to insect attack, to reduce the occurrence of gummosis, and to improve the economic gains from the use of its wood (Wang and Pijut, 2014). However, at the present time, there are no published studies of population genetic structure across the natural geographic distribution range of the species.

Similarly, to date, there is a lack of formal *ex situ* collections of *P. serotina* germplasm. The Germplasm Resources Information Network (GRIN) on its public website (GRIN-Global; GRIN, 2015), catalogs 76 accessions of *P. serotina*, located in Ecuador (27), Mexico (25), the United States (9), Peru (4), Guatemala (4), France (3), Colombia (2), Poland (1), and the United Kingdom (1). Nonetheless, all these accessions are referenced as “Historical record only”, and therefore they are not available. These observations highlight that *ex situ* conservation is urgently needed and strongly recommended for the natural populations of black cherry.

This study was undertaken with the main goal of evaluating for first time the genetic structure within and among four subspecies of black cherry, in order to improve the understanding of the geographic and taxonomic distribution of genetic diversity within the species. To do this, 178 individuals collected in Mexico and the United States were evaluated using 16 microsatellites.

The objectives of this study were to determine if intraspecific genetic groups of black cherry clearly correspond to the four subspecies studied, if the genetic structure within black cherry is explainable by geographic distribution, and if there is evidence of gene flow among subspecies and/or other (e.g., geographically based) genetic clusters. Understanding the patterns of genetic diversity of black cherry can shed light on its intraspecific genetic variation and will also guide decisions about sustainable conservation and use of genetic resources of the species.

2. Materials and methods

2.1. Field sampling and DNA extraction

In 2015 and 2016, 178 individuals representing four black cherry subspecies were collected (Table 1). The determination of the 18 collection sites and the taxonomic identities were aided by consulting herbarium specimens from Herbario del Instituto de Ecología A.C. – IEBAJÍO (IEB, Pátzcuaro, Mexico) and The University of Texas at Austin Herbarium (LL, Austin, U.S.A.) and botanical reports (e.g., McVaugh, 1951; Rzedowski and Calderón de Rzedowski, 2005; Fresnedo-Ramírez et al., 2011). At least three individuals per site were collected and labeled, pressed, and mounted as herbarium voucher specimens, which were deposited at Universidad Autónoma Chapingo Herbarium (CHAP, Chapingo, Mexico). Young leaves were collected directly in the field, immediately put in silica gel and later lyophilized for at least 60 h using the Labconco Freezone 2.5 Lyophilizer (Labconco Corporation, Kansas City, Missouri). For DNA extraction, 30–40 mg of desiccated leaf tissue was pulverized with a Mini- Beadbeater-1 (BioSpec Products Inc., Bartlesville, Oklahoma). DNA samples were obtained with *DNEasy Plant Mini Extraction* (Qiagen Inc., Valencia, California) following the directions provided by the manufacturer. Modifications suggested for improving this method of extraction by Costa and Roberts (2014) were implemented.

2.2. Microsatellites amplification

Twenty-two microsatellite markers from five economically important *Prunus* species were PCR amplified with FAM-, NED- or HEX-labeled forward primers and unlabeled reverse primers (Table 2). The primers were run in triplexes, based on their fluorescence dye and allele sizing. PCR conditions were 0.06 ul of each primer (10 pmol stock), 1.5 ul of 10X buffer, 1.5 ul of $MgCl_2$ (25 mM), 1.2 ul dNTPs (2.5 mM each nucleotide), 0.15 ul of Taq polymerase (5000 U/ml, New England Biolabs), and 15 ng of DNA in a 15.0 uL volume. The PCR conditions were as follows: one initial denaturation step of 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 40 s at 72 °C; and a final extension of 7 min at 72 °C. Replicate samples were tested in separate experiments to confirm repeatability of results. Negative controls were run with every PCR to check for DNA contamination.

Out of the 22 SSR markers, six were dropped after preliminary evaluation because they produced poor amplification (Table 2). PCR products were run on an ABI PRISM 3130 Fragment Analyzer (Applied Biosystems, Foster City, California) using the size standard 400H ROX, and allele sizes were determined by means of GeneMapper Software Version 4.0 (Applied Biosystems, Foster City, California). The observed allele sizes were then adjusted for the discrete allele sizing using the bins databased in the laboratory of the USDA National Clonal Germplasm Repository in Davis (California). Alleles sizes were scored as di-allelic for each assigned locus (1 = band present, 0 = band absent), and a binary matrix was produced.

2.3. SSR genetic diversity analysis

Each band was considered to represent a di-allelic locus; thus, presence of the band is scored as one allele and its absence is the alternative allele. The polymorphism information content (PIC) value for each SSR was estimated on an Excel spreadsheet using the following formula: $PIC = 1 - \sum x_i^2$, where x_i is the relative frequency of the ith allele of the SSR loci. Markers were classified as informative when PIC ≥ 0.5.

GenAlEx 6.5b3 (Peakall and Smouse, 2012) was used to assess the genetic diversity of each population and subspecies by calculating the percentage of polymorphic loci, allelic richness (number of alleles, number of private alleles, and number of locally common alleles), and expected heterozygosity (He). In GenAlEx, the genetic structure was studied by determining the number of private alleles for each population and subspecies, and by analyses of molecular variance (AMOVA; 999 permutations) for populations and subspecies. Additionally, correlation between geographic distance and genetic distance between individuals was tested in GenAlEx using Mantel tests (999 replicates).

The software package PAST (Hammer et al., 2001) was used to generate a distance matrix from the raw data matrix based on Neís unbiased measure of genetic distance (Nei, 1987), which bases on the proportion of alleles shared between two samples for all possible pairwise combinations of individuals and populations. The resultant matrix was subjected to a cluster analysis using the Neighbor-Joining method to obtain a tree that depicted the genetic relationships among individuals; a bootstrap analysis (10,000 replicates) was carried out to measure branch support by the data. Additionally, in GenAlEx, with the