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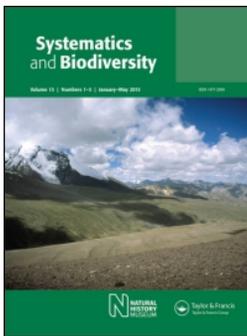


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Research Article

Using multiple sources of characters to delimit species in the genus *Crataegus* (Rosaceae): the case of the *Crataegus rosei* complex

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The aim of this study was to delimit the taxa of the *Crataegus rosei* complex using an integrative approach that incorporates a suite of molecular (cpDNA and nuclear microsatellite markers), morphological, and geometric morphometric characters. One hundred and ten plants from 19 populations that encompass the entire distribution range of the species complex were collected and examined along with herbarium specimens. Parsimony and Bayesian inference analyses were run using morphological, molecular, and both the morphological and molecular data sets combined. Analyses to determine genetic structure based on microsatellite data and multivariate analyses incorporating geometric morphometrics were also done to identify differences in leaf shape. The results supported the recognition of two taxa: *C. rosei* with high levels of gene flow among its populations, remarkable morphological variation and a wide distribution range and *C. rosei* var. *amoena*, composed of a few isolated populations in the high elevation location of Cerro Potosí; a new specific epithet will be decided for the latter in accordance with the International Code of Nomenclature for algae, fungi, and plants.

Key words: apomixis, Cerro Potosí, *Crataegus*, cryptic species, hawthorn, integrative taxonomy, tejocote, tetraploidy

Introduction

Crataegus is one of the largest genera of woody species in the Rosaceae. Distributed mostly in the temperate regions of the northern hemisphere, *Crataegus* is considered a taxonomically difficult group due to its remarkable morphological variation (Phipps, 1983; Phipps, O'Kennon, & Lance, 2003), which has been influenced by a number of processes such as apomixis, hybridization, and polyploidy (Brown, 1910; Camp, 1942a; Camp, 1942b; Phipps, 1983; Stapf, 1914). Of these, hybridization is probably one of the most important processes in this genus and has been reported to occur within and across populations of different species at various spatial scales and times, with hybrid individuals often successfully adapting to different habitats (Campbell & Dickinson, 1990; Muniyamma &

Phipps, 1979; Talent & Dickinson, 2007). Apomixis, in turn, might have two important effects on morphological variation in *Crataegus*: it can produce homogeneity within populations and differentiation among populations (Burgess et al., 2014; Dickinson & Phipps, 1985, 1986). In practice, it is impossible to tell xenogamous plants from obligate or facultative apomictic plants in natural populations based solely on morphological traits (Phipps & Muniyamma, 1980). Both apomixis and hybridization can complicate recognizing and characterizing species and intra-specific taxa in *Crataegus*, and thus obscure speciation events (Campbell & Dickinson, 1990; Dickinson & Campbell, 1991). That being said, it is estimated that there are between 140 and 200 species in *Crataegus*, divided into 15 sections and 40 series, of which more than 50% are New World endemics (Phipps, Robertson, Smiths, & Rohrer, 1990; Phipps et al., 2003).

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Modern taxonomic classifications of the genus have been proposed by Phipps (1983) and Phipps *et al.* (2003), chiefly based on the phenetic analysis of morphological characters and the geographic distribution of species. Since most of the *Crataegus* species in the Old World are diploid and possess distinctive morphological traits, their taxonomy is thought to be more straightforward (Christensen, Zarrei, Kuzmina, Talent, Lin, & Dickinson, 2014; Gu & Spongberg, 2003; Phipps *et al.*, 1990). In contrast, most American species are polyploid (Talent & Dickinson, 2007), with apomictic or vegetative reproduction (Dickinson, Lo, Talent, & Love, 2008; Phipps & Muniyamma, 1980; Phipps *et al.*, 1990) and notable morphological variation (Phipps, 1983; Phipps *et al.*, 2003). As a consequence the taxonomy of the American *Crataegus* is more problematic.

An important source of characters used to recognize species in *Crataegus* has been morphological data (Dickinson & Phipps, 1985; Dickinson, Belaussoff, Love, & Muniyamma, 1996; Macklin & Phipps, 2006; Phipps, 1997; Phipps & Dvorsky, 2008; Sinnott & Phipps, 1983), in particular the geometric morphometrics of the leaves has proven to be a valuable source of data for evaluating differentiation within *Crataegus* (Delgado, Jain, Sánchez, & Reyes, 2013; Dickinson & Phipps, 1984; Dickinson, 1986). Foliar morphological variation and leaf shape within and across species of *Crataegus*, along with other foliar traits, have been widely used for classification (Albarouki & Peterson, 2007; Depypere *et al.*, 2006; Dönmez, 2007; El-Gazzar, 1980; Phipps, 1983, 1997). Moreover, in species such as *C. mexicana*, which has edible fruit and is widely let-stand cultivated, variation in foliar traits has been used as a marker to recognize individuals with agricultural potential (López-Santiago *et al.*, 2008; Nuñez-Colin *et al.*, 2009). To recognize and characterize *Crataegus* species, foliar traits have been analysed using traditional morphometric methods on continuous, quantitative, or qualitative characters alone or in combination (Albarouki & Peterson, 2007; Depypere *et al.*, 2006; Dickinson *et al.*, 1996; Dönmez, 2007; López-Santiago *et al.*, 2008; Nuñez-Colín *et al.*, 2009; Phipps, 1983, 1997). Molecular characters such as cpDNA sequences and nuclear microsatellite markers allow for a better understanding of hybridization among individuals in *Crataegus* species, allow us to determine their origin and maternal input and to identify the genetic lineages of populations (Christensen *et al.*, 2014; Lo, Stefanovic & Dickinson, 2009; Zarrei, Stefanović, & Dickinson, 2014). Additionally, the study of such traits has helped elucidate genetic diversity and structure among populations or species (Dai, Guo, Zhang, Li, Chang & Zhang, 2009; Ferrazzini, Monteleone, & Belletti, 2008; Lo *et al.*, 2009; Lumley & Sperling, 2011; MirAli, Al-Odat, Haider, & Nabulsi, 2011; Yilmaz, Yanar, Ercisli, Sahiner, Taskin, & Zengin, 2010). Despite efforts in the search for informative

characters to define species or species limits within taxonomic complexes in the genus *Crataegus*, there are still some cases for which this task is daunting due to limited infra-specific or specific molecular variation (Albarouki & Peterson, 2007; Lo *et al.*, 2009; Phipps, 1997).

Among the Mesoamerican species of *Crataegus*, the *Crataegus rosei* complex stands out as the most widespread and variable endemic group (Nuñez-Colín *et al.*, 2008; Phipps, 1997). This species complex, together with *C. crus-galli* L., *C. reverchonii* Sargent, *C. gracilior* J.B. Phipps, *C. engelmannii* Sargent, *C. berberifolia*, *C. fecunda* Sargent, and *C. x persimilis* Sargent, comprises the *Crus-galli* series according to the latest classification by Phipps *et al.* (2003). *Crataegus rosei* is considered a taxonomic complex because of its enormous morphological variation, mainly in fruit colour and shape and in leaf margin, and as a result there is much controversy over the group. Eggleston (1909) described two species based on differences in fruit colour: *Crataegus rosei* (red fruit) and *C. parryana* (yellow fruit) from specimens collected by E. Palmer in 1902 in the Sierra de Alvarez, San Luis Potosí. Later, Phipps (1997) found that fruit colour, calyx and leaf shape, as well as leaf pubescence varied between *C. rosei* and *C. parryana* and that particular character states were not restricted to defined geographic areas. Accordingly, Phipps (1997) concluded that the taxonomic status should be intra-specific and recognized them as *C. rosei* subsp. *rosei* (Eggl.) J.B. Phipps and *C. rosei* subsp. *parryanana* (Eggl.) J.B. Phipps. These taxa are widely distributed in association with pine-oak forests in the mountains of the Sierra Madre Oriental (SMOr) and the Sierra Madre Occidental (SMOc), on the Mexican Plateau (MP), along the Trans Mexican Volcanic Belt (TMVB), and in the Highlands of Chiapas (HCh) in Mexico (Fig. 1). Additionally, Phipps (1997) analysed the variation within *C. rosei* subsp. *rosei* and proposed dividing this taxon into three groups: *C. rosei* subsp. *rosei* var. *rosei*, var. *mahindae* J.B. Phipps, and var. *amoena* J.B. Phipps. The division was based on differences in the number of stamens, anther colour, leaf length, foliage colour, and distribution area (SD1, see online supplemental material, which is available from the article's Taylor & Francis Online page at <http://dx.doi.org/10.1080/14772000.2015.1117027>) (Fig. 1) with var. *amoena* J.B. Phipps restricted to only two known localities (Phipps, 1997).

The aim of this study was to delineate the species of the *Crataegus rosei* complex using an integrative taxonomic approach that incorporates molecular (cpDNA, nuclear microsatellite markers), traditional morphological and geometric morphometric characters, separately or in combination. In addition, climate data were also incorporated to elucidate whether variation in climate has an effect on genetic isolation. Phylogenetic, microsatellite analyses, and morphometric multivariate analyses were run using an eclectic approach, as suggested by Sites and Marshall

(Albarouki & Peterson, 2007; Lo *et al.*, 2009). PCR conditions for *trnH-psbA* are described in Shaw *et al.* (2005), and in Vaillancourt and Jackson (2000) for *trnH-rpl2*. Due to the fact that the two regions are adjacent in the chloroplast genome, they were combined and treated as a single marker for analysis. We sequenced 110 individuals of the ingroup and used six species (*Mespilus* sp., *Crataegus punctata*, *C. mexicana*, *C. crus-galli*, *C. engelmannii*, and *C. gracilior*) as outgroup. The samples of *C. mexicana* and *C. gracilior* were collected in the field for this study; the other species were obtained from herbarium specimens from the MEXU herbarium (SD3, see supplementary material online). The sequences were edited and assembled using Sequencher 4.1 (Gene Codes, Ann Arbor, MI, USA). The *trnH-PsbA* and *trnH-rpl2* matrices were initially aligned with Muscle (Edgar, 2004) and later edited by eye with Se-AL v.2.0a11 (Rambaut, 2002).

Microsatellite markers

We tested 16 microsatellite loci (CH01d03, CH01f02, CH01f07a, CH01f12, CH02a08, CH03a02, CH03d08, CH03h06, CH04e05, CH04f06, CH04g12, CH05a09, CH05d04, CH05g03, CH05g11, MS14h03). Primers were developed following the protocols described by Gianfranceschi, Seglias, Tarchini Komjanc, and Gessler (1998). Seven of these loci (CH01f02, CH01f07a, CH03a02, CH03d08, CH04f06, CH05d04, CH05g11) were selected because they have been used in *Crataegus* (Lo *et al.*, 2009). The remaining loci were selected because they have been used in related genera such as *Eriobotrya*, *Malus*, and *Pyrus* (Gisbert, Martínez-Calvo, Llácer, Badenes, & Romero, 2009; Liebhard *et al.*, 2002; Yamamoto *et al.*, 2007). All selected loci were reported as perfect dinucleotide tandem repeats and together, they represent at least eight different linkage groups (LG) F3, F4, F5, F7, F10, F12, F14, and F17 with several located on unknown LGs (SD4, see supplementary data). PCRs were performed on a total volume of 8 μ L containing 5.0 \times reaction buffer (Promega), 2.5 mM MgCl₂, 0.2 mM of each dNTPs, 10 μ m of each primer, 1 U of Taq DNA polymerase (Promega) and 10 ng/ μ L of genomic DNA. Forward primers were end-labelled with fluorescent dyes 6-FAM, NED, ROX, and VIC. Although all SSR primers used the same annealing temperature, we could only combine CH05d04 and CH01f07a in the PCR amplification, all other loci were amplified separately and then combined (multiplexing) in sets of two or three loci. Fragment sizes were determined using Gene Mapper version 4.1 (Applied Biosystems).

Morphological characters

We analysed 17 morphological characters: six quantitative and 11 qualitative (SD5 and SD6, see supplementary

data). Continuous quantitative character measures were taken using a digital caliper. All characters were coded from the material collected for this study, except when it was in poor condition, in which case we used herbarium specimens from MEXU and XAL. Leaf angle between the main and secondary veins was obtained from digital images taken from the leaves of all sampled specimens using a Nikon D5100 camera and calibrated with ImageJ 1.37v (Rasband, 2006) software. Leaf surface pubescence was recorded by estimating the percentage of the surface in the visual field of a microscope covered with hairs (SD5, see supplementary data).

Phylogenetic analysis

Three analyses were done, one using molecular characters, one using morphological characters and one using a combination of molecular and morphological characters. Gaps were coded in SeqState ver. 1.4.1 (Müller, 2005) following the method of Simmons and Ochoterena (2000). Parsimony analyses were run using a new technology search approach: the ratchet algorithm with 500 iterations (Nixon, 1999). Clade support was Jackknifed (JK) with 1000 replicates with 25% deletion on a traditional search in TNT (Goloboff, Farris, & Nixon, 2008). Bayesian inference was performed with MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003). The model of molecular evolution was determined with jModelTest v.0.1.1 (Posada, 2008) using the Akaike Information Criterion (AIC); the model that best fit the molecular data was F81+G. For morphological data we used the Markov *k* (Mk) model of Lewis (2001) as recommended in Nylander, Ronquist, Huelsenbeck, and Nieves-Aldrey (2004). The Mk model is a generalized Jukes–Cantor model (JC69). In the JC69 model, the states evolve according to a continuous-time, stationary Markov process, in which each state occurs with equal probability at the root, and the transition rate on any edge of the tree is the same for each possible substitution (Steel & Fu, 1995). For morphological data the Mk model assumes that lineages are always in one of *k* possible states ($k \geq 2$), with no state considered plesiomorphic or apomorphic a priori (Lewis, 2001). Bayesian inference was carried out with two independent runs, each with three hot and cold chains, running 10,000,000 generations, sampling a tree every 1000 generations for each analysis. Stationarity was determined based on the likelihood scores for time to converge, and sample points generated prior to stationarity were eliminated as burn-in (20%). The posterior probabilities (PP) of the clades were determined by a 50% majority consensus of the retained trees.

Microsatellite marker analysis

Due to the tetraploid nature of *C. rosei* complex we converted the codominant microsatellite allele patterns into

“allele phenotypes” and analysed them as dominant markers. Alleles were entered as present (1) or absent (0). Although some information is lost (Bockelmann, Reusch, Bijlsma, & Bakker, 2003; Markwith & Parker, 2007; Rodzen, Famula, & May, 2004; Sampson & Byrne, 2012; Teixeira, Rodriguez-Echeverría, & Nabais, 2014), this method is commonly used for the microsatellite data of polyploid organisms (Kloda, Dean, Maddren, MacDonald, & Mayes, 2008; Lo et al., 2009). To estimate the number of genetic clusters or subpopulations (K) we used a Bayesian clustering approach implemented with BAPS v. 5.2 (Corander, Marttinen Siren, & Tang, 2008) and Structure v. 2.3 (Pritchard, Stephens, & Donnelly, 2000; Falush, Stephens, & Pritchard, 2007) software. With BAPS, the search was conducted using from two to 19 groups (K), with ten replicates for each K value using the spatial cluster individual functions. With Structure, we first performed an exploratory analysis run with 1 to 20 groups (K) with 10 replicates. The run conditions were: a burn-in period at 100,000 followed by 10^6 iterations of the MCMC. To find the most likely value of K , $\text{LnP}(D)$, a measure of the natural log of the posterior probability (P) of the data (D), and $D(\Delta K)$, an empirical statistic based on the second-order rate of change and the variance of $\text{LnP}(D)$ (Evanno, Regnaut, & Goudet, 2005), were calculated using Structure Harvester (Earl, 2012). The optimal value of K based on $\text{LnP}(D)$ was between three and four groups (K), and thus a second analysis was run using the admixture model from two to five groups (K) with 40 replicates each. The run conditions were: a burn-in period at 300,000 followed by 10^6 iterations of the MCMC. Clumpp and Distruct software (Jakobsson & Rosenberg, 2007) were used to combine the Structure group-membership output data for each genetic cluster from the 40 replicates for the genetic cluster for $K = 3$ in the second analysis, as shown in the Structure results.

Based on the binary data set, an analysis of molecular variance (AMOVA) was performed to evaluate the hierarchical partitioning of genetic variance among the lineage groups of populations defined with BAPS (BAPS A: population LS group 1; population LT group 2; populations CJ, LP and AC group 3; all the rest group 4) (BAPS B: population LS group1, population LT group 2; populations CJ and LP group 3; all the rest group 4) and Structure (Structure A: populations LS+LT group1; populations CJ, AC, LP, VA+EP group 2; all the rest group 3) (Structure B: populations LT group1, populations CJ+LP group 2; all the rest group 3) using Arlequin ver 3.1 software (Excoffier, Laval, & Schneider, 2006).

Morphometric analysis

Three to seven leaves were randomly selected from each of the herbarium specimens. All leaves were individually

photographed using a digital Nikon D5100 camera mounted on a tripod at approximately 30 cm from the base. Leaves were photographed beside a graduated ruler for scale. A total of 673 leaves were digitalized and included in the analysis. Because the leaves of plants in the *Crataegus rosei* complex do not have natural marks that can be used as a shape reference, we created a fan of 44 radial guidelines covering the whole leaf contour using MakeFan6 software from the "Integrated Morphometrics Package" IMP series (<http://www.canisius.edu/~sheets/morphsoft.html>). The lamina base and apex were used to construct the “fan” (radial guidelines with equal angular spacing on images). Then for each leaf image we recorded 24 homologous anatomical marks using TpsDig (Bookstein, 1991; Rohlf, 2005), and 22 semi-landmarks, along the leaf contour which represent morphological points that incorporate all of the structure information and can be considered homologous to a morphometric analysis (Zelditch, Swiderski, Sheets, & Fink, 2004).

A Procrustes superimposition analysis was run to analyse the configuration of the 24 landmarks and semi-landmarks to obtain the leaf shape variables using CoordGen6 software in the IMP series (<http://www.canisius.edu/~sheets/morphsoft.html>). The first step of Procrustes superimposition calculates the generalized least squares of the landmark and semi-landmark configurations ($x_1, y_1, x_2, y_2 \dots x_{24}, y_{24}$, plus centroid size) that minimizes the differences between landmark configurations by translation, scaling, and rotation to remove all information not related to shape, and to obtain the Procrustes coordinates of the shape variables (Rohlf, 1990). After superimposition, the shape variables (48 Procrustes distances; [i.e. 24 x and 24 y coordinates] plus centroid size) of each of the 110 specimens were averaged across all leaves measured per individual.

To explore differences in the leaf shape variation of the Procrustes coordinates in all individuals of the *C. rosei* complex, we performed a principal component analysis (PCA) using PAST v.2.12 software (Hammer, 2011). To delimit morphological variation among the different taxa of the *C. rosei* complex, we ran a canonical discriminant analysis with SPSS ver. 17.00 software, following Ferrán (2001). The grouping variables were the taxonomically identified varieties *C. rosei* subsp. *rosei* var. *rosei*, var. *mahindae* and var. *amoena*, and the subspecies *C. rosei* subsp. *parryana*. To visualize shape variation among the taxa of the *C. rosei* complex, the Procrustes coordinates were averaged by taxa and subsequently displayed on an XY graph with PAST v. 2.12 software (Hammer, 2011).

Climate variables

To explore whether the variation observed among taxa of *Crataegus rosei* complex was associated with

environmental factors, we ran a principal component analysis (PCA) using environmental variables data from WorldClim 1.4 (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005) for each population studied. First, a correlation analysis was performed to eliminate correlated environmental variables using PAST v. 2.12 software (Hammer, 2011) and then only the least correlated variables (Pearson ≤ 0.7 based on all sample locations) were used (BIO 3; BIO 9; BIO 11; BIO 12; BIO15). The PCA was run in PAST v. 2.12 (Hammer, 2011).

Results

Phylogenetic analysis

The data matrix for the Parsimony and Bayesian analyses returned 121 individuals as terminals (110 individuals as the ingroup and 11 individuals as the outgroup), and included 680 characters, including 641 bp (289 *trnH-rpl2*, 352 *psbA-trnH*), 22 gaps (9 *trnH-rpl2*, 13 *psbA-trnH*) and 17 morphological characters.

The parsimony analysis of the molecular data retrieved seven most parsimonious trees (MPT) (L = 116, CI = 0.776, RI = 0.981). The strict consensus tree recovered nine clades with JK support values above 50%. The *C. rosei* complex was not retrieved as a monophyletic group; most of the clades that received support comprised individuals from the same or geographically close populations (Fig. 2). The morphological data matrix retrieved a single most parsimonious tree (MPT) (L = 607, CI = 0.202, RI = 0.696) but none of the clades received good support (JK $\geq 50\%$) (not shown). Only 40 (5.88%) characters were parsimony-informative in the combined matrix of molecular and morphological data sets. This analysis retrieved five most parsimonious trees (MPT) (L = 465, CI = 0.355, RI = 0.858). Although eight clades received good support (JK $\geq 50\%$), only one, clade III, corresponds to *C. rosei* var. *amoena*, the rest of the *C. rosei* complex was retrieved as a paraphyletic group. Most clades with support comprised individuals from the same population (Fig. 3a).

Bayesian inference based on molecular characters identified seven clades with good support (posterior probabilities PP ≥ 0.90), however the individuals of the *C. rosei* complex were not recovered as a monophyletic group. Within this clade five groups (A, B, C, D, and E) correspond to individuals of the same population or to individuals located in nearby areas (Fig. 2). The combined Bayesian inference analysis retrieved seven well supported groups (PP $\geq .90$). Individuals of the *C. rosei* complex did not comprise a monophyletic group. Three clades (A, B, and C) received good support with individuals from the same population or from nearby populations (Fig. 3b).

Microsatellite marker analysis

Of the 16 microsatellite loci analysed, 11 loci were amplified and polymorphic (CH01d03, CH01f02, CH01f07a, CH02a08, CH03h06, CH04f06, CH04g12, CH05a09, CH05d04, CH05g03, CH05g11). Locus CH01d03 was excluded from the analyses because we were not able to amplify all samples. A total of 220 alleles (10–29 per locus) were detected and analysed (SD4, see supplementary data). According to the Bayesian clustering approach of BAPS, populations of *C. rosei* complex are clustered in four genetic groups ($K = 4$). Populations LS, LT, CJ and LP form three different groups LS = group I, LT = group II, CJ and LP = group III, the rest of the populations – VA, EP, HZ, JM, EV, JL, GD, VF, SA, SR, IX, ZA, SD and SC – form another group IV with some individuals with mixed genotypes. Only AC population has a mixture of individuals with two different genotypes (Fig. 1). Three genetic clusters ($K = 3$) were found with Structure software. Only LT population represents a genetically distinct group, the rest of the populations are comprised of a mix of two or three genotypes (2, 3) in different proportions (Fig. 1). Individuals of subsp. *parryana*, var. *rosei*, and var. *mahindae* were included in the four different genetic groups in the BAPS analyses, and different combinations of three genetic groups in the Structure analyses. Nevertheless, all *C. rosei* var. *amoena* specimens (CJ and LP populations) were in the same genetic group according to both BAPS and Structure (Fig. 1).

According to the AMOVA performed on all populations, the percentage of variation found within populations was 1.04%, while the variation found within populations was 98.96% ($F_{ST} = 0.010$, $P = 0.002$). An AMOVA on the partitioning of genetic variance within genetic groups and among genetic groups defined with BAPS A (population LS group 1; population LT group 2; populations CJ, LP and AC group 3; all the rest group 4) was 31.05% among groups and 68.95% within groups ($F_{ST} = 0.310$, $P = <0.000$, Table 1). For the BAPS B grouping (population LS group 1, population LT group 2; populations CJ and LP group 3; all the rest group 4) genetic variance was 37.07% among groups and 62.93% within groups ($F_{ST} = 0.370$, $P = <0.000$, Table 1). The groups defined with Structure revealed that for the Structure A group, 20.40% of the variation occurred among groups and 79.60% of the variation, within groups ($F_{ST} = 0.204$, $P = <0.000$, Table 1). In Structure B group 29.55% of the variation was among groups and 70.45% of the variation within groups ($F_{ST} = 0.295$, $P = < 0.000$, Table 1). The main differences among genetic groups were in LS and LT; LS and CJ-LP, LT and CJ-LP. These groups included four populations from the northern part of the Sierra Madre Oriental, in the locality of Cerro Potosí, *C. rosei* var. *rosei* plants and all var. *amoena* plants.

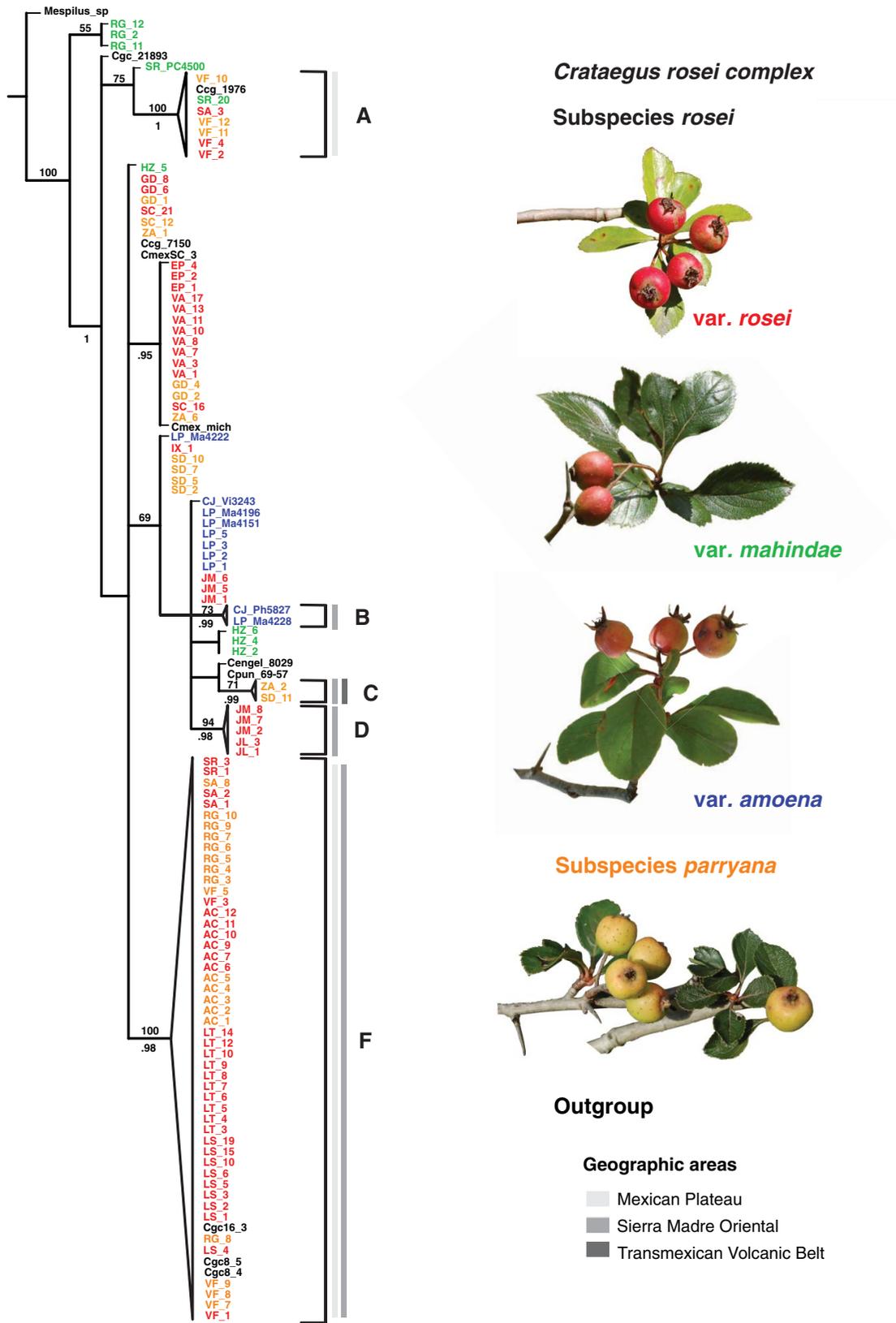


Fig. 2. Phylogenetic analyses for varieties of *Crataegus rosei* based on the combined cpDNA data (*psbA-trnH-trnH-rpl2*). The 50% consensus Bayesian tree and the strict consensus tree of the seven most parsimonious trees found were similar. Posterior probabilities are indicated below the branches and jackknife percentages, above branches. Grey bars indicate the geographic distribution area of the clades.

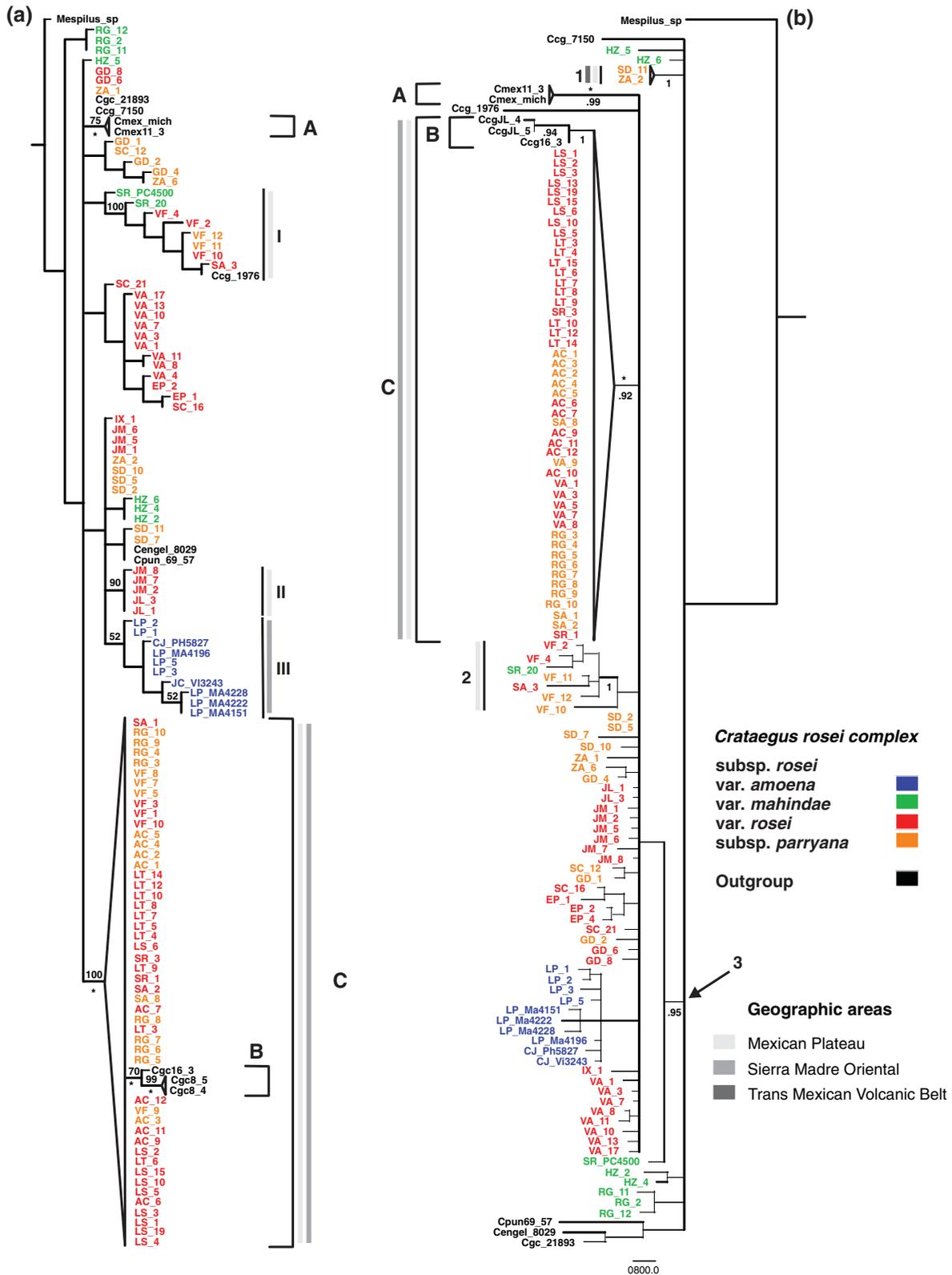


Fig. 3. Phylogenetic analyses for varieties of *Crataegus rosei* based on the combined matrix of molecular and morphological data sets. 3a corresponds to the strict consensus tree of the five most parsimonious trees and 3b to the 50% consensus Bayesian tree. The posterior probabilities are indicated below the branches and jackknife percentages, above branches. Individuals and colours represent varieties of the *Crataegus rosei* complex (*rosei* red; yellow *parryana*; green *mahindae* and blue *amoena*). Grey bars correspond to the geographic distribution areas of the clades. Asterisks represent clades supported by both analyses.

Table 1. Population differentiation in the *Crataegus rosei* complex. F_{ST} estimates from the AMOVA test are based on the binary data set of SSR data. The groups are those detected by the BAPS and Structure analyses. All values were statistically significant.

Baps A	LS	LT	CJ-AC-LP	Structure A	CJ-AC-LP-VA-EP	LS-LT
LT	0.677			LS-LT	0.339	
CJ-AC-LP	0.470	0.576		All rest	0.122	0.247
All rest	0.290	0.378	0.1678	Total	0.204	
Total	0.310					
Baps B	LS	LT	CJ-LP	Structure B	CJ-LP	LS
LT	0.677			LS	0.7133	
CJ-LP	0.7133	0.795		All rest	0.2817	0.266
All rest	0.290	0.3811	0.295	Total	0.295	
Total	0.370					

Morphometric analysis

PCA eigenvalues indicated that leaf shape explained 74.52% of the variation. The PCA graph displays different groups of individuals (Fig. 4a). Canonical discriminant analysis of leaf shape (Table 2) of the 110 specimens of the *C. rosei* complex yielded three functions that explained 79.5%, 12% and 8.5%, respectively, of total leaf shape variance and the discrimination among the three varieties and the subspecies was highly significant (Wilks' λ of function 1 to 3 = 0.198; $x^2 = 274.530$; $df = 141$; $P = 0.000$, Fig. 4b). Standardized canonical discriminant function coefficients reveal that 12 landmarks and semi-landmarks had the highest values and differentiate the three varieties and the subspecies (Table 2). The plot of discriminant functions 1 and 2 shows that leaf shape within the *C. rosei* complex differs among subspecies (Fig. 4b). The configuration of these landmarks indicates that the main differences within the complex are in the shape of maximum width and in the shape of the apex (Fig. 4c).

Relationship between ecological variables and taxonomic morphotypes

The five climate variables with the least correlation were Isothermality (BIO2/BIO7) (* 100) (BIO3); Mean Temperature of Driest Quarter (BIO9); Mean Temperature of Coldest Quarter (BIO11); Annual Precipitation (BIO12); and Precipitation Seasonality (Coefficient of Variation; BIO15). The first two principal components of the climate variables of the *C. rosei* complex explain 77.3% of the variation observed (PCA1 = 49.00% and PCA2 = 28.33%) (Fig. 5). The PCs show that Isothermality clusters population CJ with LP, LT with LS, and ZA with SD. The rest of the populations were not grouped in clusters.

Discussion

Phylogenetic analysis

Molecular analysis. It has been reported that plastid loci such as *psbA-trnH* and *trnH-rp12* can be variable at low taxonomic levels for angiosperms (Li et al., 2014; Peterson, John, Koch, & Peterson, 2004; Vaillancourt & Jackson, 2000); however, in our study, they did not possess sufficient variation to group individuals in well supported clades.

The Parsimony and Bayesian analyses using the molecular data matrix with the chloroplast regions coincide in recognizing five clades with acceptable support values (71 to 100 JK values and 0.95 to 1 posterior probability values) (Fig. 2). Groups formed by these clades include plants distributed in the same or close geographic locations with a mixture of individuals belonging to the different varieties that comprise the *C. rosei* complex. The *Crataegus rosei* complex clade was not identified as monophyletic; individuals from the outgroup, such as *C. gracilior*, *C. punctata*, *C. mexicana*, *C. crus-galli* and *C. engelmannii*, are immersed in groups that include populations from the *C. rosei* complex. In general, the parsimony strict consensus and Bayesian trees coincide in their support values for the same clades. In both analyses, the resulting trees retrieve clade "C" comprising two individuals belonging to var. *parryana*. The rest of the clades (A, B, D, and E) from the Sierra Madre Oriental and the Trans Mexican Volcanic Belt are comprised of individuals originating from the Sierra Madre Oriental and the Mexican Plateau; the boundaries of these geographic regions representing the zone with greatest abundance and widest distribution of the *C. rosei* complex.

The low resolution of the trees retrieved by the parsimony and Bayesian molecular phylogenetic analyses could be the product of the retention of ancestral polymorphisms (Brower, DeSalle, & Vogler, 1996), due to recent divergence/differentiation events (Maddison & Knowles,

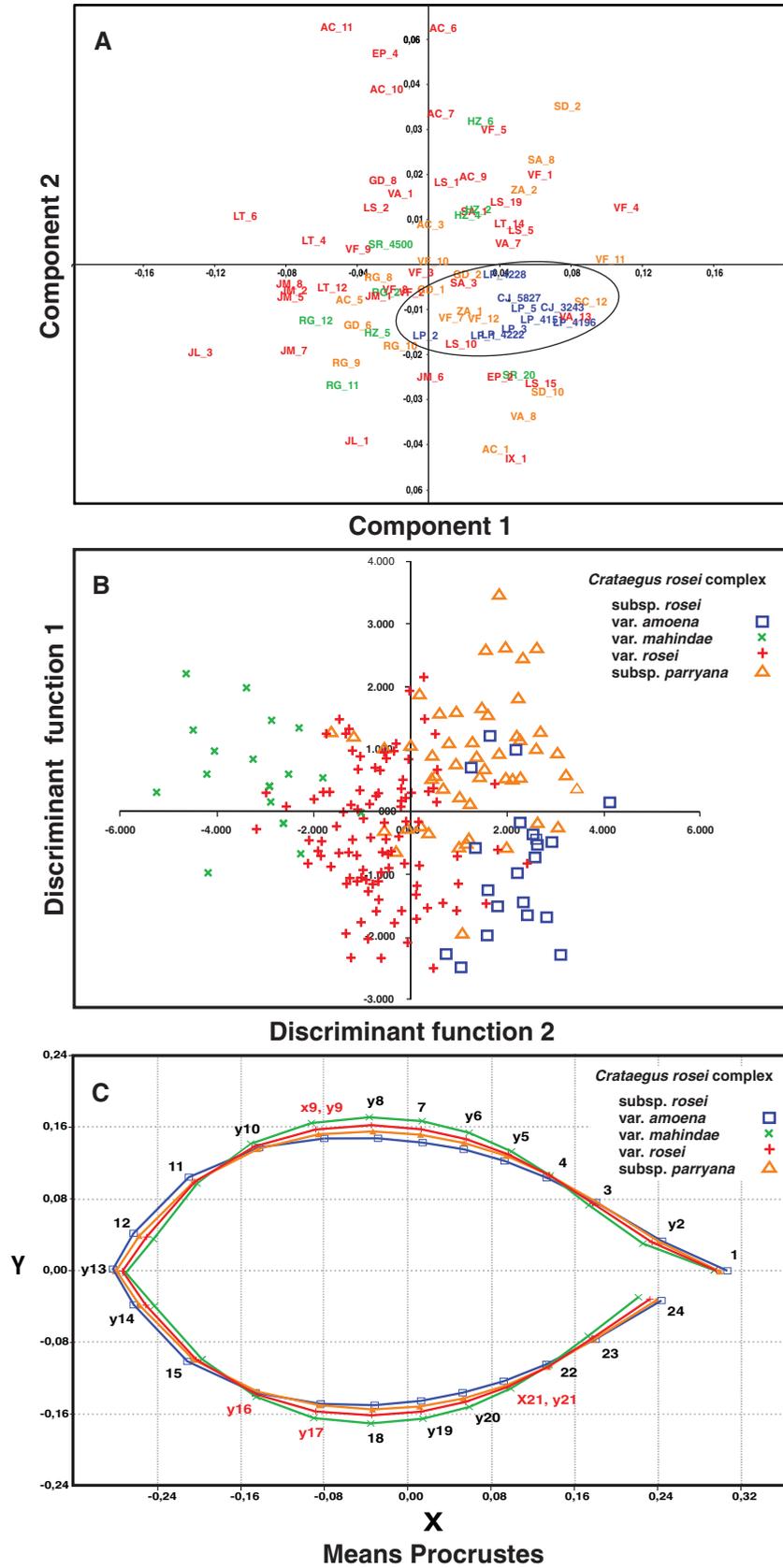


Fig. 4. Geometric morphometrics analysis plots for leaf shape in *Crataegus rosei* varieties. (A) PCA analysis. (B) Plot of canonical discriminant functions. (C) Graph of mean shape variation among the taxa of the *C. rosei* complex. Colours represent varieties of the *Crataegus rosei* complex (*rosei* red; yellow *parryana*; green *mahindae* and blue *amoena*).

Table 2. Standardized canonical discriminant function coefficient of the landmarks that explain the differences within the *C. rosei* complex for the leaf shape analysis.

Leaf shape variable	Function	
	1	2
Landmark		
y2	2.831	4.403
y5	1.804	2.250
y6	3.749	.785
y7	2.258	1.028
y8	4.185	2.734
x9	-.815	3.356
y9	1.549	4.626
y10	2.103	-.192
y13	2.245	.888
y14	2.756	1.271
y16	1.248	3.303
x17	1.885	2.130
y19	2.761	1.520
y20	2.016	3.419
x21	-1.114	3.283
y21	.658	4.346

2006; Rokas, Kruger, & Carroll, 2005). It has been estimated that diversification within *Crataegus* began 32 ± 8 MYA (Lo & Donoghue, 2012). Also, several authors have suggested that lineages with recent genetic divergence tend to display morphological differences, but not genetic differences (e.g. Shaffer & Thomson, 2007; Wagner, Harmon, & Seehausen, 2012; Witter & Carr, 1988). This pattern has been found repeatedly at different taxonomic levels in other Rosaceae groups of subtribe Malinae (Campbell, Evans, Morgan, Dickinson, & Arsenault, 2007; Zheng, Cai, Potter, Postman, Liu, & Teng, 2014), as well as in various series and American sections of *Crataegus* including the *Crus-galli* series (Lo et al., 2009) for which the lack of resolution and extremely short internal branches have been attributed to rapid radiation resulting from extensive hybridization (Lo & Donoghue, 2012; Zheng et al., 2014). Our results display a similar pattern at a finer scale.

Combined analysis with molecular and morphological data sets. Trees retrieved by parsimony and Bayesian analyses based on molecular and morphological characters produced an even lower resolution compared with the molecular data alone. A relevant result of the analyses

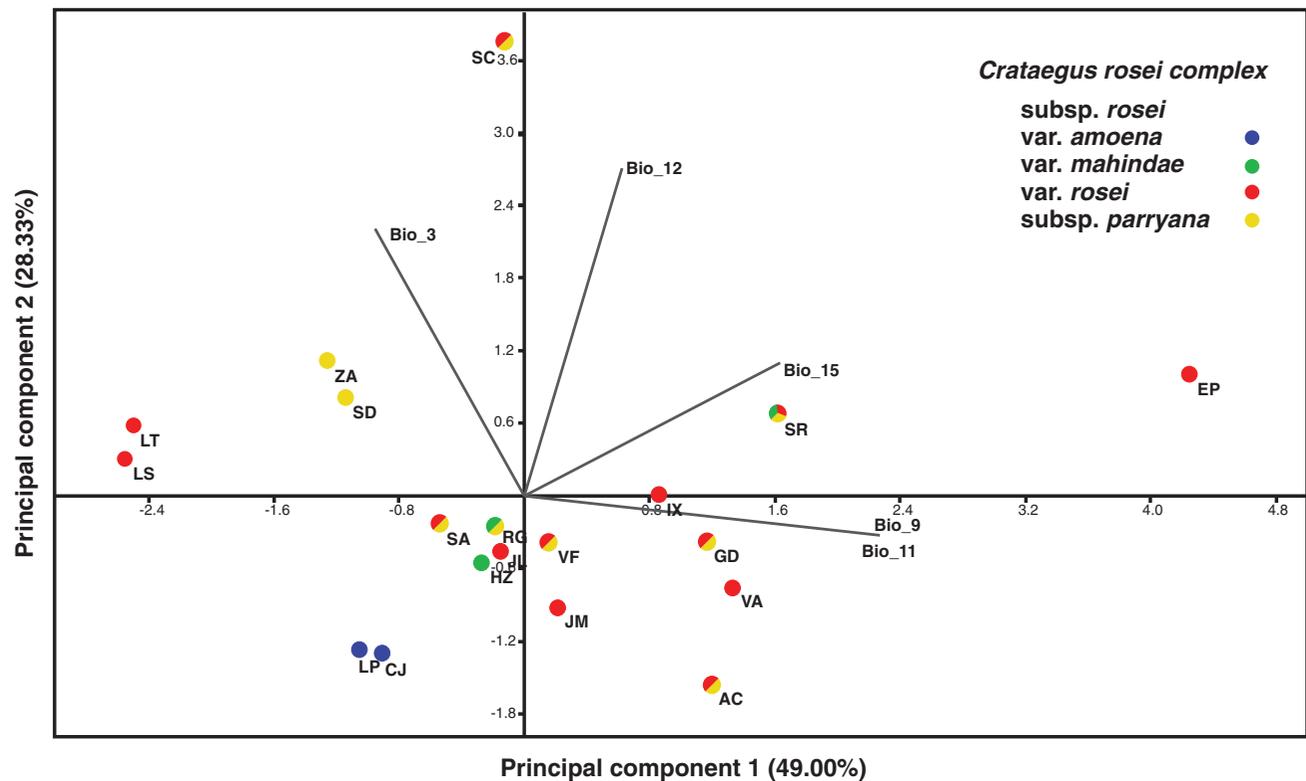


Fig. 5. PCA analysis of environmental variables derived from temperature and precipitation data (BIOS) from WorldClim 1.4 (Hijmans et al., 2005) and populations studied. Circles represent populations and abbreviations are those used in Supplementary Data SD2 (see supplemental material online). The colours of the circles represent the varieties collected from each population of the *Crataegus rosei* complex (*rosei* red; yellow *parryana*; green *mahindae*; and blue *amoena*). The circle with two or more colours means sympatric taxa at the population.

based on the combined matrix was that a small clade (clade III), with more than 50% Jackknife support, embedded in a larger group with poor support, groups specimens considered by Phipps (1997) to be var. *amoena*.

The lower level of resolution found in the parsimony and Bayesian phylogenetic analyses of the combined matrix compared with the molecular matrix could be due to the homoplasy of morphological characters. Doyle (1995) suggests that hybridization is a source of homoplasy because the observed morphological and genetic variation can be mutually contradictory and may not be the result of shared characters from a common ancestry (e.g., Lihová, Kučera, Perný, & Marhold, 2007; Pan, Zhang, & Sang, 2007). Although we do not know whether the *C. rosei* complex is of hybrid origin, as mentioned above it has been suggested that rapid radiation in *Crataegus* is the result of extensive hybridization (Lo & Donoghue, 2012; Zheng et al., 2014). Moreover, in at least three populations (SD, ZA, and SR) we were able to detect individuals with intermediate morphological characters between the taxa of the *C. rosei* complex and *C. mexicana*, and we therefore suggest that past historical hybridization events and recent introgression (Nason, Ellstrand, & Arnold, 1992; Seehausen, 2004) could be shaping the genetic and morphological variation in populations of the *Crataegus rosei* complex.

Microsatellite markers. The levels of genetic variation revealed by the nuclear microsatellite markers of the *Crataegus rosei* complex (SD4 and Table 1) were higher than those reported for other species of tetraploids (Björn et al., 2010; Kloda et al., 2008; Matesanz, Theiss, Holsinger, & Sultan, 2014) but similar to those found in other tetraploid populations of *Crataegus* (Lo et al., 2009). The AMOVA results suggest that there is little genetic differentiation among populations of the *C. rosei* complex ($F_{ST} = 0.010$, $P = 0.002$, Table 1). However, for the analysis of differentiation among genetic groups identified with BAPS and Structure, F_{ST} values change from little differentiation in Structure group A ($F_{ST} = 0.204$, $P < 0.000$, Table 1) to moderate differentiation in BAPS B ($F_{ST} = 0.3707$, $P < 0.000$, Table 1). Furthermore, the F_{ST} values within BAPS and Structure groupings are more strongly differentiated, mainly among LS and LT ($F_{ST} = 0.677$, $P < 0.000$, Table 1), LS and CJ-LP ($F_{ST} = 0.713$, $P < 0.000$, Table 1) and LT and CJ-LP ($F_{ST} = 0.795$, $P < 0.000$, Table 1). Microsatellites were very useful for distinguishing *C. rosei* var. *amoena* from the other taxa of the *C. rosei* complex proposed by Phipps (1997), because the specimens that belong to this taxon can be identified based on their genetic combination. The other taxa (subsp. *parryana*, var. *rosei*, and var. *mahindae*) proposed by Phipps (1997) do not exhibit a genetic combination or genetic group associated with any particular taxon. The

groups of genetic lineages detected for these taxa are mainly associated with geography rather than taxonomy.

It has been demonstrated that a high proportion of observed genetic differentiation in plants is the product of their reproductive system and their mechanisms of pollen and seed dispersal (Hamrick & Godt, 1996). In accordance with this, the genetic differentiation observed among the populations of the *C. rosei* complex could result from differences among populations in their modes of reproduction. Several authors (Cosendai, Wagner, Ladinig, Rosche, & Hörandl, 2013; Hörandl, 2006, 2009; Maynard Smith, 1978) have suggested that individuals with asexual reproduction (apomictic), polyploids, and/or of hybrid origin possess better establishment and colonization strategies than their sexual parents. This phenomenon, known as geographic parthenogenesis, occurs mainly in herbaceous plants (Consendia & Hörandl, 2010; Paun, Greilhuber, Temsch, & Hörandl, 2006; Puente-Molins et al., 2014), although it has been recently detected in tetraploid species of *Crataegus* (Lo et al., 2009; Lo, Stefanović & Dickinson, 2012). Another possible explanation for the structuring of the *C. rosei* complex population may be the lack of effective barriers to gene flow among neighbouring populations.

Geometric morphometrics

This analysis has been useful for delimiting species in several groups of organisms (Duminil, Kenfack, Viscosi, Grumiau, & Hardy, 2012; Silva, Andrade, & Mayo, 2012; Terral et al., 2012). Here, multivariate analyses based on geometric morphometric characters yielded significant differences in leaf shape variation among members of the *C. rosei* complex. The PCA graph shows a tendency for most of the individuals of var. *amoena* to group, as opposed to the rest of the varieties. These differences can also be observed in the linear representation of leaf form, where the individuals of var. *amoena* possess leaves with the most acute apex and the narrowest central part, coinciding with the principal deformation plot from the mean shape.

Relationship between ecological variables and taxonomic morphotypes

Climate could influence the distribution of the *C. rosei* taxa. The environmental conditions under which individuals were collected differ among taxa. Most of the populations of the *C. rosei* complex inhabited landscapes at elevations of 700–1900 m asl., while the var. *amoena* population was located at 2300–2600 m asl. Isothermality was the most important variable for separating the populations collected of *C. rosei* var. *amoena* in environmental space (Fig. 5).

Low isothermality values suggest that the temperature differences are greater or extreme between monthly day-night and warm-cool months, whereas high isothermality values indicate smaller differences between extreme temperatures of monthly day-night fluctuation and warm-cool months (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005).

Taxonomic implications

Is it possible to recognize more than one species within the *Crataegus rosei* complex? When species are old and well differentiated, taxonomic decisions are easy to make, because different methods and sources of data can converge, leading to a unique conclusion (Schlick-Steiner, Arthofer, & Steiner, 2014). However, when species origins are more recent, and there is slight differentiation, species delimitation can be challenging and this can be further complicated by ecological and evolutionary particularities (De Queiroz, 1998; Maddison & Knowles, 2006; Hey, Waples, Arnold, Butlin, & Harrison, 2003). Such is the case for the *C. rosei* complex. While there is no clear correspondence in the groups detected with the different data sets across all analyses, we can make the following observations: (1) the main groups detected by the parsimony and Bayesian analyses are a mix of var. *rosei* and var. *parryana* and of var. *rosei*, var. *parryana*, and var. *mahindae*, while var. *amoena* individuals were retrieved in a separate clade; (2) analyses based on microsatellites appear to indicate high levels of gene flow among populations; (3) these analyses also revealed elevated genetic differentiation between the lineages located in the northern SMO and the other populations; (4) the var. *amoena* populations (CJ and LP) share the same allelic proportion; (5) the results of the geometric morphometric leaf shape analysis suggest that there are differences in leaf shape among varieties, with var. *amoena* possessing the longest and widest leaves compared with other varieties; and (6) the PCA analysis performed with ecological variables and taxonomic morphotypes confirmed that var. *rosei*, var. *mahindae*, and subsp. *parryana* inhabit a broad range of environmental conditions as well as coexisting in sympatry, while var. *amoena* inhabits different environments and does not share their wide distribution range. Based on the above, we suggest that var. *amoena* sensu Phipps (1997) should be considered a separate species. While apomixis, hybridization, and polyploidy among species in *Crataegus* make species delimitation difficult, in the case of var. *amoena* we think there is sufficient evidence to indicate that a speciation process has begun and that the diagnostic morphological characters (fruit shape and size, leaf shape, anther colour) previously proposed by Phipps (1997) set it apart from the other varieties. Moreover, var. *amoena* has a restricted distribution and only a few isolated populations (CJ and LP) on Cerro Potosí, a mountain

that reaches 3800 m asl and is located on the west-central flank of the Sierra Madre Oriental range, in Nuevo Leon (Beaman & Andresen, 1966). This area is remarkable for its high number of endemic species, such as *Pinus culm-nicola*, and it has been suggested that it is a main area of endemism in Mexico (Sosa & De-Nova, 2012). The flora of the Cerro Potosí was isolated and the area provided refugia for several species during the Pleistocene (e.g. Ruíz-Sanchez, Rodriguez-Gómez, & Sosa, 2012). Several species of *Crataegus* have been reported from Cerro Potosí as well, such as *Crataegus aurescens*, *C. cuprina*, *C. grandifolia*, and *C. sulfurea* (Phipps, 1997), none of which belong in the *Crus-galli* series. How all these species maintain genetic and morphological integrity is still unknown. According to the International Code of Nomenclature for algae, fungi, and plants (McNeill et al., 2012) a new specific epithet should be designated for this species, because the name “*amoena*” was previously assigned to a species from the Niagara River in the USA (Sargent, 1908). We will address this nomenclatorial change in the future.

In conclusion, the conflict between the evidence provided by the different data sets we compiled for the *Crataegus rosei* complex and the difficulty in clearly identifying all of the groups in this species complex confirms that the task of delimiting species is challenging. However, the morphological and genetic evidence suggests that *C. rosei* var. *amoena* should be recognized as a separate species with its distribution restricted to Cerro Potosí and adapted to high elevations and specific environmental conditions. Further research should be conducted to identify whether sympatric populations of *Crataegus* in Cerro Potosí are able to hybridize, or whether polyploidy has favoured genetic isolation, given that we have detected that the *rosei* group is tetraploid.

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Disclosure statement

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Supplemental data

Supplemental material for this article can be accessed here: <http://dx.doi.org/10.1080/14772000.2015.1117027>

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