Phenotypic and Start Codon Targeted (SCoT) polymorphism reveals the genetic variation of *Crataegus bretschnideri* C. K. Schneid. and related species in China

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Research Article

Keywords: *Crataegus bretschnideri* C. K. Schneid., Related *Crataegus* species, Genetic variation, Phenotypic trait, SCoT marker, Population structure

Posted Date: February 1st, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1272845/v1>

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Abstract

Crataegus bretschnesideri C. K. Schneid. originated in Northeast China, it has excellent characteristics of cold resistance and early maturity. However, the taxonomic status of *C. bretschnesideri* has been controversial due to the intra-specific hybridization and apomixes. Comparative assessment of phenotypic and molecular evaluations used to explore genetic diversity of *C. bretschnesideri* and relative species. Qualitative and quantitative traits of *C. bretschnesideri* and relative species were evaluated. Genetic diversity and population structure of several *Crataegus* species were analyzed by thirteen reported and five newly designed SCoT markers. Leaf traits of *Crataegus* had a higher Shannon-wiener index than fruit and flower traits; Fruit contained diverse traits among seven *Crataegus* species by principal component analysis. SCoT markers amplified 148 polymorphic bands with 94.87% polymorphic loci. *Crataegus* individuals clustered into five clades, and *C. bretschnesideri* had a closer relationship with *C. pinnatifida* Bge. and *C. hupehensis* Sarg.. Population structure suggested that investigated the mixed gene pool appeared in *C. bretschnesideri*. There had variation among *Crataegus* species, and part of the *C. bretschnesideri* individuals may be a hybrid origin compared to the phenotypic and molecular evaluations. Newly designed SCoT markers were practical for *Crataegus* diversity analyses.

Introduction

Crataegus is an ancient plant genus belonging to Rosaceae, which originated in the Cenozoic (Zhao & Feng, 1996). *Crataegus* fruit contains several flavonoids, phenols, oligoprocyanidins, and other active substances with medicinal value (T.A. Dickinson et al., 2014). Laboratory tests and clinical trials have proved the preventive and therapeutic effects of active substances in *Crataegus* on cardiovascular diseases (Edwards, Brown, Talent, Dickinson, & Shipley, 2012). Several cultivated and wild species of *Crataegus* originated in China (Hong & Blackmore, 2015). Hawthorn cultivation in China has more than 1700 years of history, and long-term cultivation and selection have formed a rich variety of types (Zhao & Feng, 1996). There are eighteen species and six varieties of *Crataegus* widely distributed in China (Zhao & Feng, 1996). There is another view that twenty species, seven varieties, and one variant of *Crataegus* (Dong & Li, 2015). *C. bretschnesideri* is one of cultivars that originated in northeast China, which has the characteristics of early maturity and cold resistance (Dong & Li, 2015). However, since phenotypic traits of *C. bretschnesideri* are similar to *C. pinnatifida* (Fig. 1), its classification status is not very clear. It is not conducive to further conservation and utilization of *C. bretschnesideri* germplasm resources.

Phenotypic traits are essential indices to identify *Crataegus* species (Timothy A Dickinson, Belaoussoff, Love, & Muniyamma, 1996). Leaf morphological changes, leaf shape, and other leaf traits are widely used in the study of inter-specific and intra-specific relationships of *Crataegus* (J. Phipps & Dvorsky, 2008). Patical hawthorns have identified the inter-species differences of their phenotypic traits (Jiang & Dong, 2009). Dai et al. (2008) and Wu et al. (2008) analysed eight species and two varieties of *Crataegus* by three markers, including ISSR (Inter-Simple Sequence Repeat) markers, cpDNA PCR-RFLP (Polymerase Chain Reaction Restriction Fragment Length Polymorphism of chloroplast DNA), and cpSSR (chloroplast Simple Sequence Repeats). Liu et al. (2016) used ISSR markers to analyse the genetic relationships among *C. altaica* (Loud.) Lange, *C. songorica* Pall. and *C. pinnatifida*. In addition, studies have increasingly combined phenotypic traits with molecular markers to analyse the genetic diversity of *Crataegus* (Betancourt-Olvera, Nieto-Ángel, Urbano, & González-

Andrés, 2018; Sheng et al., 2017). Phenotypic and molecular evaluations of these studies revealed that there has considerable variation among different *Crataegus* species.

Extensive efforts are made to develop practical molecular tools and integrate phenotypic analysis to the molecular assessment of *Crataegus* genetic diversity. Start Codon Targeted polymorphism (SCoT) is a single primer designed for the conserved flanking sequence of the ATG initiation codon, and the amplified fragments tend to be candidate functional genes (Collard & Mackill, 2008). SCoT marker includes many advantages, such as fewer steps, a simple primer design process, strong universality, low requirements for DNA quality, rich genetic information, and high polymorphism (Long et al., 2015). As a new type of marker, it has been widely used for genetic diversity, phylogenetic relationship analysis, and even germplasm identification in diverse plant species (Igwe et al., 2017; Saboori et al., 2020; Xiao et al., 2020). The present study used practical SCoT markers and phenotypic traits to explore the genetic difference behind *C. bretschnideri* and related species.

Materials And Methods

Plant materials

During the 2019 season, we picked leaves, flowers, and mature fruits of seven *Crataegus* species native to China from the National Fruit-tree Germplasm Resources - Shenyang Hawthorn Repository (41°49'N, 123°34'E) at Shenyang Agricultural University from May to October, including *C. bretschnideri* (*C. bre*), *C. maximowiczii* (*C. max*), *C. sanguinea* (*C. san*), *C. pinnatifida* (*C. pin*), *C. hupehensis* (*C. hup*), *C. scabrifolia* (*C. sca*) and *C. altaica* (*C. alt*) (Table 1), which Prof. Dong and Ph.D. Zhang identified according to the pictures and descriptions of phenotypic traits in "China Fruit-plant Monograph·Hawthorn (*Crataegus*) Flora" (Zhao & Feng, 1996). We randomly collected twenty leaves, flowers, and fruits from three trees for each individual.

Phenotypic traits measurements

We used twenty leaves, flowers, and mature fruits for each of *C. bre* and other species for phenotypic trait measurements. Methods followed the scales for evaluating crop germplasm resources of hawthorn (Dong, 2013). Qualitative traits of *Crataegus* resources include twenty-one items such as leaf color, stipule shape, petal shape, petal color, fruit skin color, flesh color. Quantitative traits of *Crataegus* resources include twelve items such as leaf length, leaf width, petiole length, corolla diameter, flower number of inflorescence, and number of pistils and stamens, fruit weight, fruit hardness, fruit vertical diameter, fruit horizontal diameter, and fruit shape index (calculated as the ratio of fruit vertical to horizontal diameter). Scales of qualitative traits were summarized in supplementary Table S1. Fruit weight, leaf length, leaf width, petiole length, fruit vertical and horizontal diameter of *Crataegus* were measured by electronic balance (200 g ± 0.1 g), digital vernier calipers (0-150 mm ± 0.02 mm). Fruit hardness was evaluated using a computer-controlled texture analyzer TA.XT plus (Stable Micro System, Godalming, UK), according to the protocol described by Zhang et al. (Zhang et al., 2019).

Phenotypic data analysis

Phenotypic traits were evaluated achieved in random samples of twenty of each *Crataegus* resource. We used the "Tukey" method in SPSS 20.0 (IBM, US) to calculate the mean and variance of quantitative trait data. Otherwise, we applied the "vegan" package of R v4.0.3 software to analyse the Shannon-wiener index of

qualitative traits data. Cluster and principal component analyses of phenotypic traits were using the Origin 2019b software (<https://www.originlab.com/>), a proprietary computer program for interactive scientific graphics and data analysis. Phenotypic traits data were standardized by subtracting the mean and dividing the result by the standard deviation, this was to reduce the effects of different measurement scales on various traits. Hierarchical cluster analysis of standardized data used the group average's method coupled with the Euclidean distance method. The distance between two clusters was determined as the average distance between all pairs of objects in the different clusters.

DNA extraction and SCoT assays

Fresh leaves of seven *Crataegus* species were frozen in liquid nitrogen and stored at -80°C for DNA extraction. We ground one gram of frozen leaf material for genomic DNA extraction using cetyl-trimethylammonium bromide method (Du et al., 2019). Genotyping was performed using 13 SCoT primers selected from Collard and Mackill's data (Collard & Mackill, 2008). We designed five new primers (SCoTH₁₋₅) for *Crataegus* genetic diversity analyses based on the design role. All primers and *Tm* are listed in Table 2. PCR amplification was performed with a final volume of 10 µL reaction mixture consisting of 3 µL template DNA (40 - 50 ng), 5 µL of TaKaRa *Ex Taq*® (RR001A), and 2 µL primers. PCR conditions were as follows: initial denaturation at 94°C for 5 min followed by 30 cycles of 1 min at 94°C, 1 min at 54 - 64°C, 1 min at 72°C, and a final extension of 5 min at 72°C. PCR amplification was carried out in a Thermal Cycler (Applied Biosystems, USA). PCR products were separated on a 1.5% agarose gel in 5X TBE buffer.

Molecular data analysis

SCoT markers generated data matrices by scoring (1) for presence and (0) for absence (supplementary Table S2). Genetic similarities based on Jaccard's coefficients were calculated using the SIMQUAL program with the Numerical Taxonomy Multivariate Analysis System in NTsys-PC v2.0 software (Rohlf, 2000). The resulting genetic similarity indices generated the cluster tree through the Un-weighted Pair-Group Method with Arithmetic average (UPGMA). The Mantel correlation test of genetic similarity was applied to the matrix table of each marker by matrix comparison plot. Moreover, a series of diversity parameters were calculated from molecular data by referencing Bhattacharyya et al. (Bhattacharyya, Kumaria, Kumar, & Tandon, 2013) using Popgene 3.2 (Yeh, Yang, & Boyle, 1999). Diversity parameters include the Number of Polymorphic Loci (NPL), Percentage of Polymorphic Loci (PPL), the observed number of alleles (N_a), effective numbers of alleles (N_e), *Nei's* gene diversity (H), total genetic diversity of the sample population (H_t), genetic diversity within population (H_s), Shannon's information index (I), mean coefficient of gene differentiation (G_{st}), an estimate of gene flow ($N_m = 0.5 \times (1 - G_{st}) \div G_{st}$). We used *Nei's* unbiased measures of genetic identity to measure the similarity among alleles. We also used genetic distance was used to measure the genetic differentiation between groups of the same species. Principal Coordinate Analysis (PCoA) was performed using GenAlEx 6.41 software (Peakall & Smouse, 2006) and OmicStudio tools v2.1 (<https://www.omicstudio.cn/tool/24>) to reveal genetic clusters among the individuals. Structure v2.3.4 software (Hubisz, Falush, Stephens, & Pritchard, 2009) statistical tool was used with an Evanno correction using Structure Harvester (Earl & vonHoldt, 2012). The structure was run ten times for each value from 1 to 7 to determine the population's most likely number of ancestral Kinships (K).

Results

Phenotypic diversity analysis of seven *Crataegus* species

We assessed the genetic diversity of seven *Crataegus* resources (*C. bre*, *C. max*, *C. san*, *C. pin*, *C. hup*, *C. sca*, and *C. alt*) based on phenotypic traits and SCoT markers. Phenotypic traits of these *Crataegus* resources represented a high level of diversity (Fig. 1, supplementary Table S3). Several morphological traits of *Crataegus* resources were analysed statistically (supplementary Table S4). Among leaf traits of all test materials, Shannon-wiener indexes of leaf shape, leaf margin, and leaf base shape were 1.4, 1.3, and 1.4, indicating leaf traits contained highest diversity. 19.4% of leaf shape was ovate, 36.1% were wide ovate, 27.8% were triangular ovate, 11.1% were ovate-lanceolate. 5.6% of leaf base shape were truncate, 30.6% were round, 38.9% were broad cuneate, 19.4% were cuneate, and 5.6% were decurrence cuneate. The variable trait of *Crataegus* flowers was the pedicel surface. Shannon-wiener index was 1.3, 38.9% were glabrous, 13.9% were less woolly, 36.1% were woolly and 11.1% were extremely woolly. The Shannon-wiener indexes of fruit dot quantity, fruit surface trait, and calyx tube shape were 1.0, 1.1, 1.0, which showed high diversity. For fruit dot quantity, 27.8% of mature fruit had very few surface dots, 8.3% had few dots, 58.3% had intermediate number dots, and 5.6% had many dots. In general, leaf and fruit traits had relatively rich diversity among different *Crataegus* species.

Quantitative traits of these *Crataegus* resources were further analysed statistically (Fig. 2, supplementary Table S3, Table S4). Variation coefficients of twelve metric traits were 10.37% – 51.98%. The order of the degree of diversity was flower number of inflorescence > fruit weight > petiole length > fruit firmness > fruit horizontal diameter > fruit vertical diameter > pistil number > corolla diameter > leaf width > leaf length > stamen number > fruit shape index. The fruit of MSZ3H (*C. max*, 0.4 g) was the lightest, and the heaviest was MHL (*C. hup*, 6.2 g). HBSZ2HN (*C. hup*) had the highest number of flowers per inflorescence (30.25), while WTSSLH (*C. pin*) had the fewest (4.4). QJX (*C. pin*) had the longest petiole (47.43 mm), while MSZ2H (*C. max*) had the shortest petiole (9.82 mm). The hardest fruit was QJX (*C. pin*), and the softest fruit was LNSZ1H (*C. san*). Compared with other traits, the difference in stamen number or fruit shape indices among several *Crataegus* species was smaller (supplementary Table S3).

Principal component analysis transformed phenotypic traits data of leaf, flower, and fruit into fewer dimensions, which serve as summaries of features (supplementary Table S5). The contribution rate of the first principal component was 23.82%, and the main eigenvectors included fruit dots quantity. The contribution rate of the second principal component was 15.47%, and the main eigenvectors had fruit weight, fruit vertical diameter, and horizontal diameter. These two main components contained important diverse traits among *C. bre*, *C. max*, *C. san*, *C. pin*, *C. hup*, *C. sca*, and *C. alt*.

We implemented hierarchical cluster analysis to find similar groups of individuals among different *Crataegus* species using phenotypic data based on the Euclidean distance method. Leaf and fruit traits data grouped seven species into four clades (Fig. 3A, C), and all traits data grouped seven species into five main clades (Fig. 3D). Clade 1 included fourteen *C. bre* resources and NMGSLH, YP6H, and WTSSLH (*C. pin*), clade 2 included 1541SLH, MDFSLH, YR5H (*C. pin*), and HBSZ2H (*C. hup*), clade 3 included YNSZ1H, YNSZ2H, YNSZ3H (*C. sca*), and HBSZ3H (*C. hup*), clade 4 included MSZ2H, MSZ3H (*C. max*) and LNSZ1H, LNSZ2H, LNSZ3H (*C. san*), clade 5 included AETSZ2H, AETSZ3H (*C. alt*). The scale of dissimilarity values of the Euclidian coefficient ranged between 7.31 and 45.96 which reflected the high level of diversity among *Crataegus* resources. Phenotypic traits of leaf, flower, and fruit between *C. bre* and *C. pin* were similar. Thus, *C. bre* and *C. pin*

resources were clustered into the same clade. Additionally, *C. max* and *C. san* were pressed into clade 4, because the leaf and fruit traits data were very similar and different from other *Crataegus* species.

Molecular diversity analysis of *Crataegus* individuals based on SCoT data

Eighteen SCoT markers were able to amplify several bands across the 36 *Crataegus* resources, including *C. sca* and *C. alt* (supplementary Fig. S1, Table S2). According to the principle of SCoT primers, we designed five new SCoT primers (SCoTH₁₋₅) for the molecular diversity analysis of *Crataegus* (Table 2). A series of parameters were calculated from molecular data to assess the level of diversity among the *Crataegus* resources (Table 3). All the SCoT markers amplified 148 polymorphic bands, the percentage of polymorphic loci was 94.87%. In this regard, polymorphic bands ranged from 3 to 16, while the percentage of polymorphic loci ranged from 62.5% to 100%. The highest polymorphism information content (*PIC*) and resolving power (*RP*) values were 0.50 and 9.88, respectively, generated by SCoT22. Moreover, the percentage of polymorphic loci of 5 new SCoT markers was 100%, and *PIC* and *RP* ranged from 0.44 to 0.50 and 3.43 to 4.92, respectively.

Moreover, several diverse parameters were analysed from SCoT data among different *Crataegus* species (Table 4). The observed number of alleles (N_a) and the effective number of alleles (N_e) varied from 1.18 to 1.64 and 1.14 to 1.45, respectively. *Nei's* gene diversity (*H*) and Shannon's Information index (*I*) ranged from 0.08 to 0.25, with an overall diversity of 0.33 and from 0.11 to 0.36 with an average value of 0.26, respectively. The percentage of polymorphic loci (*PPL*) was expected to be in the range of 17.95% to 64.11%. The total variability (H_t) and variability within population (H_s) were 0.34 and 0.18, respectively. The diversity among populations (G_{st}) and gene flow (N_m) values were 0.58 and 0.36, respectively. *Nei's* unbiased measures of genetic identity, and genetic distance among the population have also been evaluated (Table 5). *Nei's* genetic distance ranged from 0.1069 to 0.5440, while *Nei's* genetic identity ranged from 0.5804 to 0.8986. *C. pin* and *C. hup* were closely related to 0.8986 identities and 0.1069 distance. *C. bre* and *C. pin* were also closely related to 0.8655 identities and 0.1444 distance.

Thirty-six *Crataegus* resources were clustered into four main clades using the UPGMA method (Fig. 4, supplementary Table S6, S7). Clade I contained fourteen *C. bre* resources and HBSZ2H (*C. hup*), while Clade II consisted of a total of eight *C. pin* resources and *C. hup* (HBSZ3H, XPZM, MHL). These two clades were separated at approximately 0.67 similarity. Clade III gathered four *C. sca* resources, while clade IV had two *C. max* and three *C. san* resources. Clade III was separated from Clade I - II at approximately 0.63 similarity, while clade IV was divided with Clade III at approximately 0.61 similarity. *C. max* and *C. san* were closely linked to approximately 0.75 similarity. AETSZ2H and AETSZ3H were far from other *Crataegus* resources. The closest *Crataegus* individuals were JF4H and FLH at 0.94 similarity.

Principal Coordinate Analysis (PCoA) based on SCoT data revealed the relationship among *Crataegus* individuals (Fig. 5, supplementary Table S8). The two-dimensional projection of varieties along the first two main axes revealed the genetic relationships among *Crataegus* individuals ($R = 0.8533$, $P = 0.001$). The closer distance between the two individuals represented fewer differences. The total percentage of variation resulting from PCoA was 19.49% and 17.5%, given by PC1 and PC2, respectively. Eleven *C. bre* individuals had a close relationship with one *C. pin*, four *C. bre* individuals had a close relationship with four *C. pin* and one *C. hup* individuals.

Population structure investigated the diversity among *Crataegus* individuals. ΔK analysis revealed that five populations ($K = 5$) represented the best model for 36 individuals (Fig. 6, supplementary Fig. S2). The results showed that when $K = 3 - 6$, *C. max* and *C. san* were mainly in the yellow gene pool, and *C. pin* and *C. hup* were primarily in the blue gene pool (Fig. 6). Population structures of *C. max* and *C. san* were very similar. Thus, relationships between these two *Crataegus* species were closer. Similar results were observed between *C. pin* and *C. hup*. Otherwise, a mixed gene pool appeared in *C. bre*, which mixed a small yellow and blue gene pool. *C. bre* showed partial gene exchange and revealed its possible hybrid origin.

Discussion

Phenotypic diversity analyses of *Crataegus* species

Due to long-term adaptation to environmental changes, wild species related to the cultivated species have formed and accumulated rich genetic variety and excellent gene resources in the process of evolution (Lu, 2014). Because of the importance of related species for hawthorn breeding programs, it is necessary to implement genetic diversity analysis of *Crataegus* fruit tree resources in China to provide the basis for botanical conervation, and utilization of germplasm resources.

The traditional classification of *Crataegus* species is based on morphological characteristics and geographical distribution. The phenomena of apomixes, inter-specific hybridization, and polyploidy often exist widely in *Crataegus*. These have led to high genetic differences in morphological characteristics (Dönmez, 2008) and increased difficulties in classifying *Crataegus* species. Flowering or fruiting periods could only reflect unique characteristics of some *Crataegus*, which makes their identification and classification more complicated (Christensen, 1992, Phipps, 2005). PCA of the phenotypic diversity of *C. pentagyna* Waldst. & Kit. ex Willd. and *C. monogyna* Jacq. from north-eastern parts of Iran revealed that fruit traits contributed the most to accession diversity, followed by leaf traits (Khadivi et al., 2019). Du et al. (2019) separated six *Crataegus* species into four groups based on leaf and mature fruit traits. *C. bre* were assigned to a group with deep leaves and big fruits. In this study, leaf and fruit traits had relatively rich diversity among different *Crataegus* species. *C. bre* and *C. pin* resources were clustered into the same clade based on the leaf and flower traits. However, *C. bre* individuals were clustered into a single groups according to the unique fruit traits (Fig. 3). The above results indicate that *C. bre* has unique fruit characters. However, it is not possible to directly identify *C. bre* resources only by phenotypic traits.

SCoT markers used in genetic diversity analysis

SCoT markers contain rich genetic information and high polymorphism. For the genetic diversity study of *Chrysanthemum morifolium* cultivars, 32 SCoT primers amplified 73.1% (SCoT24) polymorphic bands at least, and *PIC* values of these primers were at least 0.950 (SCoT19) (Feng et al., 2016). Igwe et al. (2017) demonstrated that SCoT markers were more efficient than ISSR for genetic diversity studies in *Vigna unguiculata* (L.) Walp. SCoT marker was more informative with higher discriminating power than RAPD, ISSR, and iPBS (inter Primer Binding Site) markers (Amom et al., 2020). In particular, SCoT13 had the highest Polymorphism and *PIC* value when amplified for accessions of landraces chickpea (*Cicer arietinum*) (Pakseresht et al., 2013). SCoT12 and SCoT16 were the most polymorphic markers among the 15 SCoT markers when 164 wild plants were amplified in fourteen ecologically different natural populations of the

Crataegus pontica. The high level of genetic variation that exists among and within 201 accessions of the genus *Crataegus* was reflected by 0.02 – 0.62 genetic similarity, 13 SCoT primers produced 115 polymorphic fragments, and the polymorphism percentage bands of SCoT1, 7, 11, 14, and 20 – 23 were more than 90% (Emami, et al., 2018). Our results indicated that 18 SCoT markers amplified 148 polymorphic bands in 36 *Crataegus* individuals, the percentage of the polymorphic loci ranged from 62.5% to 100%, with a mean value of 94.87% (Table 3). The highest *PIC* and *RP* values of SCoT were 0.50 and 9.88, respectively, generated by SCoT22. Moreover, the percentage of the polymorphic band of five new SCoT markers was 100%, and *PIC* and *RP* ranged from 0.44 to 0.50 and 3.43 to 4.92, respectively. Based on SCoT data, we generated a similar phylogenetic tree to SLAF-seq (Specific-Locus Amplified Fragment sequencing) (Du et al., 2019), clustering *C. bre* and *C. pin* into the closer Clade. Otherwise, SCoT markers reduce the analysis cost and time. These results revealed that SCoT markers, especially SCoT22, SCoT32, SCoTH₂, SCoTH₃, SCoTH₅ markers were efficient, informative, and polymorphic for molecular diversity studies in the *Crataegus* species in China's native germplasm.

Inter-specific hybridization of *Crataegus* species

Inter-specific hybridization can promote the formation of new hawthorn species (Phipps, 2014). The natural hybridization of different hawthorn species often occurs in the overlap region of geographical distribution, leading to hawthorn germplasm's genetic diversity (Talent & Dickinson, 2007). Researchers have provided evidence for natural hybridization in the evolution of hawthorn plants at the molecular level (Lo et al., 2010). In this study, *C. bre* was similar to *C. pin* on phenotypic traits. The plant list (<http://www.theplantlist.org/tpl1.1/record/rjp-18388>) and Plants of the World online (<http://www.plantsoftheworldonline.org/taxon/urn:lsid:ipni.org:names:723046-1>) had recorded: *C. bre* was a synonym of *C. pin*. Cluster results of RAPD showed that *C. bre* was a variety or subspecies of *C. pin* (Dai et al., 2008). However, Guo et al. (1995) and Wu et al. (2008) considered *C. bre* was independent *Crataegus* species that had a close relationship with *C. pin*. ISSR results confirmed this view (Han et al., 2009). Structure and TreeMix analysis showed that *C. bre* might have a hybrid origin (Du et al., 2019). Recently, chloroplast genomes of *C. pin*, *C. bre*, *C. max*, and *C. pinnatifida* var. *major* N.E.Br. were sequenced, and results revealed that *C. max* was the maternal origin of *C. bre* (Hu et al., 2021). In this study, a mixed gene pool appeared in *C. bre* containing a small amount of yellow and blue pools by population structure analysis (Fig. 6). *C. bre* showed partial gene exchange among *C. max*, *C. san*, *C. pin*, and *C. hup*. Otherwise, a total of eight *C. pin* resources and *C. hup* (HBSZ3H, XPZM, MHL) clustered into Clade II (Fig. 4). *C. pin* and *C. hup* had similar gene pools when *K* = 3 – 6 (Fig. 6). *C. hup* differentiated at 6.86 Ma, while *C. pin* differentiated at 1.23 Ma (Du et al., 2019). Thus, further studies are needed to determine the relationship between *C. pin* and *C. hup*.

Conclusions

Our results revealed that SCoT markers were informative and practical for the study of *Crataegus* genetic diversity. *C. bretschnideri* may be a hybrid origin compared to the phenotypic and molecular evaluations.

Declarations

Funding

This work was supported by “The Conservation and Utilization of Crop Germplasm Resource – Hawthorn (Project # 19190178).

Competing Interests

All the author declare they have no financial interests.

Author Contributions

Conceptualization, X.Z. and W.D., methodology, X.S., software, X.Z., validation, X.Z., X.S. and J.W., formal analysis, J.W., resources, W.D., data curation, M.X. and C.S., writing—original draft preparation, X.Z., writing—review and editing, X.Z. and W.D., funding acquisition, W.D. All authors have read and agreed to the published version of the manuscript.

Data Availability

The data presented in this study are available in supplementary material.

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Tables

Table 1. Details of biogeographic information for *Crataegus* resources.

Species	ID	Biogeographic regions	Species	ID	Biogeographic regions
<i>C. bre</i> ¹	JF1H	Northeast, China	<i>C. pin</i> ⁴	NMGSLH	North, China
	JF2H	Northeast, China		WTSSLH	North, China
	JF3H	Northeast, China		YP6H	Northeast, China
	JF4H	Northeast, China		1541SLH	Northeast, China
	ZF1H	Northeast, China		MDFSLH	Northeast, China
	SF1H	Northeast, China		CZSLH	Northeast, China
	CH	Northeast, China		YR5H	Northeast, China
	FLH	Northeast, China		QJX	Northeast, China
	HRSLH	Northeast, China	<i>C. hup</i> ⁵	HBSZ2H	Central, China
	ZSSLH	Northeast, China		HBSZ3H	Central, China
	HRSZ	Northeast, China		MHL	Central, China
	555	Northeast, China		XPZM	Central, China
	82015	Northeast, China	<i>C. sca</i> ⁶	YNSZ1H	Southwest, China
	LNDG	Northeast, China		YNSZ2H	Southwest, China
				YNSZ3H	Southwest, China
<i>C. max</i> ²	MSZ2H	Northeast, China			
	MSZ3H	Northeast, China	<i>C. alt</i> ⁷	AETSZ2H	Northwest, China
<i>C. san</i> ³	LNSZ1H	Northeast, China		AETSZ3H	Northwest, China
	LNSZ2H	Northeast, China			
	LNSZ3H	Northeast, China			

¹ *C. bre* = *C. bretschneideri*; ² *C. max* = *C. maximowiczii*; ³ *C. san* = *C. sanguinea*; ⁴ *C. pin* = *C. pinnatifida*; ⁵ *C. hup* = *C. hupehensis*; ⁶ *C. sca* = *C. scabrifolia*; ⁷ *C. alt* = *C. altaica*.

Table 2. Information of SCoT primers used in the present study.

SCoT Marker	Sequences (5' → 3')	GC content (%)	<i>T_m</i> (°C)
SCoT1	CAACA <u>ATGG</u> CTACCACCA	50	54.6
SCoT3	CAACA <u>ATGG</u> CTACCACCG	56	56.0
SCoT4	CAACA <u>ATGG</u> CTACCACCT	50	54.0
SCoT14	ACGAC <u>ATGG</u> CGACCACGC	67	62.7
SCoT15	ACGAC <u>ATGG</u> CGACCGCGA	67	64.0
SCoT18	ACC <u>ATGG</u> CTACCACCGCC	67	62.2
SCoT22	AACC <u>ATGG</u> CTACCACCAC	56	56.8
SCoT23	CACC <u>ATGG</u> CTACCACCAG	61	57.6
SCoT24	CACC <u>ATGG</u> CTACCACCAT	56	56.3
SCoT28	CC <u>ATGG</u> CTACCACCGCCA	67	62.4
SCoT32	CC <u>ATGG</u> CTACCACCGCAC	67	60.9
SCoT35	C <u>ATGG</u> CTACCACCGGCCC	72	63.8
SCoT36	GCAACA <u>ATGG</u> CTACCACC	56	56.0
SCoTH ₁	AAC <u>ATGG</u> CGACCACCGAC	61	60.4
SCoTH ₂	CC <u>ATGG</u> CGGCCTCAACAT	61	60.2
SCoTH ₃	CAACA <u>ATGG</u> CCACCACCA	56	58.3
SCoTH ₄	ACA <u>ATGG</u> CTCCCACAATC	50	54.7
SCoTH ₅	CAACA <u>ATGG</u> CGACCTCAA	50	55.5

Table 3. A survey of the total number of bands, number of polymorphic bands, %*P*, *PIC*, and *R_p*.

SCoT Marker	Total No. of bands	No. of poly. bands	% of poly. Bands (P)	PIC ¹	Resolving Power (Rp)
SCoT1	8	8	100	0.43	4.29
SCoT3	6	6	100	0.44	2.70
SCoT4	12	9	75	0.46	5.18
SCoT14	16	16	100	0.39	8.64
SCoT15	13	13	100	0.37	6.04
SCoT18	8	5	62.5	0.49	1.34
SCoT22	13	13	100	0.50	9.88
SCoT23	4	4	100	0.41	1.44
SCoT24	10	9	90	0.49	5.27
SCoT28	7	6	85.71	0.47	3.08
SCoT32	11	11	100	0.46	7.35
SCoT35	8	8	100	0.43	3.37
SCoT36	3	3	100	0.43	0.81
SCoTH ₁	7	7	100	0.45	3.63
SCoTH ₂	6	6	100	0.49	3.43
SCoTH ₃	10	10	100	0.48	4.92
SCoTH ₄	8	8	100	0.44	3.99
SCoTH ₅	6	6	100	0.50	3.56
Total	156	148	94.87	–	78.92
Average	–	–	–	0.45	–

¹ PIC = Polymorphic information content.

Table 4. Genetic diversity and differentiation parameters of *Crataegus* species.

Species	N_a	N_e	H	I	NPL	PPL	H_t	H_s	G_{st}	N_m
<i>C. bre</i>	1.6282	1.4279	0.2428	0.3559	98	62.82				
<i>C. max</i>	1.1987	1.1405	0.0823	0.1202	31	19.87				
<i>C. san</i>	1.1795	1.1509	0.0804	0.1144	28	17.95				
<i>C. pin</i>	1.6410	1.4580	0.2553	0.3722	100	64.10				
<i>C. hup</i>	1.4551	1.3095	0.1747	0.2573	71	45.51				
<i>C. sca</i>	1.2500	1.1844	0.1027	0.1493	39	25.00				
<i>C. alt</i>	1.2244	1.1586	0.0929	0.1357	35	22.44				
Total	1.9487	1.5775	0.3353	0.5005	148	94.87	0.3518	0.1473	0.5812	0.3602

Table 5 *Nei's* genetic identity and genetic distance of *Crataegus* species¹.

Species	<i>C. bre</i>	<i>C. max</i>	<i>C. san</i>	<i>C. pin</i>	<i>C. hup</i>	<i>C. sca</i>	<i>C. alt</i>
<i>C. bre</i>	****	0.7826	0.7420	0.8655	0.8530	0.7624	0.6860
<i>C. max</i>	0.2451	****	0.8079	0.7480	0.7003	0.6059	0.5962
<i>C. san</i>	0.2983	0.2134	****	0.7532	0.6790	0.6268	0.7090
<i>C. pin</i>	0.1444	0.2904	0.2835	****	0.8986	0.7232	0.6881
<i>C. hup</i>	0.1590	0.3562	0.3871	0.1069	****	0.7784	0.6406
<i>C. sca</i>	0.2712	0.5011	0.4672	0.3241	0.2505	****	0.5804
<i>C. alt</i>	0.3769	0.5172	0.3438	0.3738	0.4453	0.5440	****

¹ *Nei's* genetic identity (above diagonal) and genetic distance (below diagonal). *C. bre* = *C. bretschnideri*; *C. max* = *C. maximowiczii*; *C. san* = *C. sanguinea*; *C. pin* = *C. pinnatifida*; *C. hup* = *C. hupehensis*; *C. sca* = *C. scabrifolia*; *C. alt* = *C. altaica*.

Figures

Figure 1

Phenotypic traits of leaf, flower, and mature fruit of *Crataegus* individuals *C. bretschnideri* – SF1H, *C. maximowiczii* – MSZ2H, *C. sanguinea* – LNSZ1H, *C. pinnatifida* – MDFSLH, *C. hupehensis* – HBSZ2H, *C.*

scabrifolia – YNSZ1H, *C. altaica* – AETSZ2H.

Figure 2

Quantitative traits of leaf, flower, and mature fruit of *Crataegus* individuals Error bars indicate the standard deviation of twenty biological replications. ^{abcd} Means in the bar with different superscripts differs significantly. *C. bre* = *C. bretschnideri*, *C. max* = *C. maximowiczii*, *C. san* = *C. sanguinea*, *C. pin* = *C. pinnatifida*, *C. hup* = *C. hupehensis*, *C. sca*= *C. scabrifolia*, *C. alt*= *C. altaica*.

Figure 3

Cluster analysis of *Crataegus* individuals based on phenotypic data (A) Cluster analysis using phenotypic traits data of *Crataegus* leaves, (B) Cluster analysis based on phenotypic traits data of *Crataegus* flowers, (C) Cluster analysis accord to phenotypic traits data of *Crataegus* fruits, (D) Cluster analysis depend on phenotypic traits data of *Crataegus* leaves, flowers, and fruits. The names of individuals are given next to their branches. *C. bre* = *C. bretschnideri*, *C. max* = *C. maximowiczii*, *C. san* = *C. sanguinea*, *C. pin* = *C. pinnatifida*, *C. hup* = *C. hupehensis*, *C. sca*= *C. scabrifolia*, *C. alt*= *C. altaica*.

Figure 4

Dendrogram of *Crataegus* individuals based on SCoT polymorphism The resulting genetic similarity indices were used for generating the cluster tree through the UPGMA method. The names of individuals are given next

to their branches. *C. bre* = *C. bretschnideri*, *C. max* = *C. maximowiczii*, *C. san* = *C. sanguinea*, *C. pin* = *C. pinnatifida*, *C. hup* = *C. hupehensis*, *C. sca* = *C. scabrifolia*, *C. alt* = *C. altaica*.

Figure 5

Scatter plot of *Crataegus* individuals based on Principal Coordinate Analysis (PCoA) of SCoT markers *C. bre* = *C. bretschnideri*, *C. max* = *C. maximowiczii*, *C. san* = *C. sanguinea*, *C. pin* = *C. pinnatifida*, *C. hup* = *C. hupehensis*, *C. sca* = *C. scabrifolia*, *C. alt* = *C. altaica*.

Figure 6

Population structure ($K = 3$, $K = 4$, $K = 5$, and $K = 6$) of thirty-six individuals from seven *Crataegus* species based on SCoT markers The Structure was run ten times for each K value from 1 to 7 for determining the most likely number of ancestral kinships (K). Each vertical bar represents *Crataegus* individual. Each color represents one putative ancestral background. *C. bre* = *C. bretschnideri*, *C. max* = *C. maximowiczii*, *C. san* = *C. sanguinea*, *C. pin* = *C. pinnatifida*, *C. hup* = *C. hupehensis*, *C. sca* = *C. scabrifolia*, *C. alt* = *C. altaica*.

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