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RESEARCH ARTICLE

Genetic diversity of myrobalan (*Prunus cerasifera* Ehrh.), an overlooked plum species in Slovenia

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Abstract Slovenia has a favorable geographical location that allows the use of sustainable fruit production, which is especially beneficial in the context of global climate change. Efficient breeding programs, resulting in well-adapted cultivars, should include wide genetic resources as new sources of diversity. Due to its adaptability, myrobalan (*Prunus cerasifera* Ehrh.) is a promising alternative for plum cultivation. The main objective of this study was to collect new material and investigate the genetic diversity and structure of a local *P. cerasifera* collection prospected across the country. A total of 42 genotypes were genotyped using a set of 11 simple sequence repeats (SSRs). Relatively high diversity levels were found: the average number of alleles per locus was 10.38, the mean expected heterozygosity (He) was 0.77, the mean observed heterozygosity (Ho) was 0.64, and the mean polymorphic information contain (PIC) was 0.754. In addition, 32 private alleles were detected. A Bayesian analysis identified three ancestral populations, and a clustering analysis mainly grouped the material according to the origin of the accessions. This work provides a valuable insight into the diversity of Slovenian *P. cerasifera* genetic resources, basis for the selection of new materials suitable for a plum production to face the future global agricultural challenges.

Keywords *Prunus cerasifera* · SSR · Genetic diversity · Genetic structure · Plum · Genetic resources management · Breeding

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Introduction

Prunus cerasifera Ehrh., commonly known as cherry plum (Figure S1a, S1b) is a diploid species (2n = 2x = 16) belonging to the section *Euprunus* of the genus *Prunus* (family Rosaceae), which together with *Prunus domestica* L. and *Prunus spinosa* L., form the group of European plum species (Neumuller 2011). According to earlier reports, *P. cerasifera* has been described as a "genetic bridge" within the genus *Prunus*, due to its broad interspecific compatibility and high hybridization potential (Neumuller 2011). In addition, many authors described the involvement of this species in the evolution of hexaploid *P. domestica* (Decroocq et al. 2004; Bortiri et al. 2009; Depypere et al. 2009; Weiss et al. 2012; Ilgin et al. 2014). Cherry plum is considered a very diverse species consisting of wild and cultivated

material, consequently taxonomic subdivision have beeen a long time controversial (Woldring 1997; Wohrmann et al. 2011). Cherry plum is widespread from the Caucasus to Europe (Neumuller 2011), and is adapted to thrive in various growth conditions and a variety of sites (Hartmann et al. 2009). In Slovenia, wild populations of *P. cerasifera* occur primarily in the southeastern regions, whereas cultivated and feral forms are distributed across the country. They are commonly encountered in both urban and rural settings, including abandoned orchards, gardens, and the vicinity of farmsteads and other buildings (Martinčič et al. 2007). Regarding the plum genetic resources referenced by the European Cooperative for Crop Genetic Resources (ECPGR), *P. cerasifera* occupies second place after *P. domestica,* with approximately 669 accessions in the European *Prunus* database (Barreneche pers. comm.). The species undergoes a short dormancy phase over winter, which promotes early flower initiation and fruit development (Sancin 1988). The fruits vary in size (15–20 mm, cultivated types up to 40 mm) and shape, but are usually round. Fruit skin color ranges from yellow or red, to blue with various shades of purple. The color of the flesh can be greenish to yellow and orange, although some cultivars are notable for their red flesh. Cherry plums are characterized by a stone that more or less adheres to the flesh (Hartmann et al. 2009; Neumuller 2011). For many centuries, *P. cerasifera* has been valued as both a fresh and dried fruit crop across Western Asia (Hanelt 2001; Okie and Hancock 2008). In Europe, its fruits are mainly utilized in food processing industries, for instance in the preparation of preserves, sauces, and distilled beverages (Topp et al. 2012). Ethnobotanical records also highlight long-standing use in traditional medicine throughout the Caucasus, Central Asia, Belarus, and Ukraine (Dunaevskaya et al. 2021). Dark-skinned genotypes, in particular, contain elevated levels of bioactive molecules such as vitamins, minerals, organic acids, anthocyanins, phenolics, antioxidants, and essential amino acids (Wang et al. 2012). More recently, Liu et al. (2020) identified the presence of constituents with strong antioxidant properties in the leaves and branches, including tannins, flavonoids, phenolic acids, and natural pigments relevant for the beverage sector. Moreover, extracts derived from *P. cerasifera* fruits were shown to suppress pathogenic microorganisms and exhibit antimicrobial properties, supporting their potential application in biomedicine and agriculture (Bibi Jaffri and Ahmad 2018).

P. cerasifera is widely recognized as a versatile rootstock for plum, peach, apricot, and other commercially important *Prunus* species, combining resistance to pests and pathogens with tolerance to abiotic stresses (Sancin 1988; Moreno 2004; Das 2011). In addition, interspecific hybrids with *P. cerasifera* have been developed through breeding programs to transfer PPV resistance to related stone fruit species (Topp et al. 2012). Some of the successful rootstocks that were developed were clonal or seed selections of myrobalan (such as 'GF31', 'H29C' and 'B'), preselected clones ('Ademir' and 'Adara') and interspecific crosses such as *P. munsoniana*×*P. cerasifera*, called Marianna rootstocks (Moreno 2004; Reig et al. 2018; Korkmaz et al. 2023).

Beyond its role as a rootstock and its limited use in fruit production, cherry plum is often cultivated as an ornamental tree, valued for its decorative bark, foliage, and blossoms. Certain cultivars, such as 'Nigra', 'Pisardii', and 'Woodii', are especially notable for their purple leaves and pink flowers (Sedaghathoor et al. 2009). All these desirable/important traits and the adaptability of the species make it an ideal source for breeding to obtain new cultivars and rootstocks for fruit production as well as ornamental use.

In recent years, there have been a few investigations focused on evaluating the genetic diversity of wild and cultivated cherry plums using molecular markers. Although *P. cerasifera* represents an important component of the Slovenian plum gene pool and has recognized ecological and horticultural importance, a comprehensive assessment has remained lacking. Therefore, our current work focused on the systematic investigation of the *P. cerasifera* genetic resources: the new prospected collection (in situ material) and their comparison with the existing collections preserved ex situ in different institutes in Slovenia and abroad, in order to gain better knowledge of the diversity of cherry plums in the region and identify valuable material for selection.

Material and methods

Plant material

The studied accessions are part of a wider research focused on the study of genetic diversity and genetic structure of three related plum species (*P. domestica*, *P. cerasifera* and *P. spinosa*) (Ternjak et al. 2023). In this paper we present analyses specific to diploid species, hence in our case, specific to *P. cerasifera*. A total of 42 *P. cerasifera* accessions were studied, including five improved cultivars with known breeding or selection history, seven wild-collected accessions of natural origin, and 30 traditional landraces, generally maintained through propagation without documented pedigree information (Table S1). Of these, 23 were collected in situ from different Slovenian regions- Styria, Central Slovenia and Istria, while the remaining 19 originated from ex situ sources. Within the ex situ group, 11 accessions are conserved in the Slovene Plant Gene Bank Collection (SPGB), and the other eight (99, 143, 148, 163, 185, 187, 188, 190) were obtained from various collections, including botanical gardens (BG Maribor, BG Ljubljana, BG Graz), one private collection (Austria), and the 'Centre de Ressources Biologiques—CRB *Prunus Juglans*', French National Research Institute for Agriculture, Food and Environment, (INRAE) (Nouvelle Aquitaine Bordeaux). The ex situ accessions, especially from the botanical gardens and foreign collections, have been added to allow the positioning of the collected Slovenian traditional material and its comparison with well-known *P. cerasifera* genetic resources. The analyzed accessions displayed considerable phenotypic diversity (Table S1). Leaf coloration ranged from green to light red, bronze, and purple-leaved ornamental types. Fruit morphology varied in shape (round, elliptic, and pear-shaped), size (ranging from very small to small), and skin color, including yellow, orange, red, dark red, bluish-violet, and nearly black.

DNA extraction and SSR genotyping

DNA was isolated from the leaf material using the CTAB protocol described by Doyle and Doyle (1987) with some modifications, concentration was quantified using a fluorimeter and products were visualized under UV light according to Ternjak et al. (2023). The 11 SSR markers used in this study and extensively described by Ternjak et al. (2023), were developed from various *Prunus* species (Table 1). The selection criteria included their position on the *Prunus* reference map (linkage group), their amplification quality and polymorphic nature. Polymerase chain reactions (PCR) were performed using primers labelled with Cy5 or Cy5.5 dye (Sigma-Aldrich, Saint Louis, USA) and the HotStarTaq® Plus Master Mix Kit (Qiagen, Hilden, Germany), with annealing temperatures optimized for each marker at approximately 5 °C below the respective melting temperature (Tm). The PCR protocol consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 1 min, extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min, with a hold at 4 °C. The Beckman CEQ 8000 DNA sequencer (Beckman Coulter Inc., Brea, USA) was used to analyze fragment sizes following the manufacturer's guidelines. Each accession was analyzed and allele fragments were evaluated. During allele scoring, peak profiles obtained from the sequencer were automatically compared with internal size standards.

Data processing and genetic diversity parameters

For each locus, several parameters of genetic diversity were evaluated. The number of alleles per locus (Na), observed heterozygosity (Ho), expected heterozygosity (He), and inbreeding coefficient (FIS) were calculated with the "adegenet 2.1.0" R package (Jombart 2008). We did not calculate the F_{ST} parameter (measure of genetic differentiation), which indicates the proportion of the total genetic variance in a subpopulation relative to the total genetic variance. Given the sample size, potential subpopulations would not have enough individuals to accurately estimate this parameter. Although the number of studied accessions was relatively low, we calculated the number of private alleles (present only in a single accession) and effective alleles (Ne) with GenAlEx 6.5 software (Peakall and Smouse 2012). In addition, polymorphism information content (PIC), and significance of deviation from Hardy–Weinberg equilibrium (HW) using a chi-square goodness-of-fit test were calculated with Cervus 3.0 software (Kalinowski et al. 2007). According to Serrote et al. (2020), PIC values greater than 0.5 are considered to be highly informative for

Table 1 Details of 11 simple sequence repeat (SSR) primers, including their linkage groups (LG), repeat motifs, melting temperatures (Tm) and references *

SSR Locus	LG	Source species	Primer sequence (5′-3′)	Repeat motif	Tm(°C)	References
UDP96-005	1	*Prunus persica* (L.) Batsch	For: GTAACGCTC GCTACCACAAA Rev: CCTGCATAT CACCACCCAG	$(AC)_{16}TG(CT)_2CA(CT)_{11}$	62.8	Cipriani et al. (1999)
BPPCT034	2	*Prunus persica* (L.) Batsch	For: CTACCTGAA ATAAGCAGA GCCAT Rev: CAATGGAGA ATGGGGTGC	$(GA)_{19}$	62.3	Dirlewanger et al. (2002)
EMPAS12	3	*Prunus avium* L	For: TGTGCTAAT GCCAAAAATACC Rev: ACATGCATT TCAACCCACTC	$(TG)_{10}A/GA)_{10}AA(GA)_{13}$	61.3	Vaughan and Russell (2004)
UCD-CH17	4	*Prunus avium* L	For: TGGACTTCA CTCATTTCAGAGA Rev: ACTGCAGAG AATTTCCAC AACCA	$(CT)_{11}$	61.4	Struss et al. (2003)
EMPAS06	4	*Prunus avium* L	For: AAGCGGAAA GCACAGGTAG Rev: TTGCTAGCA TAGAAAAGAATT GTAG	$(CT)_{12}$	62.0	Vaughan and Russell (2004)
EMPAS11	5	*Prunus avium* L	For: ACCACTTTG AGGAACTTGGG Rev: CTGCCTGGA AGAGCAATAAC	$(TC)_{25}$	62.9	Vaughan and Russell (2004)
EMPAS14	5	*Prunus avium* L	For: TCCGCCATA TCACAATCAAC Rev: TTCCACACA AAAACCAATCC	$(TC)_{10}CCAT(TC)_5CCA T(TC)_8$	63.3	Vaughan and Russell (2004)
BPPCT014	5	*Prunus persica* (L.) Batsch	For: TTGTCTGCC TCTCATCTTAACC Rev: CATCGCAGA GAACTGAGAGC	$(AG)_{23}$	62.1	Dirlewanger et al. (2002)
BPPCT025	6	*Prunus persica* (L.) Batsch	For: TCCTGCGTA GAAGAAGGTAGC Rev: CGACATAAA GTCCAAATGGC	$(GA)_{29}$	62.4	Dirlewanger et al. (2002)
CPSCT026	7	*Prunus persica* (L.) Batsch	For: GTAACGCTC GCTACCACAAA Rev: CCTGCATAT CACCACCCAG	$(AC)_{16}TG(CT)_2CA(CT)_{11}$	64.1	Mnejja et al. (2004)
CPPCT006	8	*Prunus persica* (L.) Batsch	For: CTACCTGAA ATAAGCAGA GCCAT Rev: CAATGGAGA ATGGGGTGC	$(GA)_{19}$	58.8	Mnejja et al. (2010)

* Table taken from Ternjak et al. (2023) and completed including melting temperature (Tm) applied for the amplification of each SSR

co-dominant markers such as SSRs. The first set of analyses was carried out for the 42 *P. cerasifera* accessions, while the second set was performed separately for the in situ and ex situ accessions based on the conservation type.

Genetic structure analysis (Bayesian clustering and PCoA)

Population structure of *P. cerasifera* collection was assessed with two different methods. A Principal Coordinate Analysis (PCoA), conducted with DARwin 6.0.21 software (Perrier and Jacquemoud-Collet 2006), was complemented with the Bayesian method performed with STRUCTURE V2.3.4 software (Pritchard et al. 2000). The ΔK method described by Evanno et al. (2005), implemented in the Structure Harvester V0.6.94 application (Earl and von Holdt 2012), indicated the most relevant number of populations (K). The detailed description of the settings and construction of the bar plots of the Bayesian analysis have been previously described by Ternjak et al. (2023).

The accessions with coefficient of membership (ql) > 0.8 were assigned to a certain population, while the accessions with threshold below estimated membership, were considered admixed. However, the structure of the collection was also observed with the (ql) > 0.6 in order to gain a complete perspective of the genetic constitution of populations.

Moreover, estimation of two parameters: allele frequency divergence among populations (net nucleotide distance) and the average intra-population genetic distances (expected heterozygosity), were calculated with the STRUCTURE 2.3.4 software (Pritchard et al. 2000).

Genetic relatedness and clustering

The genetic relationships among the studied materials were assessed by the Unweighted Neighbor-Joining method (NJ) with the DARwin 6.0.21 software (Perrier and Jacquemoud-Collet 2006). Dissimilarities were calculated with 30.000 bootstraps, using the Jaccard coefficient, and the Unweighted Neighbor-Joining dendrogram with all the genotypes was created.

Results

Genetic diversity parameters analysis

The assessment of polymorphism in *P. cerasifera* was carried out with 11 microsatellite primer pairs, which amplified a total of 13 loci, as two primers (UDP96-005 and EMPAS12) each amplified two distinct regions. The analysis of these loci successfully distinguished all accessions, with no duplicates identified among the 42 samples studied (Table S2). Across all loci, 135 alleles were detected, ranging from four

Table 2 Estimation of genetic diversity parameters by 13 simple sequence repeat (SSR) loci (11 primer pairs)

SSR Locus	N_a	Allele range	N_e	Private alleles	PIC	H_o	H_e	FIS
UDP96-005_1	8	96–118	4.62	1	0.785	0.51	0.78	0.35
UDP96-005_2	13	134–174	5.93	3	0.840	0.78	0.83	0.06
BPPCT034	10	217–257	4.33	1	0.739	0.52	0.77	0.32
EMPAS12_1	8	94–118	4.15	2	0.752	0.52	0.76	0.32
EMPAS12_2	10	122–148	4.4	3	0.791	0.46	0.77	0.4
UCD-CH17	11	135–167	5.13	2	0.782	0.79	0.8	0.01
EMPAS06	14	179–245	8.07	3	0.864	0.86	0.88	0.02
EMPAS11	9	57–79	2.99	4	0.621	0.55	0.65	0.15
EMPAS14	8	171–219	4.49	3	0.744	0.71	0.78	0.1
BPPCT014	4	191–199	3.16	–	0.631	0.71	0.68	− 0.04
BPPCT025	18	133–197	8.26	7	0.869	0.81	0.88	0.08
CPSCT026	5	171–211	2.07	–	0.484	0.33	0.52	0.37
CPPCT006	17	172–188	10.25	3	0.895	0.79	0.9	0.12
Total	135	–	–	32	–	–	–	–
Mean	10.38	–	5.22	2.46	0.754	0.64	0.77	0.17

N_a: number of alleles per locus; N_e: number of effective alleles; PIC: polymorphism information content; H_o: observed heterozygosity; H_e: expected heterozygosity; FIS: inbreeding coefficient

alleles for BPPCT014 to 21 alleles for UDP96-005, with an average of 10.38 (Table 2). The mean effective number of alleles (Ne) was 5.22, with values from 2.07 (CPSCT026) to 10.25 (CPPCT006); the mean observed heterozygosity (Ho) was 0.64, ranging from 0.33 (CPSCT026) to 0.86 (EMPAS06); and the mean expected heterozygosity (He) was 0.77, extending from 0.52 (CPSCT026) to 0.9 (CPPCT006) (Table 2). PIC values demonstrated the high informativeness of the SSR markers, with a mean of 0.754 (values ranging from 0.484 for EMPAS11 to 0.895 for CPPCT006); only locus CPSCT026 fell below the 0.5 threshold (PIC = 0.484) (Table 2). The inbreeding coefficient FIS ranged between − 0.04 (BPPCT014) and 0.4 (CPSCT026). Based on FIS, loci were classified into different categories: five with a high or very high values, five with average, two with low values and one with negative value. The only locus showing an excess of heterozygotes without significant deviation from Hardy–Weinberg equilibrium was BPPCT014.

Separate analyses of genetic diversity were conducted for the in situ and ex situ datasets (Table 3). In situ accessions showed greater values for the number of alleles per locus, Ne, PIC, Ho, and He, with a slightly reduced mean FIS value.

Private alleles, 32 in total, were found at most loci, excluding BPPCT014 and CPSCT026 (Table S2, Table 4). Notably, the myrobalan 'Agdzadzor' (187) carried four private alleles, and Plum yellow (133), contained three private alleles, while seven accessions contained two private alleles each (61, 134, 136, 154, 156, 163, 188), and 11 accessions carried only one. A greater number of accessions carrying private alleles (14) originated from in situ, while only six ex situ accessions contained private alleles (Table 4). In total, 20 private alleles were detected in the in situ dataset compared with 12 in the ex situ dataset. Considering the material status, 24 private alleles were identified in landraces and wild accessions, while improved cultivars comprised only eight (Table 4).

Population structure inferred from SSR data

STRUCTURE software and PCoA were applied to assess the genetic structure of the studied material. The ΔK method indicated a maximum value at K = 3 (Figure S2, Table S3), separating the accessions into

Table 3 Estimation of genetic diversity parameters in in situ and ex situ accessions

Conservation type	N	N_a	N_e	Private alleles	PIC	H_o	H_e	FIS
In situ	23	9.08	5.08	17	0.737	0.67	0.76	0.12
Ex situ	19	8	4.76	15	0.736	0.61	0.76	0.2

N: number of accessions; N_a: number of alleles per locus; N_e: mean number of effective alleles; Private alleles: present only in a single accession; PIC: polymorphism information content mean; H_o: observed heterozygosity mean; H_e: expected heterozygosity mean; FIS: inbreeding coefficient mean

Table 4 Distribution of private alleles in in situ and ex situ accessions

Number of private alleles	1	2	3	4	Number of accessions with private alleles	Private alleles found in landraces and wild species	Private alleles found in improved cultivars	Total number of private alleles
In situ	111 (L/W), 122 (L), 130 (L), 131 (L), 132 (L), 135 (L), 137 (W), 138 (W), 139 (W)	134 (L), 136 (L), 154 (IV), 156 (L)	133 (L)	–	14	18	2	20
Ex situ	55 (L), 148 (W)	61 (L), 163 (IV), 188 (W)	–	187 (IV)	6	6	6	12

L: Landrace, IV: Improved variety, W: Wild species

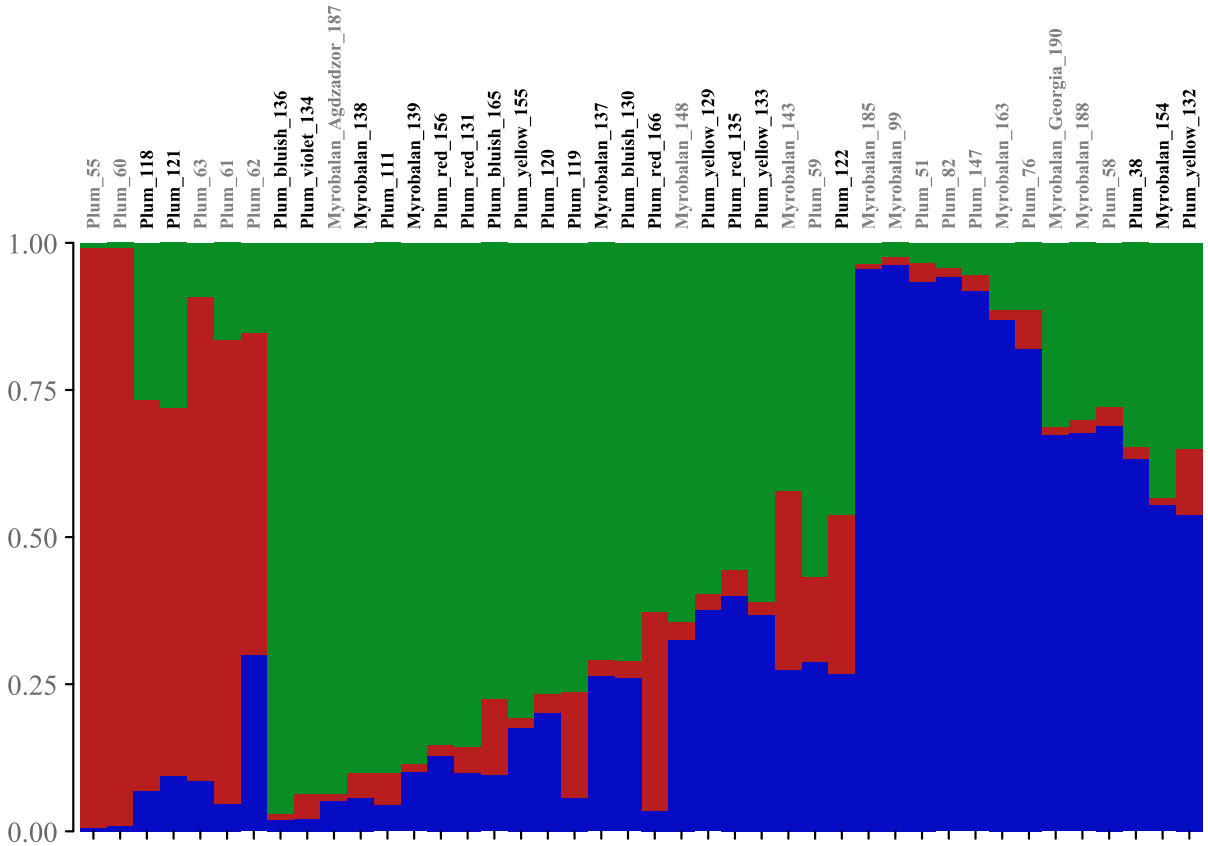


Fig. 1 Bayesian clustering of 42 *Prunus cerasifera* Ehrh. accessions (K = 3). Each genotype is represented by a vertical bar. Using a coefficient of membership ≥ 80% (q > 0.8), populations are indicated in green (K1), red (K2), and blue (K3) Conservation status of studied material is indicated by accession name color: in situ material (black) and ex situ material (grey)

three ancestral populations (Fig. 1). Applying a membership coefficient ≥ 80%, 19 accessions (45.24%) could be assigned to one of the populations, while the remaining 23 accessions were classified as admixed (Table S4). Population K1, green, comprised nine accessions, mostly in situ material with the exception of the ex situ myrobalan 'Agdzadzor' (187). Population K2, red, contained only three ex situ landraces from Lower Styria, whereas the Population K3, blue, included seven ex situ accessions of mixed status and origin. Reducing the membership threshold to ≥ 60%, resulted in 34 accessions (80.95%) being assigned to a distinct population, with eight remaining admixed (Table S4). Based on this adjustment, eight of the previously admixed accessions were classified as Population K1, three as Population K2, four as Population K3, and the rest admixed genotypes showed predominant genomic affinity to Population K1.

STRUCTURE software analysis of allele frequency divergence revealed that Populations K1 and K3 were the most closely related (0.0116), while K2 showed greater divergence from both K1 and K3, with the highest differentiation observed between K2 and K3 (0.0644) (Table S5). Mean pairwise distances between individuals indicated comparable allelic variation across populations (K1 = 0.7975, K2 = 0.7113, K3 = 0.7651) (Table S5).

The PCoA analysis results corroborated the Bayesian clustering, placing Population K2 accessions (red) in a distinct upper-left cluster, Population K1 accessions (green) mainly in the right area, and the more diverse Population K3 accessions (blue) dispersed across the lower portion of the plot (Fig. 2).

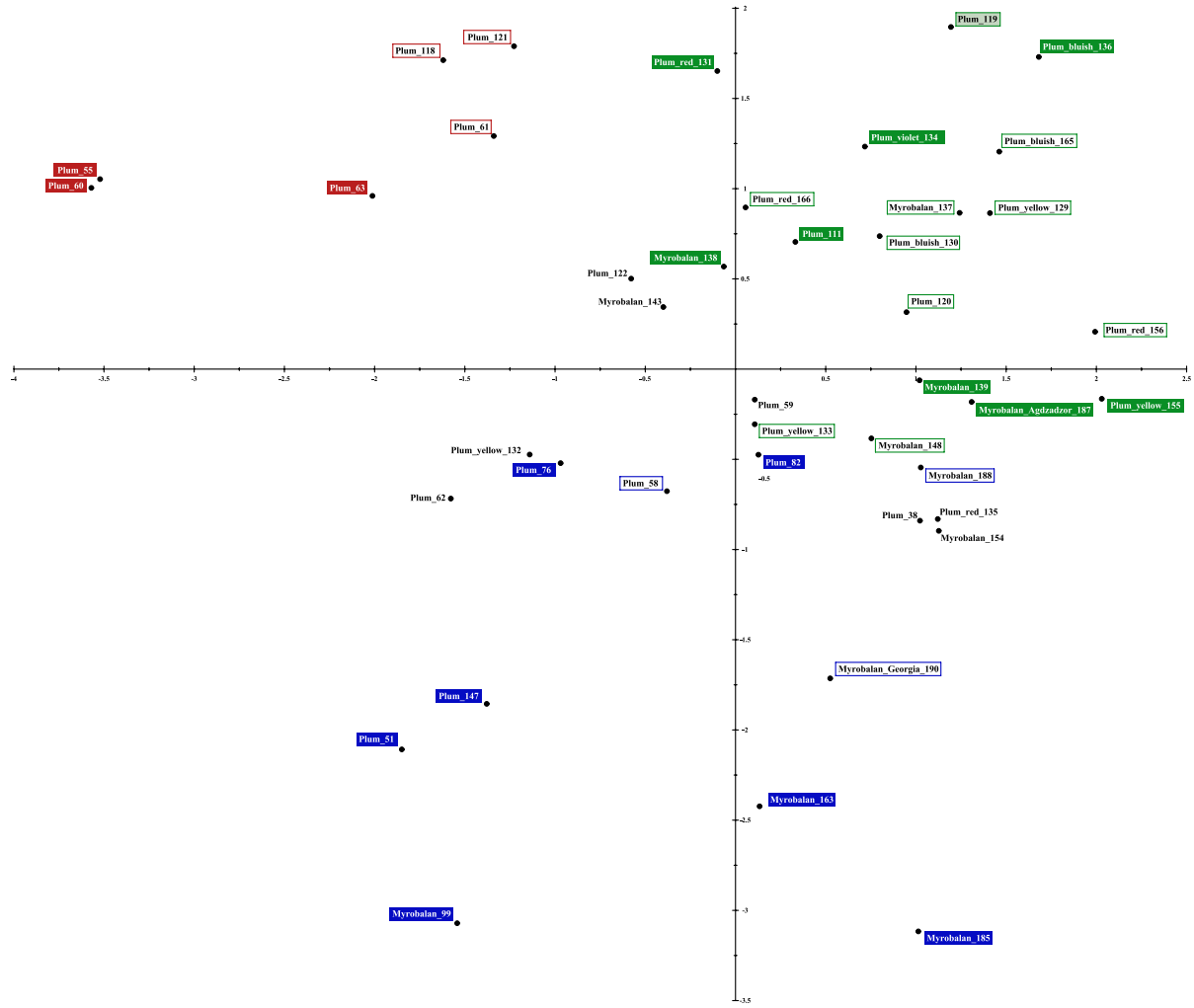


Fig. 2 Principal coordinate analysis (PCoA) based on 11 simple sequence repeat (SSR) loci. Colors correspond to STRUCTURE populations for the most relevant parameter K = 3: Population K1 (green), Population K2 (red), Population K3 (blue). Accessions assigned at $q \geq 0.8$ are shown in full color; those at $q \geq 0.6$ are lighter; admixed accessions are in black

Genetic relationships and cluster distribution

The Unweighted Neighbor-Joining tree conducted from the dissimilarity matrix grouped 42 accessions into three clusters with low bootstraps values (Fig. 3a). Within these clusters, smaller sub-clusters exhibited stronger connections, reflecting the geographic origin of the accessions.

Cluster I comprised 19 accessions, the majority of which were maintained in the ex situ collections (14) and were predominantly landraces, accompanied by three improved cultivars and two wild genotypes. This cluster was further subdivided into two sub-clusters. The first and larger sub-cluster mainly included accessions from the Lower Styria region (10 in total), seven of which originated from Brežice (55, 58, 59, 60, 61, 62, 63), while the remaining three accessions were collected in situ in Maribor (118, 121, 122). Furthermore, two in situ accessions from the south (Istria region), Plum red (131) and Plum yellow (132), along with the ornamental cultivar myrobalan 'Nigra' (143), from the Botanical Garden of the Karl-Franzens University of Graz (Austria), were also grouped within this sub-cluster. The second sub-cluster comprised only ex-situ material from various collections. It

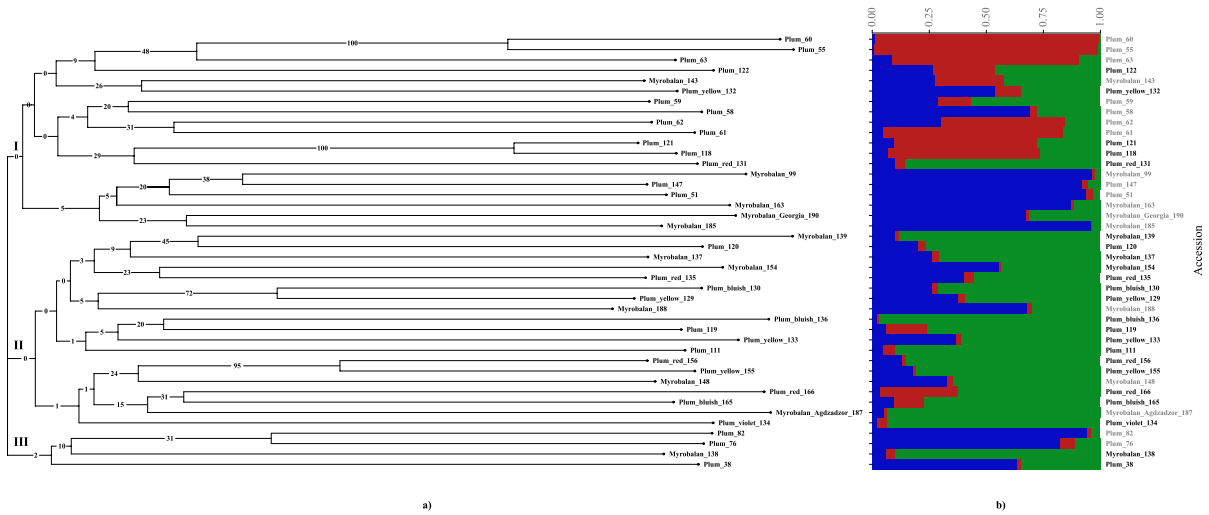


Fig. 3 Genetic relatedness of *Prunus cerasifera* Ehrh. accessions. **a** Unweighted Neighbor-Joining tree based on Jaccard coefficient. The genotyping results identified three clusters (I, II and III). **b** STRUCTURE analysis (K = 3). Each genotype is represented by a vertical bar. Using a coefficient of membership ≥ 80% (q > 0.8), populations are indicated in green (Population K1), red (Population K2), and blue (Population K3). Population assignments correspond to the clusters in the dendrogram. Conservation status is indicated by accession name color: in situ material (black) and ex situ material (grey)

included ornamental cultivars myrobalan 'Nigra' (99) from the Botanical garden of the University of Maribor and Myrobalan (163) from the Botanical Garden of the University of Ljubljana, Myrobalan Georgia (190) of Georgian origin held in a private collection in Graz (Austria), two SPGB accessions from Brežice (Plum 51, and Plum 147), and Myrobalan (185) from the INRAE CRB *Prunus Juglans* collection.

Cluster II consisted of 19 accessions with diverse status, including wild genotypes, landraces, and improved material, originating from various regions. The majority of accessions (16) were collected in situ across Slovenia, complemented by three ex situ accessions from international collections: Myrobalan (148) from Dietzen (East-Styria, Austria), French Myrobalan (188), and Armenian myrobalan 'Agdzadzor' (187), both part of the INRAE CRB *Prunus Juglans* collection. Certain pairs of accessions from the same origin exhibited stronger genetic relatedness, supported by higher bootstrap values- for instance Plum yellow (129) and Plum bluish (130) from Koper (Istria region) and Plum yellow (155) and Plum red (156) from Razvanje (Lower Styria). Other accessions from the same origins, such as Plum bluish (165) and Plum red (166) from Ljubljana (Central Slovenia), clustered together with lower bootstrap values. Notably, no accessions from Brežice were included in this cluster.

Among all clusters, Cluster III contained the fewest accessions (only four), equally representing in situ (38, 138) and ex situ (76, 82) material. Each accession traced back to the Lower Styria region: three (38, 76, and 82) from Brežice and one (Myrobalan, 138) from Kasaze.

The Bayesian clustering results were generally in agreement with the NJ analysis, with some discrepancies (Fig. 3b). In population K1 (q > 0.6) 15 of 17 accessions were collected in situ and corresponded to NJ Cluster II, apart from Plum red (131) and Myrobalan 138 that aligned with Cluster I and III, respectively. Two additional ex situ accessions of Population K1, Myrobalan (148, 187) from foreign collections, were likewise positioned within Cluster II. Population K2 included six Lower Styria accessions (four ex situ and two in situ), corresponding to the first sub-cluster of Cluster I in the NJ tree. Most accessions belonging to Population K3 were ex situ and aligned with the second sub-cluster of Cluster I (six accessions), while the remaining ex situ accessions were distributed between Cluster II (Myrobalan 188), and Cluster III (Plum 76 and 82). Only one accession from Population K3, Plum 38, was collected in situ and was positioned into Cluster III from the NJ analysis.

Discussion

In the earlier study (Ternjak et al. 2023) dealing with the three *Prunus* species (*P. domestica*, *P. cerasifera* and *P. spinosa*), complementary analyzes revealed that among the in situ collected accessions (54), a high number corresponded to *P. cerasifera* (23), demonstrating the importance of this species in the region. Furthermore, the Bayesian approach separated *P. cerasifera* accessions as an independent ancestral population, which was also confirmed by the PCoA analysis. These results have shown us the importance of studying specifically the material of this multipurpose (fruit, ornamental and rootstock) species.

In the current study focusing on diploid *P. cerasifera* material, we were able to calculate several parameters of genetic diversity. Analysis of 42 *P. cerasifera* accessions using 11 SSR markers revealed an average of 10.38 alleles per locus, similar to the 10.57 reported by Horvath et al. (2008) for a smaller dataset of 29 accessions and seven SSRs, although their origins were highly diverse. In contrast Zhao et al. (2015) observed fewer alleles on average (6.71) across 14 SSR and 40 accessions, whereas Wohrmann et al. (2011) recorded considerably higher diversity levels (16.73 alleles per locus) in a broader set of 117 individuals representing 12 different wild *P. cerasifera* populations. The increased polymorphism in the latter study likely reflects the larger sample size and inclusion of multiple wild populations. Expected heterozygosity (H_e) and observed heterozygosity (H_o) values were comparable across investigations (H_e: 0.77 in this study, 0.75, Horvath et al. (2008), and 0.73 in Wohrmann et al. (2011); H_o: 0.64, 0.65, 0.66, respectively), indicating that Slovenian myrobalan germplasm maintains a substantial level of genetic diversity comparable to or exceeding that reported in other research. This high level of heterozygosity is consistent with the predominantly outcrossing nature of *P. cerasifera*. Most diploid *Prunus* species, including *P. cerasifera*, possess a gametophytic self-incompatibility system that enforces outcrossing, which is known to maintain genetic variation in natural populations (Hegedűs and Halász 2006; Stoeckel et al. 2006). From a breeding perspective, such diversity represents a valuable resource for broadening the genetic base of future rootstock and cultivar development, as it provides a strong basis for selecting vigorous parental material and generating genetically diverse progeny.

The inbreeding coefficient (FIS) values observed in this study were greater than those reported by Wohrmann et al. (2011), with most loci showing moderate to high FIS values, showing a high level of inbreeding (Hartl 2020). Consistent with the results of Wohrmann et al. (2011) for the locus CPSCT012, the BPPCT014 locus in our dataset also showed a negative FIS, reflecting a slight excess of heterozygotes. For this marker, the observed heterozygosity (Ho) exceeded the expected heterozygosity (He), suggesting that the locus may be located in a genomic region under strong selective pressure during the evolution of *P. cerasifera* (Stoeckel et al. 2006).

Screening for private alleles highlighted 20 accessions, primarily landraces or wild types, mostly collected in situ, with three red-leaved improved cultivars: myrobalan 'Agdzadzor' (187) originating from Armenia (INRAE CRB *Prunus Juglans* collection), Myrobalan (154) from the Styria region of Slovenia, and Myrobalan (163) of unknown origin, from the Botanical Garden of the University of Ljubljana. Despite their improved status, these accessions demonstrated distinct genetic profiles. A similar pattern of higher private or unique alleles associated with traditional material, such as landraces or early selections, has been reported for Spanish European plum and apple germplasm (Urrestarazu et al. 2012), European sweet cherry accessions (Barreneche et al. (2021), traditional Croatian plum accessions (Kazija et al. 2014), Iranian apricot germplasm collection (Raji et al. 2014), Turkish walnut germplasm (Bozhuyuk et al. 2020), and local Turkish pear genotypes (Bozhuyuk and Aslantas 2020). These results emphasize the high genetic diversity and underutilized potential of traditional and wild germplasm. Comparison of in situ and ex situ *P. cerasifera* accessions revealed a difference of eight private alleles. The difference in private allele richness between in situ and ex situ material further emphasizes the need to complement ex situ collections with targeted collecting from natural and traditional growing sites. The ex situ accession with the highest number of private alleles, was the Armenian improved cultivar myrobalan 'Agdzadzor' (187), reflecting the species' native diversity in the Caucasus region (Horvath et al. 2011; Topp et al. 2012). Armenia, as part of the Near-Eastern Center of Origin of Cultivated Plants, has historically witnessed

various stages of fruit cultivation, including wild fruit trees, selected wild accesions with desirable traits, and superior wild specimens grafted onto less valuable wild forms of different *Prunus* species (Vavilov 1949). From a breeding standpoint, accessions carrying private alleles, such as myrobalan 'Agdzadzor' (187) and Plum yellow (133), may represent particularly valuable candidates for pre-breeding, as private alleles can be associated with rare or locally adapted traits. The higher number of private alleles detected in in situ accessions highlights their importance as reservoirs of novel diversity and supports prioritizing these populations for both conservation and breeding purposes. The practical value of genetically diverse *P. cerasifera* germplasm for breeding has been demonstrated in recent breeding-oriented research, where integrated molecular and phenotypic evaluation enabled the identification of promising candidates (Korkmaz et al. 2023). Such approaches illustrate how genetically diverse germplasm, similar to the material characterized in the present study, can provide as a foundation for targeted rootstock selection programs.

Genetic analyses of the local myrobalan collection identified three main groups, each highlighting distinct material that could serve as a valuable source of diversity for future research, germplasm conservation and breeding efforts. Population K1 consisted of heterogeneous and highly diverse in situ myrobalan materials, clearly distinct from those maintained in other genetic resource centers and botanical gardens. This population included the greatest number of accessions (11) exhibiting private alleles and represented an ancestral group, containing genotypes related to myrobalan 'Agdzadzor' (187) from Armenia. The Neighbor-Joining dendrogram further highlighted the uniqueness of certain accessions, such as Myrobalan (139) and myrobalan 'Agdzadzor' (187), which was evident from their extended branch lengths, reflecting substantial genetic dissimilarity. Population K2 (red), largely composed of local landraces from the Styria region, formed a distinct ancestral group with the fewest accessions but exhibited greater divergence relative to Populations K1 and K3. The core of this ancestral group consisted of three ex situ accessions, originally prospected from an abandoned estate, which were subsequently incorporated into the SPGB collection as unique germplasm. Population K3 displayed the highest heterogeneity in terms of both- status, encompassing wild types, landraces, and improved cultivars, and geographic origin, including accessions from Brežice, the Botanical Gardens of Ljubljana and Maribor, France, and a Georgian accession (190), of which 70% of its genome was assigned to this population. This complex genetic structure, where local cultivars clustered alongside a diverse set of international accessions, mirrors observations reported in apple and plum germplasm research (Urrestarazu et al. 2012).

Several investigations have reported associations between geographic origin and genetic structure in plant germplasm, including walnut (Bernard et al. 2018) and various *Prunus* species such as sweet cherry (Barreneche et al. 2021) and apricot (Bourguiba et al. 2020). Similarly, Urrestarazu et al. (2012) found that apple accessions clustered according to their geographic origin. Even though our work included a smaller number of accessions compared to previous research, a clear correspondence between geographic origin and genetic structure was apparent. Notably, Population K2 comprised only accessions from Brežice, while Population K1 predominantly consisted of material originating from the Lower Styria region.

Eleven ex situ accessions from Brežice exhibited notable genetic diversity, being assigned to two ancestral populations (K2 and K3). The accessions originated from three nearby sites—a farm, an abandoned property, and a private collection (separated by roughly six kilometers), highlighting the thoroughness of the prospecting and the richness of the local germplasm. The historical situation of Brežice, a center for plum cultivation and plum drying in eastern Slovenia, may explain the high diversity observed in this small area (Mišić, 1979). This suggests that the region could serve as a valuable source for further germplasm collection to enrich existing genetic resources.

The second sub-cluster of Cluster I comprised purple-leaved myrobalans, including wild genotypes, landraces and two cultivars, 'Nigra' (99) and 'Woodii' (185), with the sole exception of the green-leaved Myrobalan Georgia (190). This latter accession originates from Georgia, which is, such as Armenia, located in the Caucasus region, center of origin of plum but also other important fruit species. As for Armenian accession 'Agdzadzor' (187), it is interesting to observe its specific genetic background.

Purple-leaved myrobalans are valued as ornamental trees, for their vibrant foliage, pink flowers and dark red fruits. Moreover, accessions bearing dark fruits are rich in health-promoting compounds, such as anthocyanins, potassium, copper, zinc, phenolic compounds, and flavonoids, which exhibit antioxidant activity (Dunaevskaya et al. (2021). These traits make them a promising resource for functional foods, a healthy diet, and as an alternative for future plum production.

A group of eight in situ accessions from the Istria region clustered together in Cluster II of the NJ tree, except for accessions (Plum 131 and 132), which were placed in Cluster I. These accessions were collected from five distinct locations and showed considerable morphological variation in fruit and leaf traits. The sub-Mediterranean climate of Istria, with high temperatures and water scarcity, poses a major constraint on all forms of agriculture (Vrščaj et al. 2017; Repe 2018). The material studied from this region may therefore harbor traits related to drought tolerance, consistent with the recognized resilience of *P. cerasifera*. Previous investigations have highlighted its potential for breeding drought-resistant rootstocks, with ranking *P. cerasifera* among the most tolerant of ten evaluated *Prunus* species Cochard et al. (2008). Taken together, these findings underline the relevance of locally adapted germplasm for future breeding efforts. The importance of exploring such material has also been noted across other *Prunus* species. In sweet cherry, the characterization of Azerbaijani materials has been addressed as an approach to broaden the narrow genetic base of modern cultivars and to identify traits relevant for climate resilience and reduced pesticide dependency (Brault et al. 2024). In their preliminary study, Brault et al. (2024) confirmed the high genetic divergence between the prospected landraces from Azerbaijan and a set of accessions representative of the diversity maintained in INRAE collection and previously studied (Campoy et al. 2016).

Beyond drought adaptation, myrobalan is characterized by good productivity, early maturity, and wide ecological adaptability, including the ability to grow in diverse soil types, waterlogging tolerance and resistance to various pests and diseases (Xiloyannis et al. 2007; Hartmann et al. 2009; Dunaevskaya et al. 2021; Ling et al. 2025). In particular, *P. cerasifera* demonstrates phenological adaptability to temperature changes, maintaining stable timing of developmental stages under variable climatic conditions (Petrov et al. 2024), further supporting its suitability for cultivation in contrasting environments. These characteristics have been successfully utilized in rootstock development. For example, the 'Adara' rootstock (Estación Experimental de Aula Dei—Consejo Superior de Investigaciones Científicas—EEAD-CSIC, Spain) illustrates these advantages in practice. It is currently the most widely used rootstock in Spain due to its hardiness, broad adaptability to different soil types, including calcareous and compact soils, and tolerance to iron chlorosis and root asphyxia. It also exhibits immunity to root-knot nematodes, resistance to soil-borne pathogens, and supports high fruit quality in terms of size, firmness, and sugar content, combined with medium-to-high vigor (Moreno 2004; Reig et al. 2018).

Conclusion

This investigation has yielded novel insights into the genetic composition of Slovenian *P. cerasifera* germplasm applying SSR marker analyses. Our data demonstrate a considerable degree of genetic diversity within the sampled material, which predominantly included local/traditional landraces and wild accessions. Importantly, the study uncovered genetically distinct groups, including an ancestral population not previously documented in Slovenian ex situ collections. These results highlight the critical role of systematic germplasm collection, targeted conservation, and strategic long-term gene bank management to safeguard genetic resources relevant for future use. The presence of genetically differentiated populations indicates that Slovenian *P. cerasifera* germplasm represents a valuable reservoir of rare and potentially adaptive alleles. From an applied perspective, this documented genetic diversity has important implications for fruit production, ornamental and rootstock myrobalan breeding, particularly under the conditions imposed by global warming. Accessions originating from environmentally challenging regions, such as Istria, may be especially promising for the development of drought-tolerant and climate-resilient rootstocks. Even though comprehensive morphological

analyses are not yet complete, preliminary observations identified accessions with particularly promising phenotypic traits that merit further agro-morphological evaluation. These results will provide a basis for developing new cultivars and for designing sustainable breeding strategies that broaden the genetic base of myrobalan, reduce vulnerability to abiotic stress, and enhance long-term productivity. Looking forward, a significant next step would involve launching a follow-up study with more extensive sampling and in-depth agro-morphological and stress-response evaluations. This study should include both Slovenian *P. cerasifera* germplasm and representative accessions from neighboring Balkan countries to fully capture regional diversity and support breeding efforts. Such an approach will be essential for translating genetic diversity into practical solutions for myrobalan cultivation and plum production under changing climatic conditions.

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Author contributions All authors contributed to the study conceptualization and design: M.Š., A.Š. and T.T. designed the initial research, T.B. and J.Q-G suggested complementary research and methods in data analysis; M.Š., A.Š. and T.T. conducted the field work and sampling; T.T. carried out SSR molecular analyses; T.T. and T.B. curated the data; T.T. analyzed the data; T.B. and J.Q-G. contributed to the data analysis; T.T., T.B. and J.Q-G. wrote the manuscript, M.Š., A.Š. edited and reviewed the original manuscript. All authors contributed to the article and approved the submitted version.

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Data availability The datasets generated during and/or analyzed during the current study are available as supplementary data.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Appendix

The supplementary data for this article can be found online at:

Additional file 1 contains following tables: Table S1: The detailed list of the studied materials, providing name, status, origin and other passport data. Table S2: Raw SSR data (42 accessions), shown in bold the private alleles. Table S3: Summary of results obtained with the method of Evanno et al. (2005), generated with the Structure Harvester tool. Table S4: List of accessions including membership values to subgroups using STRUCTURE (Pritchard et al. 2000). Table S5: Estimates of allele frequency divergence among populations (net nucleotide distance) and the average intra-population genetic distances (expected heterozygosity), calculated with STRUCTURE 2.3.4 software (Pritchard et al. 2000).

Additional file 2 contains following figures: Figure S1a and S1b: Colorful fruits of several *Prunus cerasifera* Ehrh. accessions maintained in the SPGB collection. Figure S2: Graphical method, allowing the detection of the number of groups K using ΔK (Evanno et al 2005).

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