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Intergeneric Transfer of Simple Sequence Repeat Molecular Markers for the Study of *Chaenomeles* as Fruit Crop Breeding Material

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Abstract: The genus *Chaenomeles*, part of the subfamily *Maloideae* within the *Rosaceae* family, comprises five recognized species and has long been valued for its ornamental qualities. However, the use of *Chaenomeles japonica* as a fruit crop is relatively recent, with its introduction into targeted breeding activities only occurring in the 1950s. Due to this, genetic information on the genus remains limited, and the application of molecular markers in crop breeding and further development have been narrow, relying primarily on non-specific marker applications in germplasm analysis. One potential solution is the transfer of molecular markers between genera, specifically from the related *Maloideae* genera. This study aimed to test the transferability of SSR markers developed for *Malus* to *Chaenomeles*, and to analyze the structure of available *Chaenomeles* germplasm. By including 74 *Chaenomeles* genotypes, 95 SSR markers originally developed for *Malus* were tested, with 25 proving effective for characterizing *Chaenomeles* germplasm. These adapted SSR markers successfully differentiated among *Chaenomeles* species, including *Chaenomeles japonica*, *Chaenomeles speciosa*, *Chaenomeles cathayensis*, and hybrids like *Chaenomeles* × *superba* and *Chaenomeles* × *californica*. The markers demonstrated high stability and repeatability, indicating their suitability for large-scale genetic research, species composition assessment, and breeding material evaluation. Given the limited studies on SSR markers in *Chaenomeles*, this research lays the foundation for further exploration, potentially expanding into the genetic diversity assessment and trait screening for breeding. As genetic research on *Chaenomeles* is still in its early stages, the development of additional markers will be crucial for advancing this crop.

Keywords: *Chaenomeles*; fruit crops; germplasm; breeding; molecular markers; introduction

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1. Introduction

The genus *Chaenomeles* belongs to the subfamily *Maloideae* in the *Rosaceae* family with five recognized species: *C. japonica* (Thunb.) Lindl. (Japanese quince), *C. cathayensis* (Hemsl.) Schneider (Chinese quince), *C. speciosa* (Sweet) Nakai (flowering quince), *C. thibetica* Yü (Tibetan quince) and *C. sinensis* (Thouin) Koehne (Chinese quince) [1]. The species *C. cathayensis*, *C. japonica* and *C. speciosa* have been used to create interspecific hybrids, named *C.* × *superba* (*C. speciosa* × *C. japonica*), *C.* × *vilmoriniana* (*C. speciosa* × *C. cathayensis*), *C.* × *clarkiana* (*C. japonica* × *C. cathayensis*) and *C.* × *californica* (*C.* × *superba* × *C. cathayensis*), resulting in several hundreds of ornamental cultivars. All five *Chaenomeles* species are native to central and east Asia; however, they are also cultivated in northern Europe [2–4]. While the genus is known for its ornamental value, *Chaenomeles* domestication in the Baltics was focused on breeding cultivars of which the fruit could be used in food processing [5].

The fruits of *C. japonica* are valuable for their high organic acid, C vitamin, phenolic compound content and antioxidative properties [6–10]. Furthermore, their seeds are used in oil production [11] as a source of unsaturated and saturated fatty acids, as well as α-tocopherol, phytosterols and β-carotene [12]. To ensure that *Chaenomeles* plants yield

frequently and with high-quality fruit, it is necessary to develop efficient breeding programs to develop new cultivars. Given that *Chaenomeles* breeding, like all fruit crop breeding, is a long-term process, molecular methods are valuable for both germplasm research and the selection of breeding material.

In Latvia, the breeding of *Chaenomeles japonica* as a fruit crop began in the 1950s. The first large-scale plantations of Japanese quince were established in the 1970s, reaching approximately 300 hectares by the 1980s. All these commercial plantations were started from seedlings, resulting in highly diverse plant material. At the Institute of Horticulture (LatHort), Japanese quince breeding efforts started in the 1990s intending to develop cultivars adapted to the Latvian climate. Only *Chaenomeles japonica* was used in breeding, as other species lack the winter hardiness for northern Europe. Between 1998 and 2002, LatHort collaborated with Swedish and Lithuanian scientists to evaluate a wide range of *Chaenomeles* germplasms as part of the EU Project "Japanese quince (*Chaenomeles japonica*)—a New European Fruit Crop for Produce of Juice, Flavour and Fibre" (EUCHA) [13]. This project aimed to identify and evaluate desirable biological and agronomic traits necessary for the development of promising cultivars, including the strong winter hardiness of both plants and flower buds, thornless plants, high productivity with consistent yields, strong resistance of plants and fruits to diseases, high fruit quality with a rich biochemical content, early ripening, and erect or semi-erect shrub structure. Following this evaluation, three cultivars were selected and registered in Latvia: 'Rasa' from Latvian breeding material, and 'Darius' and 'Rondo' from the EUCHA program. These cultivars are thornless and productive (yielding 4–8 kg per bush), with uniform fruits (40–60 g) that ripen in early to mid-September [4]. Among these, 'Rondo' is best suited for commercial cultivation due to its upright, easy-to-prune structure and high yield of quality fruit. It can also be grown using methods where plant strips are cultivated rather than mulched. The 'Darius' cultivar is spreading and high-yielding, but on poorer soils or in less-maintained plantations, fruit size may decrease, impacting product quality. 'Rasa' is recommended for cultivation with an agro-textile cover over plant strips, as branches tend to bend under high yield in later years, complicating care. In 2023, two new cultivars, 'Jānis' (SR 1-6) and 'Silvija' (SR 1-3), were submitted for registration in Latvia, noted for their high-quality fruits and high yield.

Japanese quince has become an important commercial fruit crop in Latvia, with the planting area expanding rapidly in recent years, reaching 723 hectares by 2022 [14]. Japanese quince breeding, including the pre-breeding and testing of new cultivars under production conditions, is now part of the LatHort Horticultural Plant Breeding Program, supported by the Ministry of Agriculture. This program's primary goal is to develop and select cultivars that are well-suited to the conditions in Latvia and Northern Europe.

Molecular markers are used in plant breeding to assess genetic diversity, DNA fingerprinting, genetic mapping, hybrid identification and marker-assisted selection [15], thus speeding up the traditional breeding process. There have been studies on *Chaenomeles* using RAPD [3,16–18] and AFLP [19,20] markers. Based on RAPDs, Bartish and co-authors [16] concluded that *C. cathayensis* and *C. japonica* are the most distantly related, whereas *C. speciosa* might have arisen due to hybridization between the two other species. Further studies have proven interspecies hybridization within the *Chaenomeles* genus [17] and analyzed the relationship between the known *Chaenomeles* species, further establishing *C. japonica* and *C. cathayensis* as distant relatives [3,21]. Whereas RAPD markers are used to determine relationships between *Chaenomeles* species, AFLP markers are utilized to characterize different cultivars, mainly those cultivated in East Asia [18–20]. However, these studies are rather incomplete, often relying on plant material from just one breeding activity.

Given the limited or even non-existent information on the *Chaenomeles* genome, previous research on *Chaenomeles* focused on utilizing RAPD and AFLP markers. However, in recent years, SSR markers have become a more favored choice in experiments due to their high reproducibility and polymorphism, as well as the relative ease of use compared to the other two marker types [22]. To date, one study has been performed to develop SSR makers, resulting in 10 markers that can distinguish between different species of *Chaenome-*

les [23]. Since de novo marker assembly is costly and time-consuming, and the sequences obtained by RNA-seq represent only the coding part of the genome, while SSR markers are mostly found in the non-coding part, thus only a small part of the genome is represented, molecular marker transfer between related species has been used as an alternative approach; a study on the chloroplast genome of *Chaenomeles* species has revealed highly polymorphic regions, repeat sequences and SSRs that are potential targets for molecular markers [1]. Several researchers have reported successful *Malus* SSR marker transfer to *Pyrus* [24], *Cydonia* [25,26] and *Chaenomeles* [27,28]. The currently available information reveals some gaps in the knowledge of *Chaenomeles* cultivars; there have been no robust studies on the genetic diversity and relationships of *Chaenomeles* developed in the Baltics, as well as more information could be gained on the transferability of *Malus* SSR markers on *Chaenomeles*, as only a relatively small number have been successfully transferred. The SSR molecular markers developed for the genus *Malus* are a promising potential source of suitable markers for *Chaenomeles* species. The genetic relatedness between *Malus* and *Chaenomeles* is supported by studies comparing both pollen morphology [29] and sequences from the nuclear DNA ITS region [30], as well as chloroplast DNA [1,31]. In all of these studies, *Malus* (apple) is identified as the closest relative among well-characterized plant genera, and it offers a broad selection of SSR markers for potential use.

Thus, this study aimed to test the transfer of SSR markers developed for *Malus* to *Chaenomeles*, and to analyze the structure of *Chaenomeles* sp. germplasm: cultivars developed at the Institute of Horticulture and hybrids grown at the National Botanical Garden of Latvia, as well as seedlings of *C. cathayensis* to assess their genetic diversity and internal relationship to increase knowledge on local germplasm and promote its use in the further breeding process.

2. Materials and Methods

2.1. Plant Material and Isolation of Genomic DNA

This study is based on 56 *C. japonica* genotypes maintained in genetic resources and breeding collections at the Institute of Horticulture (LatHort), Dobele, Latvia (56°37′0″ N, 23°16′0″ E), representing different stages of breeding of *Chaenomeles* as a fruit crop, 10 genotypes of *C. cathayense* received as seeds from the University Botanical Garden of Strasbourg (http://jardin-botanique.unistra.fr/, accessed on 10 September 2024), germinated and grown for leaf sampling, and 8 genotypes of *Chaenomeles* species and interspecific hybrids (*C. speciosa*, *C.* × *superba*, *C.* × *californica*) collected as leaf samples at the National Botanic Garden (NBD), Salaspils, Latvia (56°51′48″ N, 23°21′4″ E) (Table 1). Total DNA from young leaves of 74 *Chaenomeles* genotypes was isolated using the Genomic DNA Purification Kit (Thermo Scientific™, Vilnius, Lithuania) according to the manufacturer's methodology. Quantification and quality evaluation by NanoDrop™ 1000 Spectrophotometer (ThermoFisher Scientific™, Waltham, MA, USA) was performed, followed by the standardization of DNA sample concentrations.

Table 1. Description of *Chaenomeles* sp. plant material utilized for the transfer of SSR molecular markers from the related genera.

Sample No.	Sample Name	Species	Source
cd 087	Abava	*C. japonica*	Cultivar bred by A. Tīcs in Pūre, Latvia (started in 1951).
cd 067	Ada	*C. japonica*	
cd 084	Agra	*C. japonica*	
cd 068	Alfa	*C. japonica*	
cd 085	Anta	*C. japonica*	
cd 092	Arta	*C. japonica*	
cd 086	Tīca 45	*C. japonica*	

Table 1. *Cont.*

Sample No.	Sample Name	Species	Source
cd 072	4-6	*C. japonica*	Plant material bred as open-pollinated hybrids by S. Ruisa at the LatHort in the 1980s, using plant material sourced from the best seedlings gathered from Latvian commercial orchards, as well as plant material developed by breeder P. Sukatnieks. Cultivar 'Rasa' is registered in Latvia.
cd 070	4-22	*C. japonica*	
cd 071	7-25	*C. japonica*	
cd 078	8-139	*C. japonica*	
cd 073	9-11	*C. japonica*	
cd 075	9-44	*C. japonica*	
cd 079	10-17	*C. japonica*	
cd 081	11-45	*C. japonica*	
cd 074	17-20	*C. japonica*	
cd 076	19-94	*C. japonica*	
cd 077	19-4	*C. japonica*	
cd 080	19-94	*C. japonica*	
cd 093	Dobeles 2-29	*C. japonica*	
cd 100	Rasa (Clone 1)	*C. japonica*	
cd 103	Rasa (Clone 2)	*C. japonica*	
cd 096	Rasa (Clone 3)	*C. japonica*	
cd 088	Rasa (Clone 4)	*C. japonica*	
cd 115	C9	*C. japonica*	Plant material bred during common Latvian–Swedish–Lithuanian breeding program (1992–2002). Cultivars 'Darius' and 'Rondo' are registered in Latvia.
cd 083	C10	*C. japonica*	
cd 066	C12	*C. japonica*	
cd 082	C13	*C. japonica*	
cd 062	C16	*C. japonica*	
cd 063	C19	*C. japonica*	
cd 064	C20	*C. japonica*	
cd 069	C26	*C. japonica*	
cd 065	C27	*C. japonica*	
cd 099	Darius (Clone 1)	*C. japonica*	
cd 102	Darius (Clone 2)	*C. japonica*	
cd 097	Darius (Clone 3)	*C. japonica*	
cd 098	Rondo (Clone 1)	*C. japonica*	
cd 101	Rondo (Clone 2)	*C. japonica*	
cd 095	Rondo (Clone 3)	*C. japonica*	
cd 114	SR1-1	*C. japonica*	Plant material was bred as open-pollinated hybrids by S. Ruisa at the LatHort and selected in 2017–2023, using plant material sourced from the best seedlings gathered from Latvian commercial orchards, as well as plant material developed during common Latvian–Swedish–Lithuanian breeding program.
cd 108	SR1-1a	*C. japonica*	
cd 107	SR1-2	*C. japonica*	
cd 104	SR1-3	*C. japonica*	
cd 112	SR1-4	*C. japonica*	
cd 105	SR1-4a	*C. japonica*	
cd 110	SR1-5	*C. japonica*	
cd 109	SR1-5a	*C. japonica*	
cd 111	SR1-6	*C. japonica*	
cd 106	SR2-0	*C. japonica*	
cd 113	SR2-9	*C. japonica*	
cd 116	Brūvelis	*C. japonica*	Unknown origin genotypes from A. Brūvelis farm.
cd 117	Brūvelis B	*C. japonica*	
cd 089	R.Indrāna 1	*C. japonica*	Unknown origin genotypes from R. Indrāns farm.
cd 090	R.Indrāna 2	*C. japonica*	
cd 094	R.Indrāna 3	*C. japonica*	
cd 091	R.Indrāna 4	*C. japonica*	
cd 119	Brilliant	*C. speciosa*	Collected as a leaf sample from the National Botanic Garden (NBD), Latvia (https://nbd.gov.lv/en/, accessed on 10 September 2024).
cd 120	Scarlet	*C. speciosa*	
cd 122	C. californica	*C.* × *californica*	
cd 118	Chaenomeles superba	*C.* × *superba*	
cd 121	Pink Trail	*C.* × *superba*	
cd 123	Vermillion	*C.* × *superba*	
cd 124	Crimson and Gold	*C.* × *superba*	
cd 125	Stanford Red	*C.* × *superba*	

Table 1. *Cont.*

Sample No.	Sample Name	Species	Source
cd126	Sample #1	*C. cathayensis*	Received as a seed sample from the University Botanical Garden of Strasbourg, France (http://jardin-botanique.unistra.fr/, accessed on 10 September 2024).
cd127	Sample #2	*C. cathayensis*	
cd128	Sample #3	*C. cathayensis*	
cd129	Sample #4	*C. cathayensis*	
cd130	Sample #5	*C. cathayensis*	
cd131	Sample #6	*C. cathayensis*	
cd132	Sample #7	*C. cathayensis*	
cd133	Sample #8	*C. cathayensis*	
cd134	Sample #9	*C. cathayensis*	
cd135	Sample #10	*C. cathayensis*	

2.2. SSR Genotyping

A genomic DNA sample of 50 ng was used for PCR amplification in a 25 µL reaction containing 1× PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 pmol of dye marked forward and reverse primer, and 0.6 U Taq DNA polymerase (Thermo Scientific™, Lithuania). The SSR markers (Table S1) selected for application in *Chaenomeles* were adapted according to the published protocols [32–34], and further adaptation of the annealing temperature and PCR protocol according to the researched plant material and reagent kit. The final PCR conditions for the experimentally selected 25 SSR marker amplification were as follows: denaturation for 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 54 to 58 °C (annealing temperatures of each SSR marker are compiled in Table 2), 1 min at 72 °C and a final extension step at 72 °C for 10 min. PCR reactions were run in the Mastercycler epgradient thermal cycler (Eppendorf, Hamburg, Germany). The PCR amplification product presence and quality were checked on a 1% agarose gel in 1× TAE buffer and visualized ethidium bromide dye. The same PCR products were then analyzed on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA) and genotyped using GeneMapper® Software v4.0 (Applied Biosystems, Waltham, MA, USA).

Table 2. Characterization of the selected SSR markers amplified in the 74 tested *Chaenomeles* genotypes.

Locus	Annealing Temperature, °C	Locus Characteristics *						
		Na	Ne	I	Ho	He	F	Allele Range, bp
CH01a09	54	9	3.977	1.712	0.500	0.749	0.332	183–223
CH01d03	54	7	1.585	0.754	0.122	0.369	0.670	108–140
CH03d01	54	7	3.514	1.485	0.541	0.715	0.244	119–167
CH05e03	54	13	6.058	2.022	0.676	0.835	0.191	161–202
CH-Vf1	54	16	7.275	2.254	0.726	0.863	0.158	170–218
CH01d09	54	12	3.688	1.733	0.583	0.729	0.200	142–168
CH03d10	56	7	2.978	1.420	0.581	0.664	0.125	220–234
CH01f03b	56	11	4.348	1.702	0.676	0.770	0.122	113–136
CH04f06	56	11	3.873	1.702	0.635	0.742	0.144	107–158
CH01F02	54	11	4.751	1.847	0.563	0.790	0.286	183–205
CH01h02	56	6	3.289	1.368	0.181	0.696	0.741	88–136
CH01g05	58	11	6.088	2.013	0.784	0.836	0.062	84–106
CH02c02b	54	11	3.885	1.649	0.554	0.743	0.254	96–108
CH02g01	54	10	3.841	1.612	0.592	0.740	0.200	161–192
CH02a03	54	4	2.129	0.915	0.473	0.530	0.108	123–151
CH03b10	54	6	1.899	1.012	0.486	0.473	−0.028	168–196
CH03b06	54	12	5.012	1.868	0.703	0.800	0.122	152–172
CH03g06	54	13	5.394	2.010	0.338	0.815	0.585	106–118
CH04g07	54	10	4.817	1.807	0.250	0.792	0.684	91–153
CH05h05	54	6	3.347	1.463	0.662	0.701	0.056	92–162
CH05c06	54	11	6.301	1.998	0.592	0.841	0.297	135–174
CH05d03	54	7	3.258	1.380	0.437	0.693	0.370	156–192

Table 2. *Cont.*

Locus	Annealing Temperature, °C	Locus Characteristics *						
		Na	Ne	I	Ho	He	F	Allele Range, bp
CH05a02_a	54	5	3.877	1.442	0.699	0.742	0.059	173–229
CH05g11	54	11	2.738	1.292	0.973	0.635	−0.533	137–161
CH05g07	54	16	7.274	2.209	0.739	0.863	0.143	125–157
Average:		9.72	4.208	1.627	0.563	0.725	0.224	-

* Na—number of alleles, Ne—number of effective alleles, I—information index, Ho—observed heterozygosity, He—expected heterozygosity, F—fixation index.

2.3. Statistical Analysis

SSR amplification fragments were represented in bp and the parameters characterizing the markers were calculated by the software GenAlEx 6.5 [35]. Principal coordinate analysis (PCoA) based on genetic distance was applied to identify the genetic structure of *Chaenomeles* germplasms and evaluate relationships among genotypes. The significance of the mutual similarity among the identified *Chaenomeles* genotype groups was evaluated and characterized using an analysis of molecular variance (AMOVA).

The model-based clustering method of STRUCTURE 2.3.3 [36,37] was applied to discover the possible genetic structure of the 74 *Chaenomeles* samples and define the most likely number of clusters (*K* value). Twenty independent runs of STRUCTURE were performed for each *K* value: (i) from 1 to 10, (ii) from 2 to 11, and (iii) from 3 to 12. Each run consisted of a burn-in period of 100,000 steps, followed by 100,000 Monte Carlo Markov chain replicates, assuming an admixture model and independent allele frequencies. No prior information was used for cluster definition. The most likely K was chosen to compare the average estimates of the likelihood of the data, ln(Pr(X | K)), for each value of *K* (Pritchard et al., 2000), as well as calculating the ad hoc statistics, ΔK, based on the rate of change in the ln-probability of the data between successive *K* values [38]. The proportion of membership (*q*) of each individual in each gene pool was estimated using Structure Harvester Web v0.6.94. The cluster matching and permutation were calculated by the software CLUMPP 1.1 [39] and visualized by Structure Plot V2.0 [40].

The AmaCAID R script [41] was employed to determine the minimal number of SSR molecular markers necessary for *Chaenomeles* germplasm discrimination.

3. Results

3.1. Transfer and Application of Malus-Developed SSR Markers in Chaenomeles

To test cross-species transfer to *Chaenomeles,* an initial set of 95 SSR markers from *Malus*, was selected (Table S1). Out of them, 67 had a successful amplification at least in some of the tested *Chaenomeles* samples. A further selection of markers was made by choosing markers that provided stable amplification in all samples, shortening the list of markers to 39. The selection results are demonstrated in the Supplementary Materials, Figures S1–S3. Out of the remaining markers, 14 were excluded for the following reasons: monomorphic (CH01f09, CH02d12, CH03a09), amplified more than one locus (CH01f12, CH03g07), monomorphic or not amplified in at least two sample groups (CH02b11, CH03d02, CH01f03, CH03g12, CH04d02, CH04e02, CH05g01, CH05g03, CH05d04). The complete list of all SSR markers used in the study is compiled in Table S1, and the final set of markers is summarized in Table 2.

Among the 25 selected SSR markers, the average number of alleles was 9.72, with the minimum being 4 (CH02a03) and the maximum being 16: Na—number of alleles, Ne—number of effective alleles, I—information index, Ho—observed heterozygosity, He—expected heterozygosity, F—fixation index (CH-Vf1 and CH05g07). The average information index was 1.627, ranging from 0.754 (CH01d03) to 2.254 (CH-Vf1). The observed heterozygosity (Ho) had an average of 0.563, varying from 0.369 (CH01d03) to 0.973 (CH05g11). For all markers, the expected heterozygosity (He) was higher than the observed

heterozygosity (Ho), except for markers CH03b10 and CH05g11, where Ho was higher than He. The average marker fixation index (F) was 0.224, with values ranging from −0.533 (CH05g11) to 0.741 (CH01h02).

Principal coordinate analysis (PCoA) was conducted on the genotyping data to evaluate the effectiveness of the amplified SSR markers in characterizing *Chaenomeles* germplasm. The analysis divided the *Chaenomeles* genotypes into three distinct groups (Figure 1). Group 1 consisted of *C. japonica* genotypes. Group 2, the closest to Group 1, included genotypes of *Chaenomeles* species like *C. speciosa*, *C.* × *californica* and *C.* × *superba*, whereas Group 3 was composed solely of *C. cathayensis* and was the most distant from the other groups. There was no overlap between these three groups. The 25 selected SSR markers offered a high resolution, distinguishing nearly all 74 samples with unique genotypes, except for two *C. japonica* genotypes, 'Darius' and R. Indrāna 3, which were genetically identical. Additionally, all *C. cathayensis* genotypes were divided into two groups of identical genotypes: one including Samples #1 to #4, and the other Samples #5 to #10.

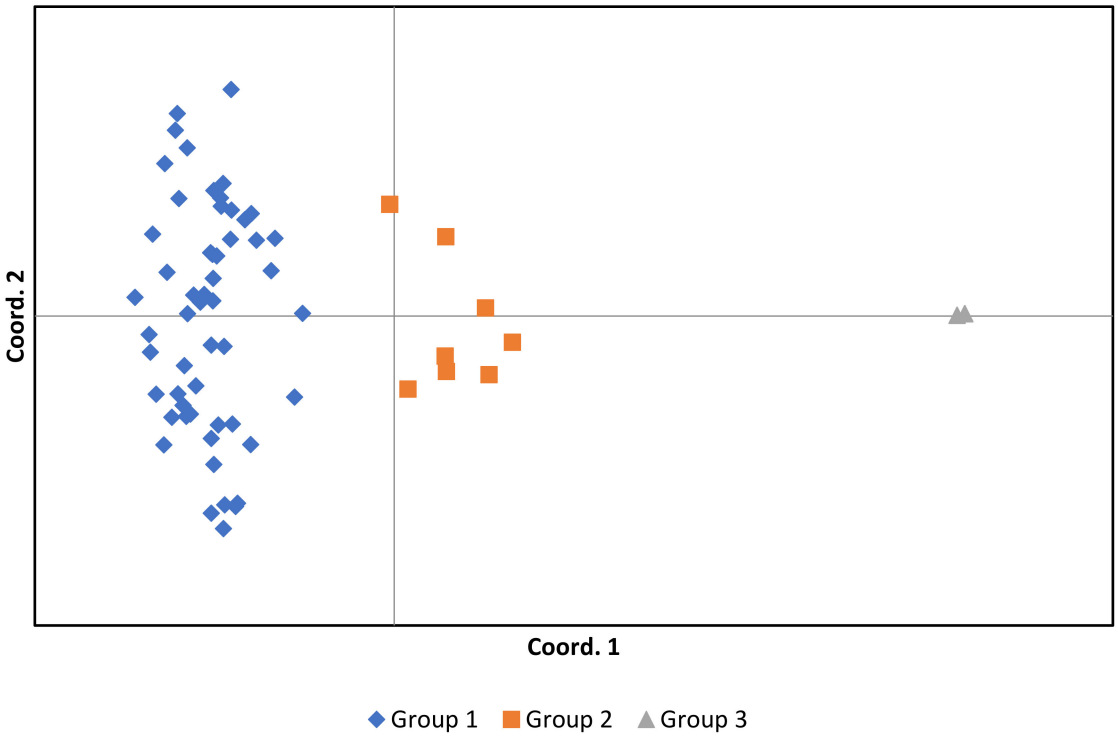
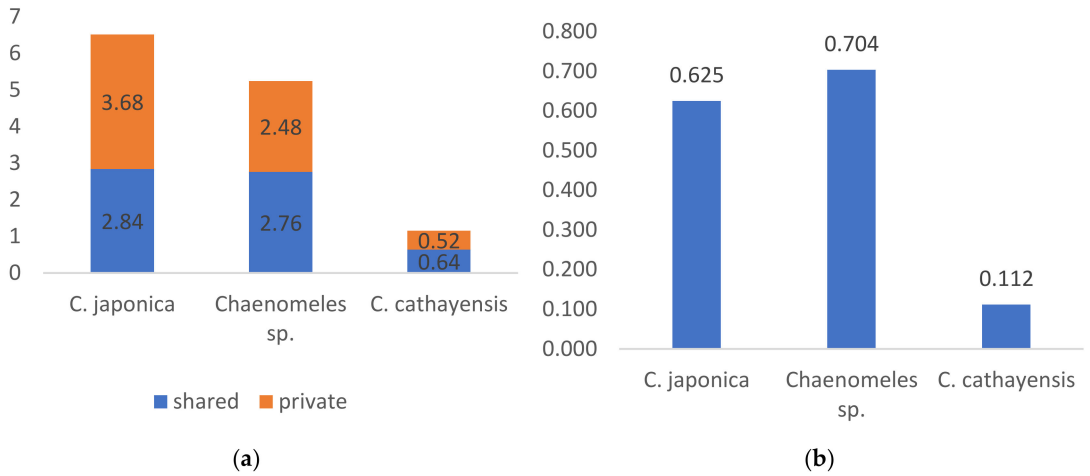


Figure 1. Principal coordinate analysis (PCoA) of 74 *Chaenomeles* genotypes based on 25 SSR markers. Group 1—*C. japonica*, Group 2—*Chaenomeles* spp., Group 3—*C. cathayensis*.

Overall, Group 1 had the highest mean values for both the total number of alleles and unique alleles (total number of alleles 6.52, 5.24, 1.16, unique alleles 3.68, 2.48, 0.52 for *C. japonica*, *Chaenomeles* spp. and *C. cathayensis*, respectively, Figure 1). In contrast, Group 2 exhibited the highest mean number of effective alleles (effective alleles 3.264, 3.801, 1.118 for Groups 1, 2 and 3, respectively) and higher values for genetic diversity parameters such as the observed heterozygosity and information index (Figure 2). Thirteen of the tested SSR markers (CH01a09, CH01d03, CH01d09, CH01h02, CH02c02b, CH02g01, CH03b10, CH03d01, CH03g06, CH04f06, CH05a02_a, CH05d03, CH05e03) exhibited a higher observed heterozygosity in *Chaenomeles* species (Group 2). Conversely, for the remaining markers, this indicator was higher for Group 1. The information index was also higher in Group 2 for markers CH01a09, CH01d03, CH01f03b, CH02c02b, CH02g01, CH03g06, CH04f06, CH05d03, CH05e03, CH05g07, CH05g11 and CH-Vf1. For the remaining twelve markers, it was higher in Group 1. All diversity parameter values were the lowest in Group 3 (*C. cathayensis*). According to the analysis of molecular variance (AMOVA), 78% of the

variance was attributed to differences among individuals, while 9% was due to differences among populations.

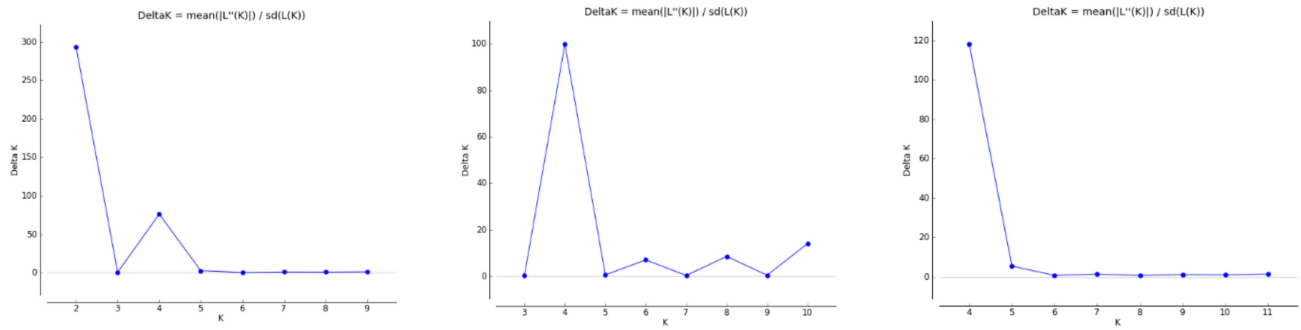


(**a**) (**b**)

Figure 2. Characterization of the *Chaenomeles* germplasm group discrimination. (**a**) Characteristics of alleles identified in *Chaenomeles* germplasm groups; (**b**) heterozygosity of the *Chaenomeles* germplasm groups.

3.2. Determining the Genetic Structure of Chaenomeles Germplasm

The Structure model approach [37] was used to analyze the *Chaenomeles* plant material internal structure. The ΔK method was employed to calculate the most likely number of clusters. To determine the optimal germplasm structure, three modeling variants were tested: K = 1 to 10, K = 2 to 11, and K = 3 to 12 (Figures 3 and 4). When analyzing K = 1 to 10, two groups of samples were identified: the samples of *C. japonica* and all other *Chaenomeles* samples. There was a slight overlap between other *Chaenomeles* species and *C. japonica*, but no overlap with *C. cathayensis*, which aligns with other studies on interspecies relationships. When analyzing K = 2 to 11, four groups were identified: *C. cathayensis*, other *Chaenomeles* species, *C. japonica* Group 1 and *C. japonica* Group 2. This grouping is best supported by the PCoA. For K = 3 to 12, the same four groups were identified, but *C. japonica* Group 1 and Group 2 appeared in a different order, with consistency within the groups. All structures confirm the markers' ability to distinguish between species and indicate the genetic differences among them, proving that all *Chaenomeles* cultivated as fruit plants are *C. japonica* without admixture from other species.



(**a**) (**b**) (**c**)

Figure 3. ΔK plot for (**a**) K = 2 (K 1 to 10), (**b**) K = 4 (K 2 to 11) and (**c**) K = 4 (K 3 to 12). calculated based on the 25 SSR marker data of the 74 *Chaenomeles* genotypes.

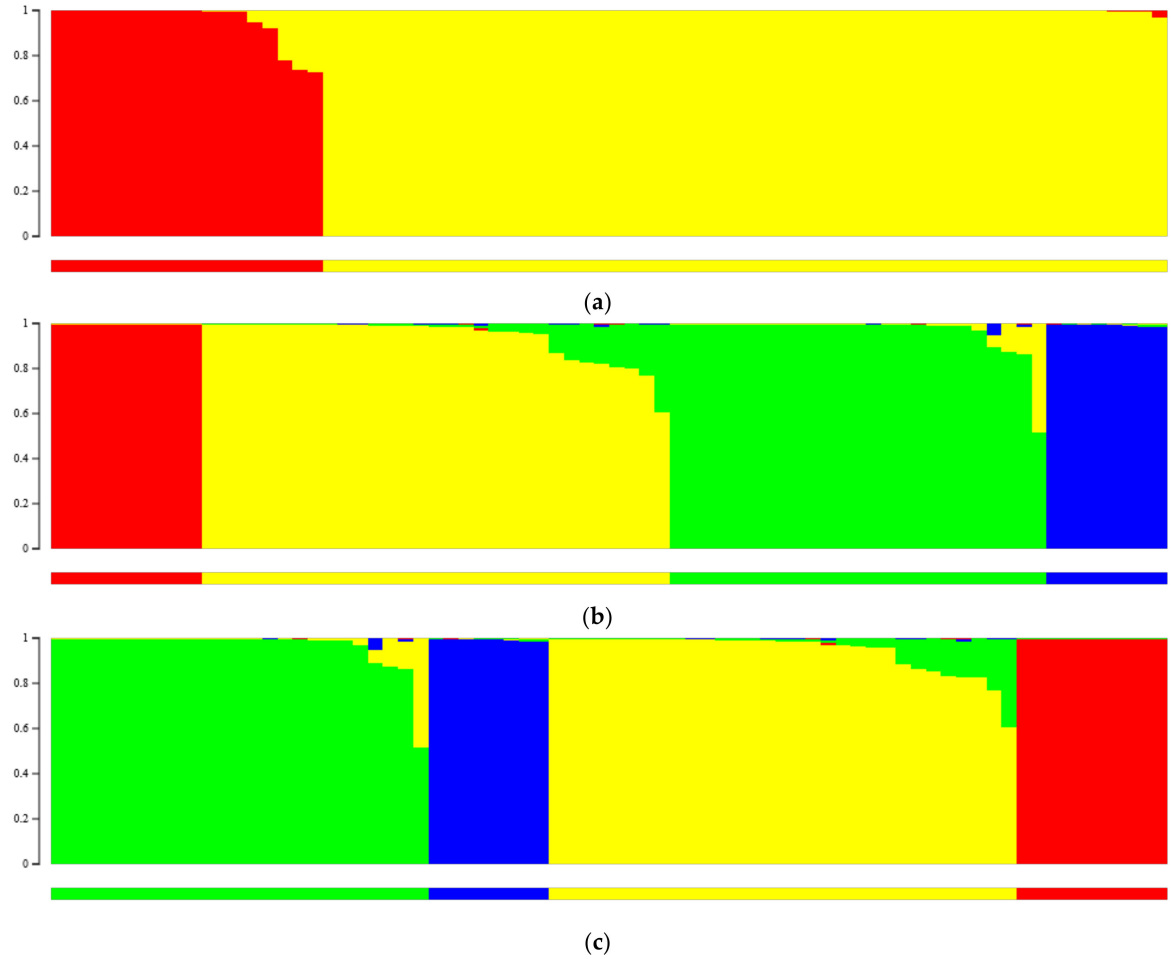


Figure 4. Structure analysis with 74 *Chaenomeles* genotypes, using 25 SSR markers, inferred at (**a**) K = 2 (K 1 to 10), (**b**) K = 4 (K 2 to 11) and (**c**) K = 4 (K 3 to 12). Each genotype is represented by a vertical line, with colors matching the K number.

The most relevant for a given germplasm was recognized as K = 4 (Figure 3b), with each cluster having 10, 31, 25 and 8 genotypes, respectively. This differed from the initial subjective grouping of the genotypes (Figure 1), which had three groups; the structure-based model split the *C. japonica* group into two sub-groups. The new groups were as follows: Group 1 (*Chaenomeles* spp.), Group 2 (*C. japonica*), Group 3 (*C. japonica*) and Group 4 (*C. cathayensis*). The sole distinction was the split of the *C. japonica* group into two segments: 25 and 31 genotypes, respectively. This division shows relatedness to the origin of the plant material. The AMOVA analysis revealed a total variance of 31% among all four sample groups. Group 2 of *C. japonica* exhibited a higher average number of alleles compared to Group 1, with values of 5.92 and 4.48, respectively, and a greater number of effective alleles, with 3.278 in Group 2 versus 2.472 in Group 1. Additionally, Group 2 showed higher observed heterozygosity, with values of 0.661 compared to 0.604 in Group 1. The primary distinctions between these groups of *C. japonica* are evident in the allele composition. These differences are observed in the length (bp) range of amplification fragments and the unique fragments specific to each group (Figure 2).

Figure 4 shows the proportions of membership (q) of each *Chaenomeles* genotype. The admixture was very low, with 85% of genotypes having a membership value above 95%. The lowest level of admixture was in the *C. cathayensis* group (Group 4): 0.3%. Similarly, Group 1 (*Chaenomeles* spp.) also had a low level of admixture: no higher than 1.8%.

Some overlap was observed between Groups 2 and 3, including *C. japonica* genotypes. The admixture rate in group 2 ranged from 12.9% (7–25) to 38.6% (C27), with genotypes

primarily having admixture from group 3. Overall, eight genotypes (9–44, 7–25, 'Abava', 'Ada', Brūvelis B, C10, C27, 'Rasa' (cuttings)) had an admixture of over 10%. In contrast, Group 3 had only three genotypes with a significant admixture from Group 2—C26 (11.8%), 'Darius' (old) (12.5%) and SR1-3 (48.1%). As per Structure analysis, Groups 1 (*Chaenomeles* spp.) and 4 (*C. cathayensis*) had the same members and genetic parameter values as in the initial grouping; thus, only the data of the *C. japonica* group were re-evaluated, which is presented in Table S4.

3.3. Selection of the Lowest Number of Primers Needed for the Discrimination of All Chaenomeles Genotypes

The AmaCAID R script was employed to determine that the germplasm used in this study consisted of 64 unique haplotypes, and the lowest number of markers to discriminate between all haplotypes was six: CH01a09, CH03d01, CH05e03, CH01d09, CH03b10, CH04g07. The markers were then used to assess the genetic diversity markers of the germplasm based on the four groups devised by Structure analysis (Figure 4). Overall, using 6 primers provided nearly the same resolution as using 25 primers, as it was still unable to distinguish between the two *C. japonica* genotypes, Darius and R. Indrāna 3. Additionally, genotypes No. 19–94 and No. 17–20 also become indistinguishable. Similar to the results with 25 markers, the 6 markers grouped all *C. cathayensis* into the same two genotype groups: Group 1 included *C. cathayensis* samples #1, #2, #3 and #4; while Group 2 comprised *C. cathayensis* samples #5 through #10. The use of the minimal six-primer set maintained the relationship structure among *Chaenomeles* species, similar to Figure 1, including *C. japonica* genotypes and other *Chaenomeles* species (*C. speciose*, *C.* × *superba* and *C.* × *californica*), and *C. cathayensis*. However, there are minor shifts in the relative positioning of the *C. japonica* groups. The PCoA based on six SSR marker data shows four groups; however, the overlap between the two *C. japonica* groups is more pronounced, and the *Chaenomeles* spp. group is closer to the *C. japonica* group (Figure 5).

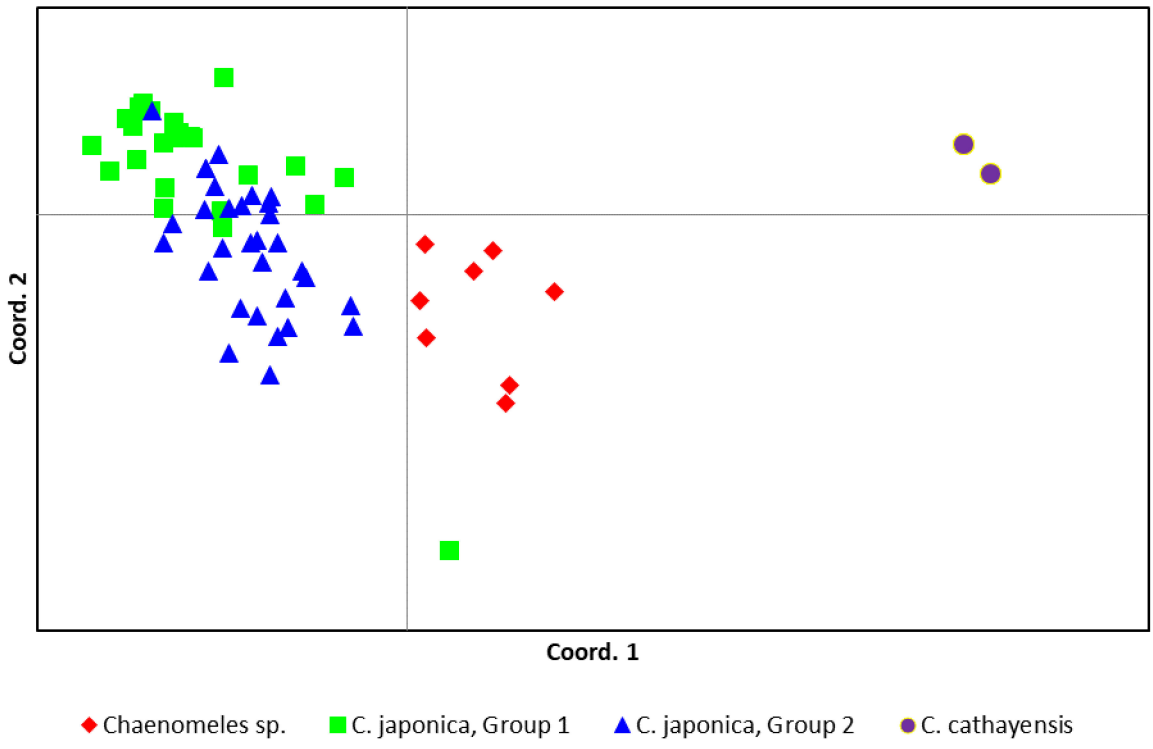


Figure 5. Principal coordinate analysis (PCoA) of 74 *Chaenomeles* genotypes based on six SSR markers grouped according to Structure-discovered genetic structure: 1—*Chaenomeles* spp., 2—*C. japonica*, 3—*C. japonica*, 4—*C. cathayensis*.

4. Discussion

A common feature of new plant crops, recently introduced or used as a food plant, has limited genomic information, which in turn limits the use of modern molecular methods in the study of genetic resources, breeding and the study of the heredity of traits. Japanese quince is one example of such a crop. The first activities for the introduction of the species of this genus as a fruit crop started only at the beginning of the 20th century [42], but targeted breeding attempts started only in the 1950s in Latvia [4,43], later continuing in Lithuania and Sweden. Researchers from these countries have provided most of the genetic research for this crop, including the use of DNA molecular markers [3,17]. In later years, similar studies were also carried out elsewhere, mainly for species systematics and chloroplast genome sequencing [1], but crop genome information is still insufficient, and the application of molecular markers in breeding and plant material studies are sporadic. Therefore, the set of SSR markers selected in this study is a significant contribution to the study of *Chaenomeles* and also allows for the application of the accumulated knowledge of systematically related crops.

Chaenomeles, similar to *Malus*, has a basic chromosome number of 17 and is diploid with 2n = 34 [44] and according to genetic studies, is more closely related to the *Malus* and *Pyrus* than to other Maloideae genera [30]. Therefore, the initial set of SSR markers created in *Malus* and mapped in *Malus*/*Pyrus* was selected for the study. Examining the linkage groups (LGs) covered by our 25 primers demonstrated that our set offers superior genome coverage compared to others. Therefore, selecting specific markers was crucial, as existing sets are insufficient. This comprehensive coverage is particularly important for analyzing breeding material and closely distinguishing related genotypes. Given the relatively low transferability of SSR markers from *Malus* to *Chaenomeles* observed in previous studies [27], a larger number of markers representing all linkage groups was selected for this study. Out of the 95 tested SSR markers developed in *Malus* and selected for this study, 25 markers, or 26% of all tested markers, provided stable and polymorphic amplification in *Chaenomeles* (Table S2), while another study on marker transferability from *Malus* to *Chaenomeles* resulted in a 58% transferability rate, which is also relatively small [27]. Despite the percentage of successful transfers in the current study being lower, it is important to note that Vanwynsberghe and co-authors (2009) looked at a smaller pool of markers (only 31) and a limited number of plant samples (8 genotypes). However, several markers that were discarded or successfully transferred from *Malus* to *Chaenomeles* were the same in both studies; for example, both studies concluded that markers CH01f02, CH01f12 and CH01h10 can be successfully transferred, and the current study also revealed 22 more markers that can be used for the assessment of genetic diversity in *Chaenomeles*, as well as identified markers that were not transferable, thus creating a framework for future studies to increase the pool of SSR markers developed for other *Maloideae* species which could be transferred to *Chaenomeles*. The study data support the argument that SSR markers first developed in *Malus* can be successfully transferred to *Chaenomeles* and utilized for genetic structure and diversity studies (Vanwynsberghe et al., 2009).

Out of all the tested *Chaenomeles* samples, representatives of species *C. cathayensis* had the highest number of loci (15 loci), of which the allele range matched up with the allele range originally described in *Malus* by Liebhard and co-authors [33] (Table S2), with *C. japonica* having the second highest number of loci (13 loci) (Tables S3 and S4). In many cases, the allele range exceeded both the lower and upper limits of the marker's original range. The dissimilarities of allele length between the study and literature call into question the reliability of the markers, as well as the biological properties of the studied plant material. However, their overall performance is further demonstrated to be sufficient in assessing the genetic diversity of the chosen germplasm.

The 25 SSR markers successfully discriminated all the *Chaenomeles* species used in the study (Figure 1) and obtained unique genotypes for all samples; the set allows us to identify the samples and validate previous knowledge on the relationship between *Chaenomeles* species [3,17,21]. *C. cathayensis* was the most genetically distant of the three

groups, whereas the *Chaenomeles* spp. group, which included *C. speciosa* and interspecific hybrids *C.* × *superba* (*C. speciosa* × *C. japonica*) and *C.* × *californica* (*C.* × *superba* × *C. cathayensis*) were closer to *C. japonica* genetically. The differences between the groups were also validated by Structure analysis, as the *Chaenomeles* spp. and *C. cathayensis* groups had very low admixture, no more than 2%. However, the genetic structure analysis also revealed that the *C. japonica* group consisted of two sub-groups with some overlap (Figure 4), and admixture ranged from over 10%, even up to almost 50% between the sub-groups, which calls for further analysis of the genetic makeup and origin of the *C. japonica* genotypes used in the study. This is also confirmed to some extent by the plant material origin: the *C. japonica* samples of both groups come from joint breeding activities, albeit at different stages of their development. The small admixture of *Chaenomeles* samples indicates relatively pure *C. japonica* material in the existing varieties and breeding material, which should still be verified by genome-wide sequencing. Another important thing to note is that *C. cathayensis* scored very low in all genetic diversity parameters, which leads to a belief that the seeds were gathered from the same plant or even the same fruit. Thus, the seedlings were genetically homogenous; however, the group also had the lowest admixture as per Structure analysis, indicating that the group was the "purest" and practically had no genetic overlap with the other *Chaenomeles* species. Overall, the genetic diversity parameters (Na, Ne, I, Ho) of the *Chaenomeles* germplasm could be considered higher than average (Table S3); however, studies on *Malus* with the same markers as used in the present study produced higher heterozygosity values [45–48], as well as a higher polymorphic information content score [49]. The discrepancy between the genetic diversity scores of *Malus* cultivars and the *C. japonica* and *Chaenomeles* spp. groups could be explained by the studies on apples having a much larger number of accessions analyzed, ranging from 273 [45] to 484 [48]; however, the sample size may not always indicate higher genetic diversity. In the present study, the *C. japonica* groups were the biggest, with 31 and 25 genotypes when split into two sub-groups. Nevertheless, the *Chaeonomeles* spp. group had the highest values in all genetic diversity parameters, despite consisting of 8 genotypes, whereas the *C. cathayensis* group had 10 genotypes but scored very low due to the group's homogeneity (Table S3). Thus, it could be argued that genetic diversity is affected by the individuals' uniqueness; the analysis of molecular variance validated this. According to the four Structure groups, the analysis of molecular variance indicated that most of the variance was explained by molecular variance within individuals. This means that most of the differences in genetic diversity were due to the variance of the loci of each genotype; thus, the overall diversity of the materials is dictated by the nature of the individuals within the group, not by the group itself. In the present study, the groups themselves are represented by the *Chaenomeles* species, which dominates in the genetic makeup; however, it does not necessarily describe the diversity of the individuals within the group, as was revealed with the *C. japonica* genotypes having some of the highest admixture percentages.

The number of private or unique alleles can also characterize genetic diversity. It is a trait that describes alleles that are unique to each of the groups in a set of genotypes and is not necessarily tied to the number of individuals in a group. Comparing a study performed on apples with a similar sample size to the groups in the present study [46] revealed that *Chaenomeles* had either a similar (*C. japonica*) or a higher (*Chaenomeles* spp.) number of private alleles. Similarly, in a study with a low sample size [50], all groups of apple genotypes had a lower number of private alleles than the *Chaenomeles* spp. group in the current study. This could be explained by the *Chaenomeles* spp. high taxonomic diversity of the group, which includes both intraspecific and interspecific hybrids. The utility in identifying private alleles in a certain group of genotypes lies in discovering new, unique traits that could be used in diversifying breeding material, which is particularly important in the case of *Chaenomeles*, as commercial breeding as a fruit crop has only been around since the 1950s [4], making it a relatively new crop compared to apples. Therefore, understanding the genetic structure of *Chaenomeles* genotypes and cultivars is important, for example, discovering the relatively high admixture of certain *C. japonica* genotypes

creates an incentive to introduce genotypes from the *C. cathayensis* group or *Chaenomeles* spp. group to diversify the breeding material; however, this would mean that further studies would be necessary to discover valuable traits that could be carried over from one species to another. As such, it is important to identify molecular markers that can efficiently screen any given group of samples.

To extract the minimum number of representative SSR markers, the AMaCAID script was applied and markers CH01a09, CH01d09, CH03b10, CH03d01, CH04g07 and CH05e03 were identified as the most relevant. An assessment of the *Chaenomeles* genotypes using 6 SSR markers produced comparable results to those using 25 SSR markers; however, several *C. cathayensis* and *C. japonica* genotypes could not be discriminated with only six SSR markers, and the overlap between *C. japonica* genotypes was more pronounced (Figure 5). The application of these six markers showed that, in the case of the *Chaenomeles* spp., *C. japonica* (Group 2), and *C. cathayensis* groups, all the genetic diversity parameter values were lower than when using 25 markers (Table S4). However, the mean values of the information index and expected heterozygosity, as well as the polymorphic information content and the number of private alleles were higher in Group 3 (*C. japonica*). Combining these two findings, a small number of SSR markers could be used for the initial germplasm screening, followed by increasing the number of markers, to ensure that the germplasm's genetic structure and genetic diversity are accurately depicted. Similar results have been obtained in other studies with SSR molecular markers [51].

Molecular markers have become essential tools in plant breeding, particularly for well-researched crops with extensive genomic data, where many marker systems exist for early trait identification and mapping. However, for minor and less-studied crops like Japanese quince, genomic information is extremely limited or non-existent, and marker systems for selecting desired traits are practically unavailable. Therefore, developing any molecular marker system is crucial. SSR markers, which bind to non-coding regions of the genome, are widely used to assess genetic diversity and population structure. For *Chaenomeles* breeding, this is especially important due to the heavy reliance on open-pollinated material in cultivar development, aiding in the selection of parent plants for further breeding stages. Genetic mapping, another key application of SSR markers, requires a substantial number of functional markers to achieve adequate map density. Reaching this goal would necessitate more comprehensive DNA sequence data of crops.

In the breeding of *Chaenomeles*, key traits have been identified for which introgression into new cultivars is essential, and for which future molecular marker development would be highly beneficial. Previous studies indicate that 80–90% of Japanese quince genotypes are self-incompatible, with yields heavily dependent on pollinator [52], which complicates large-scale cultivation. Understanding the degree of self-fertility and its genetic mechanisms has thus become a primary focus in the breeding program. Among the genotypes studied, only 'Rasa' and 'Jānis' showed partial self-fertility, while others, such as 'Ada' and 'Alfa' by A. Tīcs and hybrids SR1-1 and SR1-5, exhibited variable self-fertility across years [52,53], although the underlying mechanism remains unclear. For large-scale cultivation and mechanized management, an upright growth habit is crucial yet challenging to secure. 'Rondo' is a typical carrier of this trait, but it is less expressed in newer hybrids. Frost resistance in flower buds is another critical trait, as spring frosts increasingly impact yields, with genotype differences especially evident following harsh frosts, suggesting a genetic basis for this resilience. Limited information exists on the inheritance of these traits, and the lack of molecular markers for selection presents a significant challenge for advancing Japanese quince breeding.

5. Conclusions

- Overall, the study shows that the 25 adapted *Malus* SSR markers are suitable for the genetic characterization of *Chaenomeles* plant material, with the possibility of using a small set of 6 markers for initial screening and then increasing the number

of markers to achieve more precise data on the genetic structure and diversity of the chosen germplasm.

- The set of adapted SSR markers effectively distinguishes between *Chaenomeles* species and confirms the results previously obtained with other molecular markers that *C. japonica* is genetically closer to *C. speciosa* and interspecies crosses *C.* × *superba* and *C.* × *californica* than *C. cathayensis*. Compared to previously used ones, SSR markers provide high stability and repeatability and are suitable for large-scale genetic research of germplasm or breeding material and for assessing species composition.
- There are few studies on using SSR markers on *Chaenomeles* cultivars and species; thus, the study sets up the groundwork for further work in the field, which could include not only assessing the genetic diversity of *Chaenomeles*, but also screening for valuable traits for breeding. Given the limited level of genetic research on *Chaenomeles*, an additional set of markers will undoubtedly be valuable for the further development of this crop.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10111233/s1, Table S1: List of SSR markers selected for transfer to the application in *Chaenomeles* germplasm analysis; Table S2: Characterization of a set of 25 SSR markers based on groups of *Chaenomeles* samples identified through principal Coordinates analysis (PCoA); Table S3: Characterization of a minimal set of 25 SSR markers based on groups of *Chaenomeles* samples identified through Structure analysis; Table S4: Characterization of a minimal set of six SSR markers based on groups of *Chaenomeles* samples identified through Structure analysis. Figure S1. PCR amplification to test *Malus* marker transfer to randomly selected *C. japonica* cultivars from the Institute of Horticulture genetic resource collection. Figure S2. PCR amplification of *Chaenomeles* material from the National Botanic Garden of Latvia, using SSR markers from *Malus*. 1—*C. speciosa* 'Brilliant', 2—*C. speciosa* 'Scarlet', 3—*C.* × *californica*, 4—*C.* × *superba*, 5—*C.* × *superba* 'Pink Trail', 6—*C.* × *superba* 'Vermillion', 7—*C.* × *superba* 'Crimson and Gold', 8—*C.* × *superba* 'Stanford Red'. Figure S3. PCR amplification of *C. cathayensis* material received as a seed sample from the University Botanical Garden of Strasbourg, France, using SSR markers from *Malus*. 1—10: *C. cathayensis* genotypes #1 through #10.

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