Genetics and Biodiversity Journal

Journal homepage:http://ojs.univ-tlemcen.dz/index.php/GABJ

Original Research Paper

OPEN ACCESS

Using SSR markers from *Prunus* genus for wild cherry genotyping

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Article history: Received: September 9th 2018, Revised: December 25th 2018, Accepted: January 3th 2019

Abstract

Prunus genus include fruit and wooden species. Because its economic importance, both phenotypic and genetic markers have been developed and used in genetic studies. Among them, for their versatility, degree of transferability and repeatability, some microsatellite sets have been developed for several species from this genus. Although they have been successfully used inside the Rosaceae family, their transferability to other species and/or provenances different for what they were designed, must be first assessed. Thus, the higher the number of markers assessed, the higher their applicability for performing genetic studies. Hence, the functionality of 20 microsatellite markers designed for *P. persica* (L.) Batsch and *P.* avium
L. were tested for genotyping a wild cherry progeny for wood production. Scorable amplifications and reasonably high polymorphism were registered for 13 out of 20 SSR assessed, averaging 7.7 alleles/ locus. The observed heterozygosity ranges from 0.513 to 0.946; with allelic frequencies below 0.50, except for two loci. The individual discriminative power goes from 0.045 to 0.250; while the combined random probability of identity was as low as 1.8114×10^{-13}, allowing to identify and to differentiate unambiguously individuals in the sample formed by 36 trees. According these results, this set has showed its potential for genotyping plus trees from wild cherry for wood production.

Keywords: Microsatellite; Genotyping; Genetic identification; Timber production

Introduction

From the beginning of agriculture, markers have been used to identify and to differentiate varieties. A marker can be of phenotypic (morphological, phenological and molecular, other than DNA, characters) or from genetic (DNA) nature. While phenotypic characteristics are influenced by environment and genetic factors as intergenic interactions, genetic markers are stable, at least mutations have occurred (Staub et al., 1996). Once DNA-based markers are not determined environmentally (Struss et al., 2003), becoming them in a strong tool for genotyping, the study of flow of genes and populations and to assist the genetic improvement (Weising et al., 1995; Staub et al., 1996; Ruane and Sonnino, 2007; Kalia et al., 2011).

Different kind of DNA markers have been developed. From them, because their repeatability, which allow compare data among laboratories, their transferability, their relative low costs, their abundance, their variability, their Mendelian co-dominant way in which they are inherited and their ability to detect polymorphism, microsatellites have become in a powerful technique for genetic studies (Morgante and Olivieri, 1993; Jones et al., 1997; Glenn and Schable, 2005; Selkoe and Toonen, 2006).

As a consequence of the economic importance of Prunus genus, several SSR markers have been designed for various species. The first set of microsatellites for genus was developed for peach (Gannavarapu, 1998 cited by Downey and Iezzoni, 2000). During the next years, four new sets for peach were published (Cipriani et al., 1999; Sosinsky et al., 2000; Testolin et al., 2000; Dirlewanger et al., 2002). Downey and Iezzoni (2000) also reported the use of primers designed specifically for black cherry (P. serotina Ehrh.). The first SSR primers specifically designed for sweet cherry from genomic libraries of varieties 'Napoleon' and 'Valerj Tschkalov', respectively, were obtained by Clarke and Tobutt (2003) and Struss et al. (2003). A year later, Vaughan and Russell (2004) developed the first SSR markers from wild cherry.

Sweet cherry is mainly appreciated by its fruits; whereas the hardwood from its ancestor relative, the wild cherry, is comparable in quality and aesthetic characteristics to tropical woods even to other valuable woods from temperate-broadleaves species, as walnut (Ducci et al., 2013). Hardwood from wild cherry is characterized for its straight, fine grained, easy working timber with pinkish brown heartwood and paler sapwood is highly sought after for cabinet making, furniture, paneling, decorative joinery and turnery, becoming in one of the most important timber species from family Rosaceae (Russell, 2003).

Because of over exploitation of natural stands, production of timber of wild cherry has declined in Europe and it has been substituted by wood from black cherry (P. serotine) (Russell, 2003; Ducci et al., 2013). Hence, from last century, several programs have been initiated with the main goal to increase the productivity of wild cherry plantations for wood production and to couple to the demands of market (Kobliha, 2002; Russell, 2003; Nocetti et al., 2010). Stimulated by politics on the use and conservation of genetic resources, both public and private companies have showed interest into the noble hardwoods, including wild cherry. Based on unsuccessfully previous experiences, either local, national and programs have been promoting the establishment of highly productive exploitations, mainly, by mean of afforestation of agricultural lands and the use of selected and certified plantation materials (Russell, 2003; Ducci et al., 2013).

In this context, the use of solid markers, as microsatellites, to classify and to identify genetic resources and varieties, has become a crucial task. Although several sets of SSR markers have been developed for *P. avium* L., few researches have been published for populations and/or progenies exclusively used for timber production. Recently, De Rogatis et al. (2013) and Fernandez-Cruz et al. (2014) performed genetic studies with microsatellites for wild cherry populations and progenies, respectively. However, in both cases they used only some of primers designed by Clarke and Tobutt (2003) and Vaugham and Russell (2004).

Motivated for the necessity to evaluate a greater diversity of SSR markers for wild cherry selections, a wider range of primers were selected for genotyping a close related progeny. Thus, the purposes of this research were (1) to increase the number of microsatellites available for genetic studies in wild cherry; (2) to determine the transferability of microsatellites markers from *Prunus* genus to wild cherry trees with outstanding characteristics for wood production; and (3) to assess the possibility to use them for genotyping and for the genetic identification of individuals.

Material and methods

Plant material and selection of markers

A sample, formed by 36 wild cherry (P. avium L.) plus trees with outstanding characteristics for wood production, were used to assess the transferability of SSR markers designed for peach (P. persica (L.)

Batsch) and sweet cherry (P. avium L.). These trees were selected from the selection program of Bosques Naturales S. A. (Spain) on the base of their stem height and the diameter at breast height as well as the form of log and the response under intensive model of plantation. Green leaves, without visual damages, were collected on the spring of 2010 from each tree, individually packed and stored at -80°C until the DNA extraction.

Twenty (20) microsatellites markers were selected from literature, mainly on the base of their reported informative capacity (polymorphism) and discriminative power. Details of each pair of primers are showed in Table 1.

DNA extraction and PCR conditions

For DNA extractions, samples of 100mg of frozen leaves for each genotype were used, following the

guidelines of DNeasy Plant Mini kit (Qiagen). The quality of genomic DNA was assessed in agarose gel (0.8%, TBE buffer) and was quantified by UV spectrophotometry (Nanodrop ND-1000, NanoDrop Technologies).

PCR amplifications were performed in a final volume of 10µl, containing 1µl 10× reaction buffer (1× was 75mM Tris-HCl, pH 9, 50mM KCl, 2mM MgCl2 and 20mM (NH4)2SO4), 20ng genomic DNA, 0.5µM each primer, 200µM each dNTP, and 0.4 units Taq DNA polymerase (Biotools BandM Labs, Spain). The reaction cycles consisted of an initial step of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at the annealing temperature of each primer pair (Table 1) and 30 s at 72°C. Afterwards, an additional extension step was performed for 20 min at 72°C. Forward primers were labelled with fluorophores 6-FAM, PET, VIC, and NED (Applied Biosystems, USA) and PCR products were fractionated by capillary electrophoresis using an ABI 3730 Analyser (Applied Biosystems). Fragment sizes were assessed with the Peak Scanner 1.0 software (Applied Biosystems). To ensure consistent results, three independent amplifications per sample were performed.

Statistical analysis

Statistical estimation of the observed and expected heterozygosity, the number of alleles per locus, the allelic frequencies, the null allele frequencies and the unbiased or random probability of identity to differentiate two non-direct related trees were made using IDENTITY 1.0 software (Wagner and Sefc, 1999). A semi proper dissimilarity matrix was calculated from allelic data by simple matching using DARwin 6.0.17 software (Perrier and Jacquemoud-Collet, 2006). From this matrix, with the same software, a hierarchical tree was generated by the unweighted neighbour joining method.

Results

Choosing the suitable markers

Twenty SSR markers (Table 1) were selected from literature on the base of their polymorphism and discriminative power. Although these markers have been developed from and for several provenances and species into the Prunus genus, an initial screening to know their transferability and to optimize the conditions for PCR was performed. The objective was to determine which of them amplify correctly and would yield consistent and interpretable products. Before this step, the optimal quantity of DNA for PCR (20ng) and the most suitable temperature for each primer were determined and adjusted (Table 1).

Table 1

Table 1. Allelic range (AR) and number of observed alleles (A), annealing temperature (At), allelic frequencies (AF), observed heterozygosity (HObs), frequency of null alleles (NA) and random probability of identity (PID) registered for 36 wild cherry trees selected for wood production

Locus	AR (bp)*	A (bp)*	At (ºC)	AR (bp)	A (bp)	AF	HObs	NA	PID	Reference
UDP96-001	127-129	2	57	107-135	7	0.01-0.32	0.889	-0.092	0.121	Cipriani et al., (1999)
UDP97-402	139-151	2	60	113-143	9	0.02-0.42	0.709	0.007	0.119	Cipriani et al., (1999)
PCHGMS 1	194**	4	57	121-151	6	0.01-0.47	0.647	0.022	0.146	Sosinski et al., (2000)
UDP98-021	146-157	6	57	101-117	5	0.06-0.39	0.545	0.107	0.114	Testolin et al., (2000)
UDP98-410	146-182	8	54	123-135	6	0.01-0.51	0.889	-0.187	0.245	Testolin et al., (2000)
BPPCT 002	226-238	5	57	167-187	6	0.01-0.51	0.514	0.089	0.155	Dirlewanger et al., (2002)
BPPCT 005	157-199	6	57	140-198	10	0.01-0.30	0.861	-0.038	0.071	Dirlewanger et al., (2002)
BPPCT 026	134-145	3	54	163-185	5	0.01-0.39	0.778	-0.056	0.162	Dirlewanger et al., (2002)
BPPCT 037	146-156	5	57	133-170	10	0.01-0.26	0.942	-0.065	0.053	Dirlewanger et al., (2002)
BPPCT 038	127-143	7	57	98-133	10	0.01-0.22	0.882	-0.020	0.043	Dirlewanger et al., (2002)
BPPCT 039	148-158	2	57	130-147	4	0.01-0.44	0.606	0.030	0.187	Dirlewanger et al., (2002)
UCD-CH12	173-200	5	57	175-197	9	0.01-0.26	0.743	0.038	0.062	Struss et al., (2003)
UCD-CH18	178-188	4	54	182-211	10	0.01-0.27	0.828	-0.013	0.065	Struss et al., (2003)

*Combined PID5997 1.8114 × 10-13 ; * Data reported in the original paper; ** Predicted length*

DNA from 16 trees was used for the initial assessment. Those primers that did not amplified at all for none genotype (UDP96-015) or did it for few of them (UDP96-013) were rejected. Were also discriminated those primers that amplifying several bands per genotype (UDP98-407). For marker BPPCT034, also extra bands were observed, which could not be suppressed increasing the annealing temperature; however, it passed to next step once only a genotype showed this altered allelic profile.

For the last selection step, forward primers were labelled with fluorophores, favouring then the automation of genotyping and the increasing of resolution (number of alleles detected) of technique. However, it seems that the method used to detect the alleles also influenced the obtention of repeatable and interpretable results. Thus, when the products of amplification of markers UDP96-003, UDP98-407 and BPPCT040 were separate in agarose gels, clear bands were observed. Although the conditions for PCR were the same, after the forward primers were labelled, no peaks appeared for no one these loci, at least in the expected range to be considered as microsatellites. While, previous results observed for BPPCT034 were confirmed: several peaks appeared for all trees, hence it was rejected. The rest of primers (Table 2) offered solid and scorable products, although finally the marker UDP96-18 was also discarded because only one allele
(241) was found for all 16 trees.

Genotyping

A whole of 36 trees were randomly selected for genotyping. On average, 75.9% of loci were heterozygotes, ranging the observed heterozygosity from
0.513 (BPPCT002) to 0.946 (BPPCT037); however, the presence of negative null allele frequencies suggests an overestimation of it, especially low for locus UDP98-410 (-0.194). Although some alleles that could be considered as rare (frequencies below 0.01) were detected in 19% of loci, mostly of them were distributed with frequencies from 0.03 to 0.45. The exception were markers UDP98-
410 and BPPCT002, with frequencies above 0.5 for alleles 127 and 181, respectively.

All the screened loci showed a reasonably high polymorphism (Table 1), with up to 97 alleles detected, averaging 7.7 alleles/ locus. In general, a high polymorphism was registered regarding the original

papers, except for markers UDP98-021 and UDP98-410. Remarkable were the cases of loci BPPCT005, BPPCT037, BPPCT038 and UCD-CH18 with 10 alleles each; followed by markers UDP97-402 and UCD-CH12 with 9 alleles. These were also the loci with the higher differentiation capacity; while UDP98-410 was the less informative, likely influenced by the presence of alleles with frequencies above 0.50.

Table 2. Allelic profiles of the 36 wild cherry trees for the 13 SSR loci assessed

Gent UDP96-001	UDP97-402	PCHGMS1	UDP98-021	UDP98-410	BPPCT002	BPPCT005	BPPCT026	BPPCT037	BPPCT038	BPPCT039	UCD-CH12	UCD-CH18
KC 126/126	133/133	145/145	105/113	127/129	179/185	140/146	169/183	145/162	108/119	134/147	179/181	184/192
KF 109/109	143/143	145/145	101/103	123/127	181/185	155/159	163/183	139/152	98/108	140/147	181/187	182/190
KI 109/126	0/0	141/141	103/103	127/129	181/181	140/159	183/183	139/145	115/131	134/134	179/181	186/190
KJ 124/126	143/143	133/141	103/117	127/129	179/179	155/163	183/183	156/162	117/123	134/140	181/195	192/194
KK 124/126	123/139	145/151	103/103	125/127	181/181	161/163	163/177	154/170	98/123	130/134	181/197	182/192
KM 109/126	117/143	121/141	105/113	125/127	179/179	140/163	163/183	141/145	98/123	134/147	181/187	186/190
X1 109/126	121/133	145/145	103/103	127/129	179/185	153/155	163/169	139/162	119/121	140/140	175/195	201/211
X2 107/124	121/121	141/145	113/113	127/129	181/181	155/163	177/183	135/139	115/129	147/147	175/195	186/196
K11 109/124	121/121	141/141	105/117	127/127	168/181	146/163	163/169	139/152	108/133	134/147	181/181	184/192
K12 109/126	121/121	141/141	105/105	127/129	181/181	155/159	163/169	135/145	108/129	134/147	179/187	186/192
K14 118/135	121/137	121/121	101/101	127/127	181/181	146/146	163/163	133/133	108/108	134/147	189/191	188/194
K17 109/126	121/123	141/143	105/105	127/129	181/181	155/159	163/169	135/145	108/129	134/147	179/187	186/192
K20 124/126	121/123	133/145	103/113	127/135	181/185	155/163	163/163	139/170	121/123	134/147	175/181	182/192
K21 114/118	143/143	141/141	101/113	127/129	168/181	155/163	169/183	139/145	98/119	134/147	181/181	186/192
K23 114/124	143/143	141/145	101/113	127/129	168/181	155/163	169/183	139/145	98/119	134/147	175/181	186/192
K24 124/126	121/121	143/143	105/105	127/129	181/185	155/196	163/169	139/152	115/115	134/147	179/193	192/211
AK30 109/124	0/0	141/141	103/103	127/137	181/181	146/163	169/177	139/145	115/131	134/147	179/187	184/211
C15- 118/126	0/0	133/145	117/117	129/129	179/181	159/159	163/163	152/170	115/123	134/147	181/187	182/186
C1-12 124/124	121/123	141/145	105/113	127/129	168/179	163/163	163/169	139/141	98/98	134/147	175/175	182/182
C15- 109/124	113/125	141/141	101/103	127/127	183/183	155/198	163/163	135/145	98/115	134/147	175/189	182/188
C15-1 124/126	0/0	0/0	0/0	125/129	168/181	155/198	163/163	152/152	129/131	134/147	189/189	182/188
C15-4 126/126	0/0	133/143	103/113	123/127	168/187	155/183	163/185	135/152	108/115	134/147	175/189	182/188
G21 109/124	121/123	141/145	105/113	127/129	168/179	163/163	169/183	139/141	98/98	134/147	175/175	182/182
C9-8 109/126	121/123	0/0	0/0	127/129	181/181	155/159	163/169	135/145	108/129	140/140	179/187	0/0
C1-18 109/126	121/123	141/141	105/105	127/129	181/181	155/159	163/169	135/145	108/129	140/140	179/187	186/192
C1-3-1 109/124	121/123	141/145	105/113	127/129	168/179	163/163	169/169	135/139	98/123	134/134	175/175	182/182
C3-22 109/126	121/123	141/143	105/105	127/129	168/179	155/159	163/169	135/145	108/129	140/140	179/187	186/192
AJ10- 109/126	121/123	141/143	105/105	127/129	0/0	155/159	163/169	135/145	108/129	140/140	179/187	186/192
AI7-10 109/126	121/123	141/143	105/105	127/129	181/181	155/159	163/169	135/145	108/129	140/140	179/187	186/192
K8-14 109/124	121/123	141/145	105/113	127/129	181/181	161/163	169/183	139/141	98/123	134/134	175/175	182/182
K6-24 109/124	121/123	141/145	105/113	127/129	168/179	161/163	169/183	139/141	98/123	134/134	175/175	182/182
E4-17 109/126	121/123	141/143	0/0	127/129	181/181	155/159	163/169	135/145	108/129	140/140	179/187	186/192
J8-13 109/124	121/123	141/145	105/113	127/129	181/181	161/163	169/183	0/0	0/0	134/134	175/175	182/182
C9-2-1 109/126	121/123	141/143	105/105	127/129	168/179	155/159	163/169	135/145	108/129	0/0	179/187	186/192
C15- 114/124	121/143	141/145	101/113	127/129	181/181	155/163	169/183	139/145	0/0	0/0	0/0	186/192
G1-1 109/126	117/143	121/141	105/113	125/127	168/181	140/163	163/169	141/145	98/123	0/0	181/187	186/190

Using this set of SSR markers was possible to distinguish unambiguously all the genotypes assessed, with a combined random probability of identity as low as 1.8114x10-13. Thus, unique genetic profiles were obtained for each tree (Table 2, Fig. 1).

A whole of 630 dissimilarities values were obtained. Whereas the genetic distance averaged 0.62741, the minimum distance detected was between the trees AI7-

10 and C1-18 (d=0.03846); while the maximum dissimilarities were found among trees K14 and X1, K14 and KJ, C1-3-1 and K14, K6-24 and K14 and C15-16-3 and C15-4 (d=0.88462). K14 has the edgiest profile (Fig. 1), with alleles and/or particular allelic combinations for loci UDP001, UDP97-402, BPPCT037 and UCD-CH12 (Table 2).

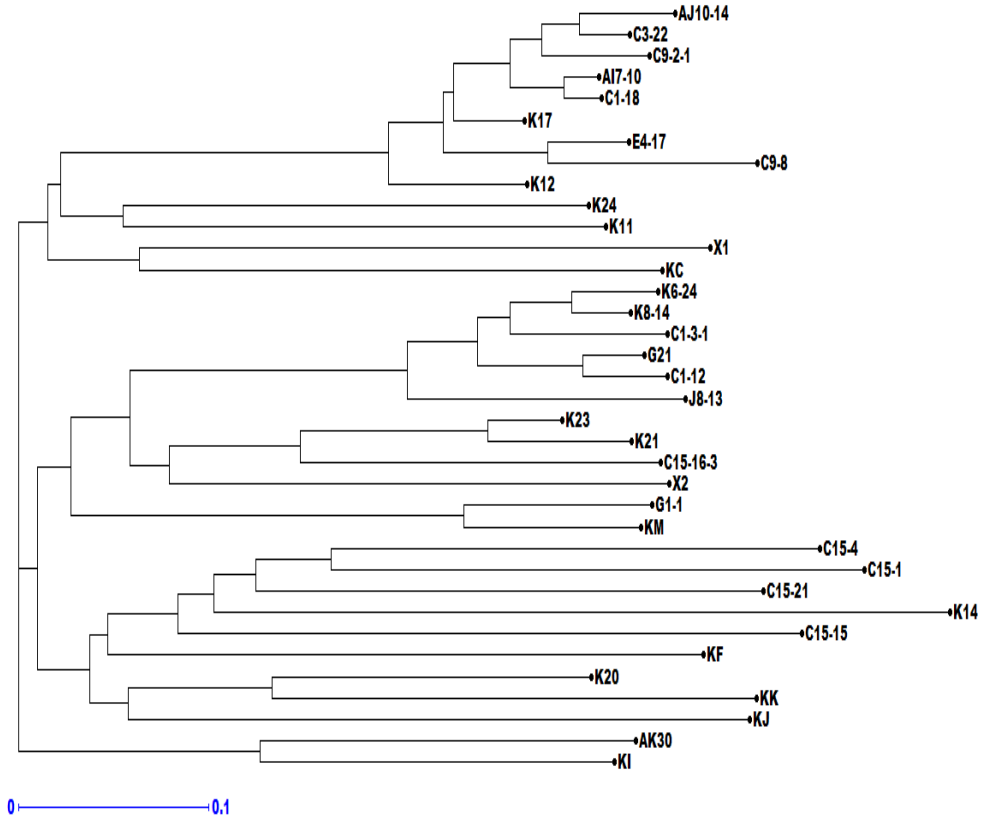

Fig. 1. Hierarchical tree constructed by the unweighted neighbour joining method using data from 13 SSR loci for a sample formed by 36 wild cherry trees selected for wood production

Discussion

The election of the right primers is a critical step to test their transferability to other species or different populations from those that they were designed. Although it is difficult to hit with the right markers that serve to the purpose pursued, some basic criteria could be followed. As some SSRs can be near to conserved regions, the closer are the species involved, the higher the possibility to use them successfully (Downey and Iezzoni, 2000). However, either because non-conserved regions have been explored and/or important genetic differences exist, the fastness of this postulate is only partial. Thus, vertical transferability has also been reported (Varshney et al., 2005; Kalia et al., 2011).

All 20 elected markers were composed by dinucleotide repeats; however, different kind of repetitions can be observed. According the classification proposed by Weber (1990) and Morgante and Olivieri (1993), 75% (15 out 20) of microsatellites here used contain regions with simple perfect repetitions and 20% (4 out 20) bear both perfect (UDP96-003, UDP98-021 and PCHGMS1) and imperfect (UDP96-013) compound repetitions; whereas only one (5%) is classified as simple imperfect (BPPCT026).

Was not possible to establish a relationship between the kind of repetition and the length of microsatellite regions with their abundance and polymorphism as was suggested by Morgante and Olivieri (1993). Thus, both primers flanking simple perfect (UDP96-015, UDP96- 018, UDP98-407, BPPCT 034 and BPPCT040), perfect

compound (UDP96-003) and imperfect repetitions (UDP96-013) were rejected. At the same time, different profiles of amplifications were observed among them, from complete absence to the presence of several bands.

Aranzana et al. (2003) found in peach that microsatellites with a high number of repeats were generally those having the largest number of alleles. Merrit et al. (2015) also concluded that as the size of the

marker increases, too does the number of possible alleles; although, the low magnitude of correlations found suggest that there is a point at which this relationship breaks down. Certainly, some of the most polymorphic loci here tested in the wild cherry progeny were those coincident with the largest alleles (BPPCT005, UCD-CH12 and UCD-CH18) but the BPPCT038 marker, with up to 10 alleles, registered the second smaller maximum allele (133, see Table 2).

In general, a high polymorphism was observed for all loci analyzed, in some cases higher than reported for the original species, which is consistent with results obtained for other authors. For example, Cipriani et al. (1999) registered only 2 alleles for locus UDP96-001 in *P. persica* (L.) Batsch, while greatest polymorphisms have been found for other peach cultivars (Testolin et al., 2000), mahaleb cherry (Godoy and Jordano, 2001), an almond x peach F2 progeny (Aranzana et al., 2003) and wild cherry (Ercisli et al., 2011). For the wild cherry progeny, outstanding were the results obtained with loci UDP96-001 and UDP97-402 registering 6 and 7 more alleles (Table 2), respectively, than those reported for the original species (Cipriani et al., 1999). For marker UCD- CH18, also 7 new alleles were observed regarding those originally registered by Struss et al. (2003).

Despite different provenances of wild cherry and species, as well as a variable size of samples and methods to detect the presence of alleles, were used, the results here presented are consistent with literature. Thus, some of the observed alleles in the assessed wild cherry progeny have also been previously reported for this species, as were the cases of alleles 179 and 185 for locus BPPCT002 (Dirlewanger et al., 2002), 126 for locus UDP96-001 (Schueler et al., 2003) and 184, 186 and 188 for locus UCD-CH18 (Struss et al., 2003). Coincidences were also observed in the allelic size regarding those registered in peach, as were the cases of alleles 139 and 156 for markers UDP97-402 (Cipriani et al., 1999; Testolin et al., 2000) and BPPCT037 (Dirlewanger et al., 2002), respectively; reinforcing the postulate that microsatellites are genetic markers that offer solid and repeatable results.

Besides Vaugham and Russell (2004), only De Rogatis et al. (2013) and Fernandez-Cruz et al. (2014) have used nuclear SSR markers to perform genetic studies in wild cherry. Analyzing the level and distribution of genetic variability of breeding zones in Italy, De Rogatis et al. (2013), using microsatellites from the libraries of Clarke and Tobutt (2003) and Vaugham and Russell (2004), detected an average of 7.53 alleles/locus; similar to those found in this research (7.7 alleles/locus). On the other side, despite a higher number of individuals were assessed by Fernandez-Cruz et al. (2014), also using markers from these libraries, registered lower polymorphism (84 versus 97 alleles) and discriminative capacities (10^{-9} versus 10^{-11}) than those found here. This suggest the necessity to assess a higher number of microsatellites from other sources, to increase the usefulness of this kind of markers for genetic studies into wild cherry.

Conclusions

Using this set of markers, formed by 13 microsatellites developed for peach and sweet cherry, was possible to identify and differentiate unambiguously among a sample formed by 36 wild cherry trees selected for wood production. Besides the clear advantage to construct the genetic profile of each trees with a low probability of error, the results here presented might also be useful for the management of productive wild cherry plantations for wood production; contributing to reduce the risks of the impact of biotic and/or abiotic factors caused for the establishment of clonal stands.

Acknowledgement

This research was performed through a collaboration agreement among Bosques Naturales S.A. (Spain), the Center for Plant Biotechnology and Genomics (CBGP, Spain) and the Universidad Politécnica de Madrid (UPM, Spain). We would like to thanks to Ignacio Urban Martinez for his help during the recollection and the election of samples. Also, thanks to the other members of Laboratory 201 at CBGP, Irene Merino and Alvaro Vinuesa for their advices and support during the execution of research.

Author's Contributions

RJLM performed the experimental work, participated in the interpretation of results and prepared the

draft. JQ and AC supported the execution of research and the interpretation of results. LG as team leader, supervised and guided the execution of the different experiments and the interpretation of results.

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