Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Available online at www.inia.es/forestsystems
doi: http://dx.doi.org/10.5424/fs/2014231-04580

Forest Systems 2014 23(1): 120-128
ISSN: 2171-5068
eISSN: 2171-9845

Molecular characterization of Spanish *Prunus avium* plus trees

J. Fernandez-Cruz, J. Fernandez-Lopez*, M. E. Miranda-Fontaiña,
R. Diaz and G. Toval

Centro de Investigación Forestal de Lourizán. Carretera de Marín, km 4. 36080 Pontevedra, Spain

Abstract

Aim of the study: The Breeding Program of wild cherry (*Prunus avium*) developed by Lourizán Forest Research Center (NW Spain), aims for the creation of the Main Breeding Population, that is formed by a large number of plus trees and for obtaining an Elite Population generated from controlled crosses of a number of plus trees selected by, at least, one trait of economic importance. The aim of this study was to genotype 131 accessions of *Prunus avium* plus trees, included in the breeding program.

Area of study: Prunus avium plus trees are located in the North, Northwest and Central Spain.

Material and methods: Prunus avium plus trees were genotyped with nine microsatellites. Several genetic parameters were calculated. Genetic data were analyzed with STRUCTURE and the genetic distance between plus trees were calculated.

Main results: A total of 122 multilocus genotypes were detected. Several accessions with the same genotype were identified, which could be due to clonality or to labelling errors. The nine microsatellites are useful for identifying individuals because the combined probability of identity was low (PI = 5.19×10^{-9}). Bayesian methods detected two genetic clusters in the sampled plus trees.

Research highlights: The unique genotypes identified in this work are suitable for being included in the elite breeding population for economic traits.

Key words: *Prunus avium*; breeding program; microsatellite; genetic distance.

Introduction

Wild cherry (*Prunus avium*) is a noble hardwood species of economic importance which is being used in clonal plantations in order to produce high quality timber. In 1998, the Lourizán Forest Reseach Center, located in Galicia, in the Northwest of Spain, started a phenotypic selection of *P. avium* plus trees. These trees were propagated by grafting to stablish clonal seed orchards. In addition, a clonal selection of other 30 individuals was developed to study their rooting ability and to select clones to be used in commercial plantations. Presently, the Innovation and Forest Tree Breeding Plan of the Galician region, aims to obtain long-term genetic gains in several traits of interest for wood production. For *P. avium*, the plan defines two different populations. On one hand, the Main Breeding Population contains plus trees from the North and Northwest coast and Central Spain, phenotypically selected on the basis of their value for timber production. On the other hand, the Elite Population has the best individuals selected by at least one of the following breeding traits: growth, resistance to *Blumeriella jaapii*, straightness and propagation fitness. These individuals are being crossed in a half-diallel mating design to obtain high multi-trait genetic gain.

The main objectives of this study were to genotype, with nine nuclear loci, 131 accessions of *P. avium* plus trees that are being used in the Main Breeding Population and in the Elite Population and to detect a clonal and genetic structure. Several analyses were performed to know the necessary number of loci to distinguish all the multilocus genotypes and its discrimination power for individual fingerprinting.

Material and methods

Plant material and laboratory methods

The samples were collected from the seed orchards of Areas and Sergude, the clonal trial of Bos, the

*Corresponding author: josefa.fernandez.lopez@xunta.es
Recibido: 06-06-13. Aceptado: 15-01-14.

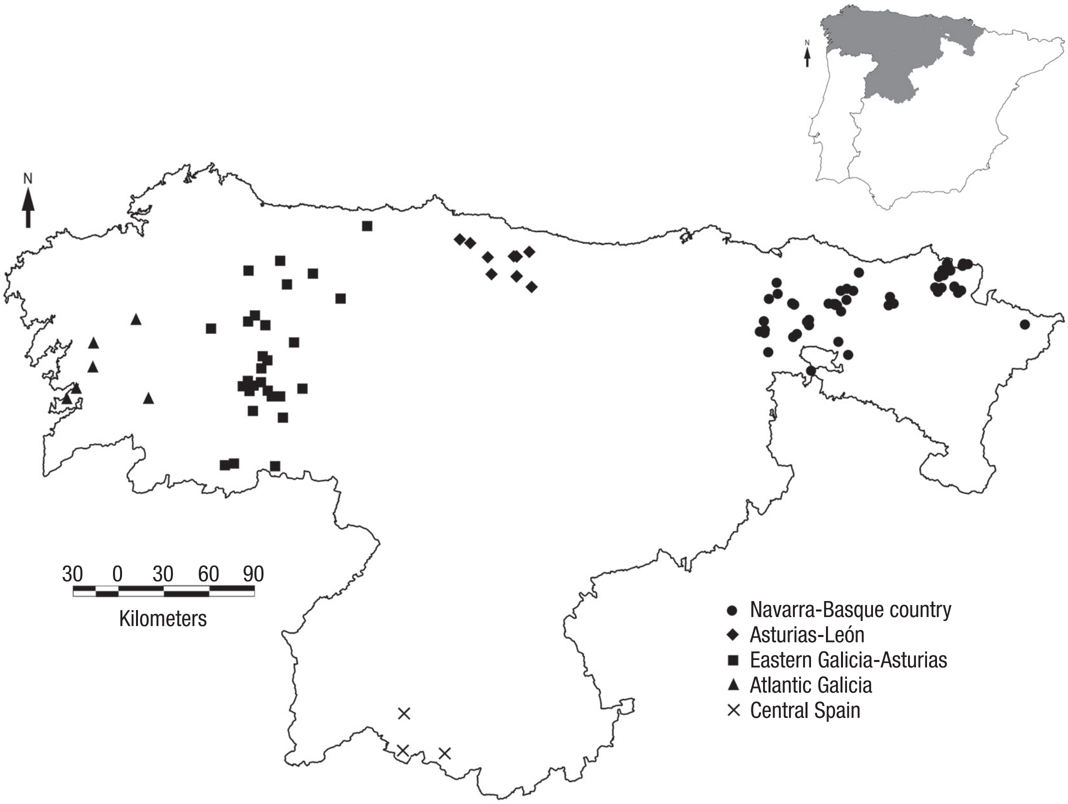


Figure 1. Geographic location of sampled wild cherry (*Prunus avium*) in Spain.

germplasm collection of Mantequera and from several micropropagules located in *in vitro* laboratory. Up to 173 samples were collected from 131 accessions of *P. avium* that were classified as plus trees in different field prospections in the North of Spain (Figure 1). Individuals were classified into five populations: Navarra-Basque country, Asturias-León, Eastern Galicia-Asturias, Atlantic Galicia and Central Spain. A total of 42 replicas were sampled from 38 accessions in order to verify the clonal fidelity.

DNA was isolated from frozen leaves or buds using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and quantified using a BioPhotometer Plus (Eppendorf, Hamburg, Germany). Nine nuclear microsatellite loci were used to genotype *Prunus avium* plus trees: EMPaS01, EMPaS02, EMPaS06, EMPaS10, EMPaS12, EMPaS14 (Vaughan and Russell, 2004) and EMPA004, EMPA005 and EMPA015 (Clarke and Tobutt, 2003). The amplified products were analyzed in an automatic sequencer, CEQ 8800 Genetic Analysis System (Beckman Coulter, Fullerton, California, USA).

Statistical analysis

The presence of null alleles was determined using Micro-Checker ver. 2.2.3 (Van Oosterhout *et al.*, 2004). Loci with estimated null allele frequencies higher than 0.19 were excluded from further analysis because from this threshold, the underestimation of the expected heterozygosity due to null alleles is significant (Chapuis *et al.*, 2008).Several standard measures were calculated with GENCLONE v2.0 (Arnaud-Haond and Belkhir, 2007) in order to detect the presence of potential clones in the defined populations: the number of samples (N), the number of multilocus genotypes (MLGs), the number of repeated MLGs (MLG_r), the number of unique MLGs within each population (MLG_I) (Ellstrand and Roose, 1987), the number of multilocus lineages (MLLs), and the modified index of genotypic richness (R) (Dorken and Eckert, 2001). The probability that two individuals with the same MLG were originated from different sexual reproductive events, Psex, was also calculated.

Once the number of MLGs was established, the probability of identity (PI) and the combined probability of identity was calculated with SPAGeDi v1.4 (Hardy and Vekemans, 2002) in order to know whether the set of loci are useful to estimate the real number of multilocus genotypes (MLGs). The probability of identity (PI) represents the average probability of a match for any genotype.

The number of alleles (n_a), the effective number of alleles (n_e), and the number of privative alleles were calculated with SPAGeDi v1.4 (Hardy and Vekemans, 2002).

STRUCTURE version 2.3.4 software (Pritchard *et al.*, 2000; Hubisz *et al.*, 2009) was used to assign the defined MLGs to different genetic clusters. Two independent analyses were performed with and without LOCPRIOR model. In both cases, a model without admixture and with correlated frequencies was assumed. A burn-in period of 50,000 iterations followed by 100,000 Markov Chain Monte Carlo (MCMC) iterations was used for K values from 1 to 10. Ten independent runs were tested for each K value. The L(K) non parametric test (Pritchard *et al.*, 2000) and ΔK approach (Evanno *et al.*, 2005) were used to identify the most likely number of clusters (K).

The number of shared alleles was used to calculate the genetic distance between clones. The NEIGHBOR package of PHYLIP software (Felsenstein, 1989) was used to construct a dendrogram following the UPGMA method and it was displayed with FIGTREE 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Flow cytometry

Prunus avium leaves of trees suspected to be triploid were sent to Centro Nacional de Biotecnología (CNB) in Madrid, Spain. A flow cytometry analysis was performed using Citomics FC500 (Beckman Coulter, Fullerton, California, USA). The intensity of the fluorescence of the cell nuclei of the putative triploid was compared with LU23 clone that was used as diploid control.

Results and discussion

The analysis with MICRO-CHECKER detected deviations from Hardy-Weinberg equilibrium for EMPA015 in Navarra-Basque country population due to the presence of null alleles. However, EMPA015 was maintained in further analysis because the estimated null allele frequency was 0.08.

The amplification with nine loci showed one or two alleles in all samples except for the accessions SA4 and SA12 (Supporting information 1).

Although the samples SA4 and SA12, were coded as different accessions, they belong to the same plus tree because they display the same genotype, the same three alleles in loci EMPaS01, EMPaS10 and EMPaS12 and the same doubled alleles in the remaining six loci. Flow cytometry revealed that the fluorescence intensity of SA12 was nearly 1.5 times higher than the control LU23 and confirms that SA12 and, therefore SA4, are triploids. Natural triploids of *P. avium* are not very common in nature. Nevertheless, one individual was detected in Germany (von Schelhorn, 1947), another one in Belgium (De Cuyper *et al.*, 2005) and eleven in France (Serres-Giardi *et al.*, 2010). In general, they are phenotypically superior trees. Triploids have significantly better height and circumference growth than diploids and therefore, they can be suitable for wood production (Serres-Giardi *et al.*, 2010).

A total of 122 MLGs were detected out of 131 accessions (Table 1). A clonal lineage (MLL) was also found; PO34 located in Areas was different from the micropropagated PO34 in one allele in locus EMPA015 (Supporting information 1) probably due to somatic mutation or scoring errors. There were 5 MLGs with at least two different accessions in the same population and one MLG, LU40-PO36, with the accessions generated from different populations. The psex values of the accessions LU40-PO36, LOURIZÁN 1-LU47, NA7-NA12, NA8-NA9-NA10-NA11, NA22-NA26 and PO33-PO34 suggest that they were produced by asexual reproduction because the probability of the genotype to be present once or more times as the result of different reproductive events are quite low (Supporting information 1). In addition, a labelling error can explain the MLG identity of LU40 and PO36, coming from different populations.

The nine loci used in this study are useful for fingerprinting because the probability of another random and unrelated individual with the same genotype is very low (Combined probability of identity = 5.19×10^{-9}) (Supporting information 2).

The genetic analysis of the 131 accesions of *Prunus avium* revealed that all microsatellite loci were polymorphic. A total of 84 alleles were detected. The number of alleles ranged from 17 in EMPA015 to 4 in EMPA014 (Supporting information 2). Nevertheless, the effective number of alleles decreased significantly

Supporting information 1. Geographic coordinates and allelic profiles of 131 accesions of *Prunus avium* plus trees genotyped with nine loci. Note that SA4 and SA12 are different accessions that belong to the same plus tree and PO34/IV is different from PO34 in locus EMPA015. Several accessions share the same geographic coordinates because it represents the parish or the council where the accessions are located. Accessions in bold belong to the same MLG and show the Psex value

Clone	Latitude	Longitude	MLG	Psex	EMPaS01	EMPaS02	EMPA004	EMPA005	EMPaS06	EMPaS10	EMPaS12	EMPA014	EMPA015
AS1	43°18'30"W	5°9'55"N	1		232	145/149	193	260	205/207	163/171	140/146	202/216	242/258
AS0103/1	43°22'50"W	5°32'43"N	2		226/232	139/149	185/193	260	205/223	157/159	125	202	229/242
AS2	43°18'40"W	5°10'0"N	3		232	147/149	193	260	205/221	163/171	140/146	202/204	215/229
AS3	43°18'35"W	5°11'20"N	4		226/232	145/149	185/193	260	205/223	157	140/146	216	215/242
AS4	43°18'35"W	5°11'20"N	5		232/242	147/149	185/193	248/260	205/215	171	138/140	202	229
AS0402/3	43°24'1"W	5°37'52"N	6		232/236	147	191	260	205/223	171	125/140	216	225/242
AS5	43°11'30"W	5°9'40"N	7		232/236	147/149	185	244/260	205/207	157	140/148	202/216	229/260
AS6	43°11'30"W	5°9'40"N	8		232	149	185	244/250	205/221	157	138/146	202	229/260
AS06/4	43°20'16"W	5°3'50"N	9		232	145/149	185	248/260	205/209	157/171	138/146	202/204	225
AS0609/1	43°20'16"W	5°3'50"N	10		226/232	149	185/193	248	207/223	157/171	125/127	202/216	225/242
AS7	43°1'30"W	6°35'0"N	11		232/236	149	191	250/260	205/207	157/187	125	202	227/242
AS0706/4	43°20'16"W	5°3'50"N	12		232/236	147/149	185	248/260	205	157/163	146/148	202	242/256
AS8	43°9'55"W	6°48'55"N	13		232/236	149	185/191	248/260	205	157/173	125/148	202/216	242
AS9	43°27'40"W	6°23'30"N	14		232	149	193/195	260	205/221	171	125/140	216	242/256
AS0903/1	43°11'25"W	5°9'47"N	15		232	147/149	185	246/260	205/207	157/163	140/146	204/216	229/242
AS1015/1	43°11'25"W	5°9'47"N	16		226/232	149	185/193	250/260	205	157/163	125/146	202	223/242
AS1017/1	43°11'25"W	5°9'47"N	17		232	145/149	191/193	248/250	205/223	157/163	125/146	216	225/250
AS11	43°18'0"W	5°24'0"N	18		232	145/149	191/193	260/264	205/221	163	138/146	202/204	225/256
AS16	43°11'30"W	5°9'40"N	19		232	149	185/191	246/250	205/221	163	140/146	202	215/256
AS19	43°12'0"W	5°22'0"N	20		232	147/149	185/193	260	221/223	171	138	202/204	225/256
AV7	40°21'14"W	5°37'19"N	21		226	143/147	185/197	244/260	205	157/187	140/146	202/216	225
C39	42°50'17"W	8°13'18"N	22		232/242	135/139	193	260	205/223	157/171	140	202/216	242/244
LE4	43°7'50"W	5°2'14"N	23		226	145/147	185/191	250/260	207/209	157	138/148	202	225/227
LOURIZÁN1	**42°51'45"W**	**7°19'7"N**	**24**		**226/236**	**135/145**	**191/193**	**260**	**205/215**	**171**	**148**	**202/216**	**240**
LU47	**42°54'1"W**	**7°16'0"N**	**24**	**2.05×10^{-9}**	**226/236**	**135/145**	**191/193**	**260**	**205/215**	**171**	**148**	**202/216**	**240**
LOURIZÁN2	42°28'42"W	7°20'9"N	25		232/238	149	193	260	205/223	163/171	140/146	202/216	225/256
LU0104/1	42°29'9"W	7°14'58"N	26		232	149	183/185	248/260	205/207	163/171	148	202/216	225/242
LU0108/2	42°29'9"W	7°14'58"N	27		232	145/149	183/193	260	205	163/171	148	202/216	240/242
LU0504/2	42°30'37"W	7°17'51"N	28		226/236	149/151	185/193	260	223	157	138	202/216	225/256
LU06/5	42°27'7"W	7°16'46"N	29		232/236	139/149	185	244/260	205/209	171	146/148	202/216	213/225
LU24	42°30'22"W	7°11'27"N	30		236	147/149	183/191	250/260	209/223	157/163	138/146	202/216	240/252
LU25	42°27'35"W	7°7'59"N	31		232/236	147/149	191/193	260	223	163	125/138	202	227/242
LU27	42°28'42"W	7°20'9"N	32		232/236	149	193	248/260	223	163/171	138/146	202	225/256
LU31	42°45'12"W	6°56'30"N	33		226/232	135/149	183/185	260	205/223	171	146	202/204	252
LU32	42°45'12"W	6°56'30"N	34		232/236	135/149	183/193	232/260	205/221	171	109/146	216	225/242
LU35	42°39'39"W	7°11'20"N	35		226/236	149	185/193	260	221/223	171	125/148	202/216	227/242
LU37	43°5'36"W	7°1'17"N	36		226/232	147	191/193	260	205	163/171	140/148	202	225/252
LU38	43°13'58"W	7°5'7"N	37		236	149	183/191	260	207/223	157/159	140/146	202	225/256
LU40	**42°38'23"W**	**7°9'1"N**	**38**		**226/232**	**135/147**	**183/193**	**248/260**	**205**	**157/171**	**109/148**	**202**	**242/256**
PO36	**42°40'57"W**	**8°32'59"N**	**38**	**6.35×10^{-8}**	**226/232**	**135/147**	**183/193**	**248/260**	**205**	**157/171**	**109/148**	**202**	**242/256**
LU45	42°48'31"W	7°36'52"N	39		232/236	135/145	183/193	260	207/215	157/171	109/148	216	223/242
LU48	43°9'52"W	7°20'12"N	40		232/238	147/149	185/195	260	205	157/187	138/140	202	225/242
LU50	42°50'46"W	7°10'46"N	41		236	135/149	185/193	232/260	205/207	157/163	109/125	202	227/252
MEZ10/1	42°0'47"W	7°2'48"N	42		232	139/149	191/193	248	205/209	157/171	138	202/216	227/258
MEZ4/2	42°0'47"W	7°2'48"N	43		232/236	147	185	250/260	207/209	187	138/140	202	225/258
MEZ8/2	42°0'47"W	7°2'48"N	44		232/236	139/149	191/193	248/250	205/223	165/171	138/140	202/216	225/227
NA1	43°14'25"W	1°38'25"N	45		226/236	145/149	185/193	260	205/207	163/171	140/146	202/216	225
NA2	43°14'18"W	1°38'25"N	46		226/236	147	187/191	260/262	205	157	138/148	202/216	223/242
NA3	43°14'33"W	1°40'20"N	47		226/242	145/149	191/193	260	205/221	157/171	140/146	202/216	252/254
NA5	43°16'51"W	1°40'0"N	48		226/242	145/147	193	232/260	205/221	163	138/146	202	242/252
NA6	43°16'52"W	1°40'1"N	49		232/236	145	185/191	250	221/223	157/187	140/146	202	225/252

Supporting information 1 (cont.). Geographic coordinates and allelic profiles of 131 accesions of *Prunus avium* plus trees genotyped with nine loci. Note that SA4 and SA12 are different accessions that belong to the same plus tree and PO34/IV is different from PO34 in locus EMPA015. Several accessions share the same geographic coordinates because it represents the parish or the council where the accessions are located. Accessions in bold belong to the same MLG and show the Psex value

Clone	Latitude	Longitude	MLG	Psex	EMPaS01	EMPaS02	EMPA004	EMPA005	EMPaS06	EMPaS10	EMPaS12	EMPA014	EMPA015
NA7	**43°12'40"W**	**1°43'52"N**	**50**		**226/242**	**149**	**193**	**260**	**205/221**	**171/183**	**138**	**202**	**223/225**
NA12	**43°12'7"W**	**1°44'2"N**	**50**	**2.42×10^{-8}**	**226/242**	**149**	**193**	**260**	**205/221**	**171/183**	**138**	**202**	**223/225**
NA8	**43°12'39"W**	**1°43'57"N**	**51**		**232/242**	**149/151**	**185**	**250/260**	**205/221**	**157**	**148**	**202**	**225**
NA9	**43°12'40"W**	**1°43'56"N**	**51**		**232/242**	**149/151**	**185**	**250/260**	**205/221**	**157**	**148**	**202**	**225**
NA10	**43°12'39"W**	**1°41'42"N**	**51**		**232/242**	**149/151**	**185**	**250/260**	**205/221**	**157**	**148**	**202**	**225**
NA11	**43°12'41"W**	**1°43'56"N**	**51**	**6.24×10^{-7}**	**232/242**	**149/151**	**185**	**250/260**	**205/221**	**157**	**148**	**202**	**225**
NA13	43°12'7"W	1°43'57"N	52		232	145/149	185/193	248/260	205/223	163/171	138	202/216	252/256
NA16	43°14'28"W	1°42'18"N	53		232/236	145/151	193	260	205	157/171	140/146	202	252/256
NA17	43°14'29"W	1°42'18"N	54		242	139/149	191	248/260	207/221	163/187	138/140	202/216	256
NA18	43°14'29"W	1°42'18"N	55		232	149/151	185/193	248/260	205/209	157/187	146	202/216	225/252
NA19	43°14'29"W	1°42'16"N	56		226/242	145/149	185	244/248	205/207	157	140/146	202	225/242
NA20	43°14'27"W	1°42'17"N	57		226/242	145/149	193	248/260	205/209	157	146	202/216	252/256
NA21	43°14'27"W	1°42'11"N	58		226/242	145	185/193	250/260	205/207	157/171	140	202	242/256
NA22	**43°14'29"W**	**1°42'12"N**	**59**		**232**	**145**	**185/193**	**244/260**	**207/223**	**171/187**	**138/146**	**202/216**	**223/225**
NA26	**43°16'32"W**	**1°29'51"N**	**59**	**4.81×10^{-8}**	**232**	**145**	**185/193**	**244/260**	**207/223**	**171/187**	**138/146**	**202/216**	**223/225**
NA23	43°15'57"W	1°32'31"N	60		232/236	145/149	185/193	248/260	209/221	157/171	138/148	202/216	225
NA24	43°16'14"W	1°32'15"N	61		232/245	145	185/193	260	209/223	157/187	140/146	202	225
NA25	43°16'47"W	1°32'6"N	62		226/242	145/149	185/193	248/260	205	157/163	140	216	225/242
NA27	43°16'34"W	1°29'48"N	63		232	145/149	193	244/260	207	171/187	146	204/216	223/225
NA28	43°7'15"W	1°33'18"N	64		232/236	145/151	185/193	260	205/209	157/183	146/148	202/216	225/256
NA29	43°6'22"W	1°34'49"N	65		232/242	149	193/195	260	209/221	163/169	138/146	202/216	225
NA30	43°6'22"W	1°34'49"N	66		232/236	145/149	185/193	250/260	205/209	157/171	140/146	202/216	225
NA31	43°6'24"W	1°34'52"N	67		226/232	147/149	185/193	244/248	205/221	169/189	140/148	202	225/256
NA32	43°8'28"W	1°36'29"N	68		232/242	145/147	193/195	244/260	221/223	157/183	140/146	202	225/256
NA33	43°8'36"W	1°36'38"N	69		232/236	149	193	250/260	205/223	157/163	138/146	202/204	256
NA34	43°8'37"W	1°36'38"N	70		226/242	149	193	250/260	223	163/169	138/146	202/204	256
NA35	43°8'13"W	1°42'49"N	71		226	145/149	191/193	248/260	223	163/187	138	202	256
NA37	43°8'25"W	1°45'51"N	72		226/242	147/149	183/193	244/260	223	163/187	148	202	223/256
NA38	43°6'53"W	1°44'33"N	73		242	147/149	185/193	244/248	221/223	163/189	125/138	202	225
NA47	42°54'35"W	1°2'46"N	74		226/232	149/151	193	248/260	207	157/159	140/146	202/216	252
OU0201/3	42°25'37"W	7°5'55"N	75		232	147/149	185/193	260	209/223	157/187	138/148	202/216	225/258
OU0202/1	42°25'37"W	7°5'55"N	76		232/242	147/149	185/193	260	209	157/187	138/148	202/216	258
OU0302/2	42°25'41"W	7°1'57"N	77		232/242	139	185/193	250/260	205	157/189	138	216	227/256
OU11	42°20'3"W	7°14'46"N	78		232/236	151	185/193	250	205/223	157/163	138/148	202	242/256
OU13/13	42°28'50"W	6°51'28"N	79		226/236	145/149	183/193	260	223	157/171	109/138	202/204	252/256
OU20	42°0'15"W	7°26'41"N	80		226/242	149	185/193	260	205/207	187	109/125	216	242/256
OU21	42°1'4"W	7°22'24"N	81		236	145/149	183/191	250/260	223	157/197	125/156	202/216	225
OU22	42°18'16"W	7°0'11"N	82		226/232	139/147	183/185	260	207/223	163/171	138/140	216	225
OU42	42°22'30"W	8°4'54"N	83		232/236	149/151	183/193	250	205/221	163/171	146	202	225/256
PO28	42°24'30"W	8°39'45"N	84		236/242	147	185	260	215/223	157	125/138	202	223
PO29	42°24'30"W	8°39'45"N	85		226/242	139/147	185/191	260	215/223	157/183	140	202	223/225
PO33	**42°20'31"W**	**8°44'1"N**	**86**		**240/242**	**139/147**	**185**	**260**	**205/223**	**157/163**	**125/138**	**202**	**242/256**
PO34	**42°20'31"W**	**8°44'1"N**	**86**	**1.13×10^{-5}**	**240/242**	**139/147**	**185**	**260**	**205/223**	**157/163**	**125/138**	**202**	**242/256**
PO34/IV	42°20'31"W	8°44'1"N	86		240/242	139/147	185	260	205/223	157/163	125/138	202	242/258
PO41	42°20'31"W	8°44'1"N	87		232	139/149	185/195	260	205/223	157	140/148	202	225/256
PO43	42°32'20"W	8°32'32"N	88		232/236	147/149	191/193	232/260	221	171/187	140/148	202	225/258
PV2	43°3'12"W	2°55'30"N	89		232	139/147	183	246/250	205	163	125/140	216	219/229
PV4	43°2'50"W	2°35'22"N	90		232/236	145/147	185/193	244/250	205/209	157/159	140	202	223/225
PV5	42°53'37"W	3°8'45"N	91		232/242	145/149	185/191	250	205/209	163/171	138/140	202/216	229/254
PV6	42°52'36"W	3°8'53"N	92		226/232	147/149	185/193	250/260	205/223	157/187	138/140	202	227/256
PV7	42°53'5"W	3°11'10"N	93		232/242	149	185/191	250/260	205/223	157	125/146	202	223/229

Supporting information 1 (cont.). Geographic coordinates and allelic profiles of 131 accesions of *Prunus avium* plus trees genotyped with nine loci. Note that SA4 and SA12 are different accessions that belong to the same plus tree and PO34/IV is different from PO34 in locus EMPA015. Several accessions share the same geographic coordinates because it represents the parish or the council where the accessions are located. Accessions in bold belong to the same MLG and show the Psex value

Clone	Latitude	Longitude	MLG	Psex	EMPaS01	EMPaS02	EMPA004	EMPA005	EMPaS06	EMPaS10	EMPaS12	EMPA014	EMPA015
PV10	42°56'44"W	3°9'22"N	94		232	147/149	185/191	260	209/223	157/163	140/146	202	229/242
PV11	42°44'44"W	2°28'29"N	95		232/236	143/149	193	250/260	207/223	157	140	202/216	225/242
PV13	42°49'24"W	2°33'12"N	96		236/242	147/149	191/193	244/260	205	157	125/140	202	252/256
PV15	43°2'42"W	2°54'30"N	97		232/242	147/149	183/185	248/250	223	157/163	125/138	202	215/225
PV17	42°57'17"W	2°47'20"N	98		226/232	145/147	185/187	248/252	205	157	140	202/216	227/256
PV18	42°56'33"W	2°48'18"N	99		232/242	149	185	250/260	205/221	157/159	146	202	227/242
PV19	42°55'23"W	2°47'23"N	100		238/242	149	185/193	248/252	205/221	157	140/148	202	223
PV21	43°4'44"W	3°6'47"N	101		232/234	135/139	173/183	248/260	207/223	163	140	204	229/242
PV22	42°51'10"W	2°55'0"N	102		232	149	193	250	205	157	138/146	202	229/242
PV24	43°5'13"W	2°8'0"N	103		232/242	147/149	183/185	244	205/207	157/163	146/148	216	223/242
PV25	42°52'12"W	2°53'15"N	104		232	149	185/193	248/260	205/223	157/187	138	202/216	242/262
PV28	43°2'53"W	2°6'8"N	105		226/242	147/149	185/187	244/260	205/207	157	140/150	202	242/256
PV30	43°6'35"W	3°2'36"N	106		232/236	149	185	250/260	205/223	157	140	202/216	242/256
PV31	42°39'4"W	2°46'24"N	107		226	145/147	185	244/260	205/209	157/163	140/146	202/216	225/252
PV32	42°45'41"W	3°7'8"N	108		232	147	185/193	250/260	223	157	138/140	216	225
PV33	43°10'31"W	3°3'7"N	109		232/242	147/149	185/193	246/248	205/207	157/187	138/140	202/216	213/256
PV35	43°2'50"W	2°34'36"N	110		232/236	147/149	185/193	248/260	205/223	157	140/146	202	225/227
PV36	43°0'14"W	2°31'46"N	111		226/232	145/149	191/193	244/258	205/207	157	140	202/216	229
PV38	43°8'16"W	2°29'0"N	112		236	147	183/185	250/260	205/223	157	140	202	227/242
PV39	43°2'30"W	2°34'29"N	113		232	147/149	191/193	244/250	205	157	140/146	202	225/227
PV40	43°4'18"W	2°29'7"N	114		226/232	147/149	185/193	248/260	205/207	159/163	140	202/216	225/242
PV41	43°2'25"W	2°33'43"N	115		226/236	147	185/193	244/260	205/223	157/163	140/146	202	215/256
PV42	43°2'14"W	2°8'44"N	116		232/242	147/149	185/191	248/250	205/207	157	146/148	202	223/256
PV43	43°7'33"W	2°25'54"N	117		232/236	149	183/185	250/260	205/207	157	138/148	202	242
PV44	43°7'33"W	2°31'54"N	118		226/232	149	183/193	244/248	223	187	138/140	202	223
PV45	43°14'0"W	2°23'0"N	119		226/236	149	183/185	244/260	205/223	163	138/140	202	223/242
PV46	43°3'0"W	2°37'48"N	120		226/232	145/151	185/193	250/260	205/223	159/163	138	202/216	256
SA2	40°34'32"W	5°57'8"N	121		232/236	145/149	185/191	244/260	205/207	157/163	140	202/216	225/229
SA4=SA12	40°20'51"W	5°57'36"N	122		228/232/236	141/141/149	185/185/193	226/260/260	205/207/207	157/171/187	123/138/148	174/202/202	225/252/252

Table 1. Clonal paramenters of 131 *P. avium* plus trees clustered in 5 populations

Population	N	MLG	MLG_r	MLG_l	MLL	R
Navarra-Basque Country	67	62	3	62	0	0.92
Asturias-León	18	18	0	18	0	1.00
Eastern Galicia-Asturias	33	32	1	32	0	0.97
Atlantic Galicia	9	7	1	6	1	0.75
Central Spain	4	3	0	3	0	0.66
Total	131	122	5	121	1	0.93

N, number of individuals; MLG, number of multilocus genotypes; MLG_r, number of repeated MLGs; MLG_l, number of local unique MLGs; MLL, number of multilocus lineages; R, genotypic diversity.

which means that only a few alleles have high allelic frequencies in each locus. The number of privative alleles ranged from 12 in Navarra-Basque country population to 1 in Asturias-León population. No privative alleles were detected in Atlantic Galicia and Central Spain populations (Data not shown). The allelic range of the loci overlaps or partially overlaps with the results of other works (Guarino *et al.*, 2009; Tanceva Crmaric *et al.*, 2011; De Rogatis *et al.*, 2013).

The minimum combination of loci to distinguish all MLGs is EMPA015, EMPaS12 and EMPaS06. However, there still were 58 MLGs that were different

Supporting information 2. Genetic parameters of nine microsatellite loci estimated from 122 MLGs

	Allelic range	n_a	n_e	PI
EMPaS01	226-245	9	3.41	0.13
EMPaS02	135-151	8	3.36	0.13
EMPA004	173-197	8	3.4	0.14
EMPA005	226-264	11	2.82	0.16
EMPaS06	205-223	6	3.76	0.11
EMPaS10	157-197	11	3.76	0.11
EMPaS12	109-156	10	4.84	0.08
EMPA014	174-216	4	1.93	0.35
EMPA015	213-262	17	6.7	0.04
Overall		9.33^a	3.77^a	5.16×10^{-9b}

n_a, number of alleles; n_e, effective number; PI, probability of identity. [a] Average of the estimated value across all loci. [b] Combined probability of identity, considering all markers.

in only one allele. To ensure that all MLGs are genetically different, five loci (EMPA015, EMPaS12, EMPaS06, EMPaS10, EMPaS01) with the lowest probability of identity, were needed to distinguish the 122 MLGs and to avoid MLLs. These five loci can be useful for the routinely identity verification of the individuals of the Breeding Program of wild cherry and for detecting labeling errors during their manipulation.

STRUCTURE software detected, with nine loci, two main clusters only when LOCPRIOR model was used. When LOCPRIOR model was disabled, STRUCTURE did not find any population structure. The LOCPRIOR model takes into account the sampling locations and it is suitable for detecting a genetic structure when the signal is too weak (Hubisz *et al.*, 2009). The least negative value of Ln*P(K)* with the lower standard deviation was at $K = 2$. All runs at K = 2 displayed the same result. From $K = 3$, Ln*P(K)* decreased and the standard deviation in the different K values was very high. In addition, the Evanno method also detected two main clusters. The 122 MLGs could be classified into two genetic clusters (Figure 2). The first group

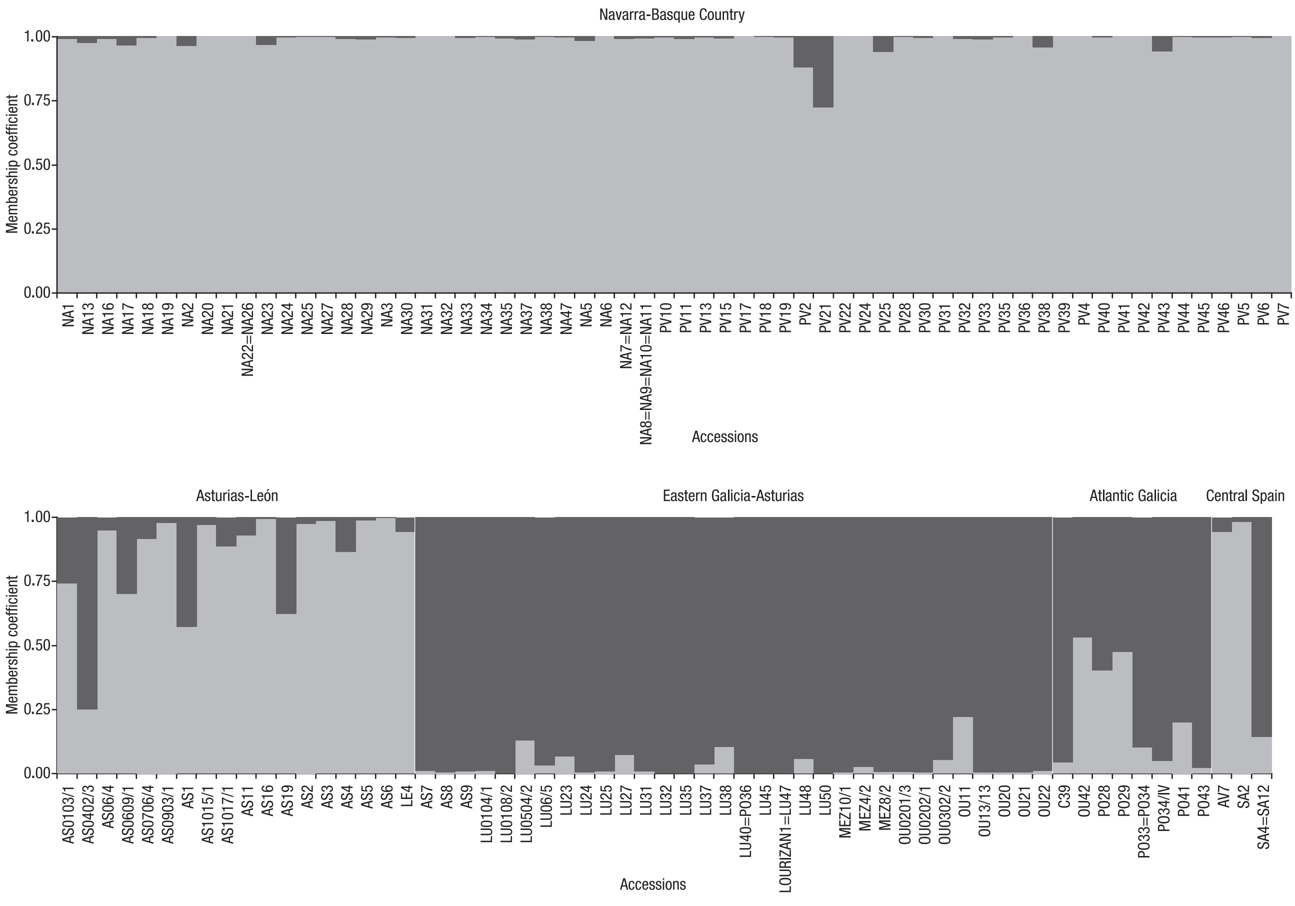
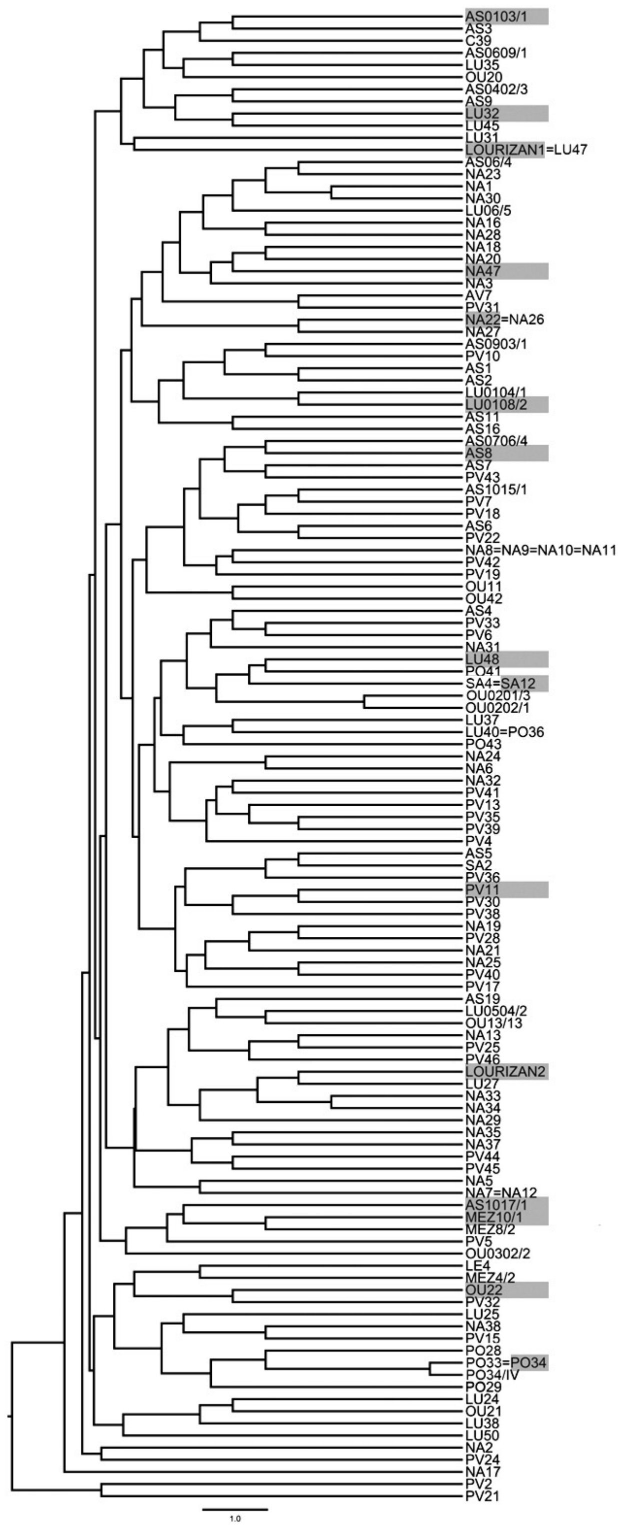


Figure 2. Membership coefficients calculated by the program STRUCTURE (Pritchard *et al.* 2000) for 122 MLGs and one MLL (PO34/IV) for $K = 2$. Each individual is represented by a vertical line partitioned into light gray and dark gray segments, the lengths of which indicate the posterior probability of membership in each group.



Supporting information 3. UPGMA dendrogram of genetic distances between 122 MLGs and a MLL (PO34/IV). A total of 6 MLGs contain more than one accession. Highlighted individuals indicate that the clone was selected for the Elite Population

comprised individuals sampled in Navarra, the Basque country, Asturias, León and Central Spain. The second genetic pool contained individuals from Atlantic and Eastern Galicia-Asturias. No clear assignation is achieved for individuals AS0402/3, AS1, AS19 (Asturias-León), OU42-PO28-PO29 (Atlantic Galicia). Notably, the triploid SA4 = SA12 is different from the other individuals of the Central Spain population and it was assigned to the Asturias-Galicia group. The two genetic clusters detected in this study can be explained by population fragmentation and reproductive isolation. The information provided by STRUCTURE can be useful for the selection of stands for the production of seeds for conservation plantations.

A dendrogram constructed according to nine nSSR data (Supporting information 3) of 122 MLGs and 1 MLL divided them into two main clusters. The accession PV2 and PV21 have the most different genotypes. The highlighted individuals are being used in the elite population for crosses. They are genetically quite different among them and it is expected that the crosses between these parental lines will produce individuals with a superior expression of economic traits.

Acknowledgments

This study was funded by the research project: "Selección de cerezos por caracteres adaptativos y de crecimiento y creación de nuevos clones para la obtención de una población de mejora de Galicia - 10MRU502022PR" funded by Xunta de Galicia. We thank M. Carmen Moreno-Ortiz for the flow citometry analysis.

References

Arnaud-Haond S, Belkhir K, 2007. GENCLONE: A computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. *Mol. Ecol. Notes* 7: 15-17.

Chapuis M-P, Lecoq M, Michalakis Y, Loiseau A, Sword GA, Piry S, *et al.*, 2008. Do outbreaks affect genetic population structure? A worldwide survey in Locusta migratoria, a pest plagued by microsatellite null alleles. *Mol. Ecol.* 17: 3640-3653.

Clarke JB, Tobutt KR, 2003. Development and characterization of polymorphic microsatellites from Prunus avium "Napoleon." *Mol. Ecol. Notes* 3: 578-580.

De Cuyper B, Sonneveld T, Tobutt KR, 2005. Determining self-incompatibility genotypes in Belgian wild cherries. *Mol. Ecol.* 14: 945-955.

De Rogatis A, Ferrazzini D, Ducci F, Guerri S, Carnevale S, Belletti P, 2013. Genetic variation in Italian wild cherry (Prunus avium L.) as characterized by nSSR markers. *Forestry* 86: 391-400.

Dorken ME, Eckert CG, 2001. Severely reduced sexual reproduction in northern populations of a clonal plant, Decodon verticillatus (Lythraceae). *J. Ecol.* 89: 339-350.

Ellstrand NC, Roose ML, 1987. Patterns of genotypic diversity in clonal plant species. *Am. J. Bot.* 74: 123-131.

Evanno G, Regnaut S, Goudet J, 2005. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.* 14: 2611-2620.

Felsenstein J, 1989. PHYLIP - Phylogeny Inference Package (Version 3.2). *Cladistics* 5: 164-166.

Guarino C, Santoro S, Simone LD, Cipriani G, 2009. Prunus avium: nuclear DNA study in wild populations and sweet cherry cultivars. *Genome* 52: 320-337.

Hardy OJ, Vekemans X, 2002. SPAGeDI: A versatile computer program to analyse spatial genetic structure at the individual or population levels. *Mol. Ecol. Notes* 2: 618-620.

Hubisz MJ, Falush D, Stephens M, Pritchard JK, 2009. Inferring weak population structure with the assistance of sample group information. *Mol. Ecol. Resour.* 9: 1322-1332.

Pritchard JK, Stephens M, Donnelly P, 2000. Inference of population structure using multilocus genotype data. *Genetics* 155: 945-959.

Serres-Giardi L, Dufour J, Russell K, Buret C, Laurens F, Santi F, 2010. Natural triploids of wild cherry. *Can. J. For. Res.* 40: 1951-1961.

Tanceva Crmaric O, Stambuk S, Satovic Z, Kajba D, 2011. Genotypic diversity of wild cherry (Prunus avium L.) In the part of its natural distribution in croatia. *Sumar. List* 135: 543-555.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P, 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* 4: 535-538.

Vaughan SP, Russell K, 2004. Characterization of novel microsatellites and development of multiplex PCR for large-scale population studies in wild cherry, Prunus avium. *Mol. Ecol. Notes* 4: 429-431.

Von Schelhorn M, 1947. Über eine triploide Vogelkirsche. *Der Züchter* 17-18: 232-235.