Plant Omics Journal POJ

POJ 6(3):165-174 (2013) ISSN:1836-3644

Establishment of molecular markers for germplasm management in a worldwide provenance *Ribes* spp. collection

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Abstract

The variability of a *Ribes* spp. germplasm collection was characterized using molecular markers as a complementary strategy to traditional phenotyping approach to redefine the plant genetic resources within the Fondazione Edmund Mach (FEM) *Ribes* collection. With 91 accessions, the FEM *Ribes* spp. collection contains a major part of the European and some extra-European germplasm. Molecular markers were used for its characterization and to study possible genetic relationships between species and cultivars. A total of 138 blackcurrant (*Ribes nigrum* L.), six jostaberry (*R. nigrum* L. × *nidigrolaria* Bauer and *Ribes nidigrolaria* Bauer × *nidigrolaria* Bauer) and 147 red, white and pink currant (*Ribes rubrum* L., *R. petraeum* Wulf., *R. longeracemosum* Franch., *R. multiflorum* Kit.) clones belonging to the 91 accessions, were genotyped using ten simple sequence repeat (SSR) markers. Seventy-five of these accessions were evaluated using 17 phenological descriptors. The SSR characterization identified 87 unique multilocus genotypes (MLGs), allowing 96% of samples analyzed to be uniquely discriminated. Phenotypic and genetic diversity were related to the taxonomy and geographical origin of provenience. We demonstrated that a limited set of markers has proved highly informative for studying the diversity and relationships within our collection and to identify synonyms, homonyms and mislabeling. The knowledge derived from genotyping associated with phenological and agronomic traits will be the background for the development of marker-assisted breeding strategies for *Ribes*, to improve fruit quality and to expedite the process through which new cultivars are developed.

Keywords: *Ribes* spp., SSR, Co-dominant markers, germplasm.
Abbreviations: AFLP- amplified fragment length polymorphism; EST- expressed sequence tag; FEM- Fondazione Edmund Mach; ISSR- inter-simple sequence repeat; PIC- polymorphic information content; RAPD- random amplified polymorphic DNA; SAF- shared allele frequency; SNP- single nucleotide polymorphism; SSR- simple sequence repeat; UPGMA- unweighted pair group method with arithmetic mean analysis.

Introduction

The *Ribes* L. genus includes more than 150 described species of shrubs mostly found in temperate regions of eastern and northern Europe, Asia, North and South America, and northwestern Africa (Brennan, 1996). In the past, *Ribes* spp. were classified in the family Saxifragaceae, but currently taxonomic treatments place them in Grossulariaceae because of some phenotypic characteristics. The main *Ribes* crop is *R. nigrum* L. (blackcurrant), followed by *R. rubrum* L. (red, white and pink currant) and some *R. uva-crispa* L. (gooseberry). The basic chromosome number of all species is x=8 (Zielinski, 1953), cultivars are diploid ($2n = 2x = 16$), and the mean genome size was estimated to be 2C = 1.91 pg in *R. petraeum* Wulf., *R. rubrum* and *R. grossularia* L. (Chiche et al., 2003). The practice of *Ribes* breeding and domestication has evolved only within the last 450 years. *R. nigrum* isolates were originally collected from the wild by herbalists, for their medicinal properties. Blackcurrants were imported from Holland to the United Kingdom in 1611 (Brennan et al., 1996) and in the 1800s began to be cultivated to obtain processed products such as jelly. The breeding of currants, and in particular of blackcurrant, now focuses on the production of fruits with elevated levels of nutritional components such as flavoniods, as well as resistance to major pests and diseases (Brennan, 2006). Moreover, other characteristics such as response to declining levels of winter chilling (Sunley et al., 2006) are likely to increase in importance in the future. To facilitate modern breeding of new varieties incorporating these and other characteristics, morphological descriptors are necessary. Knowledge of the genetic diversity of the different currants collections and gene banks is to some extent inadequate and the accessions need to be better characterized at phenotypic level (Pluta et al., 2012). However, such phenotypic descriptors, that are referred to major morphological characteristics related mainly to fruit attributes and agronomic related traits, need to be supported by genetic data since they are susceptible to environmental effects and to different evaluation methods used in describing varieties. A reliable, unambiguous genetic identification of *Ribes* accessions is mandatory for biodiversity preservation programs, managing breeding programs and for nursery and germplasm plant certification. Furthermore, the legal right to market a newly-bred cultivar requires information to distinguish it from all other previously released genotypes in order to guarantee its authenticity as a new variety to producers and the consumers.
The establishment of PCR-based molecular markers, such as random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs) has created the opportunity for fine-scale genetic

characterization of germplasm collections in different plant species (Baldoni et al., 2009). In the case of *Ribes*, several molecular markers, such as RAPDs (Korbin et al., 2002), inter-simple sequence repeats (ISSRs) (Lanham et al., 2000; Korbin et al., 2002), anchored microsatellites (Lanham & Brennan, 1998), SSRs (Cavanna et al., 2009), expressed sequence tags-SSRs (EST-SSR) and SNPs (Brennan et al., 2008; Russell et al., 2011) were already successfully developed to assess the relationships among different species, cultivars and selections and to obtain genetic linkage maps. In general, SSRs have been demonstrated to be the most suitable markers for genetic diversity evaluation in germplasm collections in different plant species (Belaj et al., 2012). In the present work, a number of SSR markers and 17 phenotypical descriptors were selected for fingerprinting and evaluating a collection of *Ribes* accessions from the Fondazione Edmund Mach (FEM), representing the majority of the genotypes cultivated at European and extra-European level. These markers and descriptors were used to assess the diversity, relationship and differentiation within and between different *Ribes* species and to obtain individual fingerprints for accessions and selections. The presence of some mislabeling within the investigated germplasm was also evaluated. These results will be of interest for the conservation of genetic resources and germplasm management.

Results

SSR polymorphism

All ten SSR loci analyzed were polymorphic in both species. The number of alleles amplified in *R. nigrum* (blackcurrant and jostaberry), *R. rubrum* (red, white and pink currant) and the total sample analyzed was 56, 66 and 99, with mean values of 5.6, 6.6 and 9.9 respectively, ranging from 3 (g1-I02, g2-J08 and gr2-J05**)** to 16 (e4-D03) alleles per locus. Thirty alleles were restricted to *R. nigrum* germplasm and 43 to the *R. rubrum* germplasm. Sixteen alleles were unique to a specific cultivar for blackcurrant and ten for red, white and pink currant accessions. Nineteen alleles were unique to a single cultivar when the overall germplasm was analyzed. The loci with the highest number of alleles were e4-D03 (11, 10 and 16 respectively) for all germplasm and e3-M04 (10) for the *R. rubrum* germplasm. The locus g2-J08 showed the lowest number of alleles in blackcurrant (3) and the total germplasm (6), as well as the locus g1-I02 in blackcurrant (3). The locus gr2-J05 showed the lowest number of alleles in the combined red, white and pink currant accessions (3) (Table 3). The expected heterozygosity averaged 0.58, 0.62 and 0.72 in *R. nigrum*, *R. rubrum* and whole germplasm, respectively. Moreover, it ranged between 0.77 (g2-G12) and 0.35 (e1-O21) with regard to the blackcurrant, 0.77 (e4-D03 and e1-O20) and 0.20 (g1-K04) with regard to the red, white and pink currant and finally 0.85 (e3-M04) and 0.61 (3-B02) in the total accessions. A tendency towards lower observed (H_o) than expected (H_e) heterozygosity was apparent at most loci (g1-K04, e3-M04, e4-D03, e1-O2, g2-J08, e3-B02, gr2-J05) when the whole germplasm collection was evaluated, and at two (g1-K04, e3-M04) and four (e3-M04, e4-D03, g2-J08, e3-B02) loci when the blackcurrant and white/red currant germplasm was analyzed separately. Based on PIC (from 0.60 to 0.83) and Dj (from 0.61 to 0.85) values, all analyzed loci were classified as informative markers (PIC and Dj > 0.5) for the whole germplasm analysis. For two (e1-O20 and e1-O21) and three (g1-K04, e3-B02, gr2-J05) out of ten loci analyzed in blackcurrant and white/red currants, respectively, the information level decreased when the two species were considered separately (Table 4). The inbreeding coefficient (f) ranged from – 0.04 to 0.53, with an average of – 0.05 for blackcurrants and from – 0.07 to 0.75, with an average of 0.06 for white/red currants.

The combined probability of identity (PI) from all 10 loci was 3.29×10^{-11} and ranged from 0.039 for e3-M04 to 0.191 for e3-B02, with an average of 0.1. (Table 4). Using the 10 selected markers, 87 unique multilocus genotypes (MLGs) were described from the 91 clones in the dataset, allowing 96% of samples analyzed to be uniquely discriminated. The box plot presented in Fig. 3 shows the improvement in discriminating power gained by increasing the number of SSR markers analyzed and demonstrates that an accurate determination of the number of genotypes in the samples is reached with at least 6 markers.

Genetic relationship among Ribes cultivars

The SSR data obtained were used to establish genetic relationships among the cultivars. The cluster analysis, carried out using shared allele genetic distance, grouped most of the clones belonging to the same accession, with the exception of a single clone belonging to Rosetta, Hosszufuvtu, Ben Nevis, Rosenthal S4 and Zitavia accessions (data not shown), indicating these clones were rogues. These clones were discarded from further statistical analysis. As far as the similarity analysis carried out on 91 accessions was concerned, the cophenetic correlation coefficient between the dendogram and the original similarity matrix was significant and high (r = 0.94). The dendrogram topology (Fig. 4) showed a clear separation of accessions into two major clusters (I and II), with four (1-4) and two (5 and 6) sub-clusters, respectively. The first cluster (I) contained all blackcurrant accessions plus the jostaberry varieties. In this group most of the accessions of blackcurrant that were developed in Germany, France, Netherlands and the single variety derived from Sweden grouped together in the sub-cluster 1, including two accessions developed in the UK (Baldwin Hilltop and Wellington), one from Romania (Record) and one selection with unknown provenance (Ecm 3/5). The accessions derived from Romania (Ronix, Abanos, Deea, Geo, 124_3) clustered in sub-cluster 2 together with three accessions derived from the UK (Ben More, Bdown and Ben Torn), one from the Netherlands (Tenah), France (Troll) and Poland (PC96). Another sub-cluster (3) grouped most of the accessions developed in UK (Ben Sarek, Ben Loyal, Ben Nevis, Ben Tirran, Ben Adler, Ben Connan) with four geographically unrelated accessions (Tines, Gofert, Sanijuta and Climax). Finally sub-cluster 4 included all the accessions developed in the Russian Federation, with the exception of the Sanjiuta, which was included in sub-cluster 3. All *R. rubrum* accessions were grouped in cluster II (Fig. 1). All the white accessions (Witte Parel, Zitavia, Werdavia, Bar le Duc, White Versailles, Viktoria, White Hollander, Blanka, Oroin, Primus, Jantar) and the pink variety (Rosa Sport) grouped together into the sub-cluster 5. Moreover, the analysis highlighted a clustering (sub-cluster 6) of all the accessions derived from USA (Perfection, Fay's Prolific, Red Lake), the red accessions of Netherlands (Stanza, Rotet, Rote Vierlander, Rolan, Roodneus, Augustus, Cassa, Rosetta, Jonkeer Van Tets, Rovada), the Slovenian and Romanian red accessions (Morawan, Koral, Trent, Losan, Vitan, Tatran, Rosu Timpuriu, 11_50, Abundent), two German accessions (Heinemann's Rote Spatlese and Heros), the English accession Lopper Summer and the French accession Junifer.

Table 1. List of *Ribes* accessions analyzed.

Accession	Pedigree	Provenance	Colour
Climax	selection of *R. nigrum* L.	Canada	black
Josta	F2 o.p. selection	Germany	black
RosenthalS4	selection of *R. nigrum* L.	Germany	black
RosenthalS1	selection of *R. nigrum* L.	Germany	black
Jostabes	(*R. nigrum* L. × *R. divaricatum* Douglas) × (*R. nigrum* L. × *grossularia* L.)	Germany	black
Tifon	Øjebyn × Tsema	France	black
Andenga	Noir de Bourgogne × Tsema	France	black
Troll	Tsema × Bdown	France	black
Noir de Bourgogne	selection of *R. nigrum* L.	France	black
Bdown	Baldwin × Brodtorp	UK	black
Davison eight	selection of *R. nigrum* L.	UK	black
Ben Torn	(ND12/26 × (('Vistavotnjaja' ×('Mendip Cross' × *R. dikuscha* Fisch. Ex Turcz.) × ('GoliathxØjebyn')) × 'Westra')	UK	black
Ben Sarek	(Goliath × Øjebyn)o.p.	UK	black
Ben Loyal	parentage unknown	UK	black
Ben More	Goliath × Øjebyn	UK	black
Ben Nevis	(Consort × Magnus) × (Brodtorp × Jaslunda)	UK	black
Ben Lomond	(Consort × Magnus) × (Brodtorp × Jaslunda)	UK	black
Baldwin Hilltop	Seedling selction of cv. Baldwin	UK	black
Wellington	Boscoop Giant ×Baldwin	UK	black
Ben Tirran	Ben Lomond × (Seabrooks B × Amos B × *Ribesia spp*)	UK	black
Ben Adler	(Goliath × Øjebyn) × Ben Lomond	UK	black
Ben Connan	Ben Sarek × Ben Lomond	UK	black
Tenah	selection of *R. nigrum* L.	Netherlands	black
Tsema	selection of *R. nigrum* L.	Netherlands	black
B Reward	Haarsteeegse o.p.	Netherlands	black
PC 96	Titania × Consort	Poland	black
PC 425	Titania × Ben Hope	Poland	black
Ruben	Beloruskaja Slodkaja × Ben Lomond	Poland	black
Gofert	Golubka × Fertodi_1	Poland	black
Tines	Titania × Ben Nevis	Poland	black
Ores	(Øjebyn × S_{24}) × Ceres	Poland	black
Ronix	parentage unknown	Romania	black
Abanos	Tsema × Kantata50	Romania	black
Deea	Tsema × Kantata50	Romania	black
Geo	Tsema × Kantata50	Romania	black
Record	parentage unknown	Romania	black
124_3	Tsema x Kantata50	Romania	black
Sjuta Kiyew	(Snostb × Zoya) ×Minaj Shmyrev	Russ.Fed.	black
Chernieca	Novosti Prikarparaja × Minaj Shmyriov	Russ.Fed	black
Sanjiuta	(Snostb × Zoya) × Minaj Shmyrev	Russ.Fed	black
CzornjCzemeczuc	Minaj Shmyriov × Brodtorp	Russ.Fed	black
Titania	Altayskaya Desertnaya ×(Consort × Kajanin Musta)	Sweden	black
Ecm 3/5	parentage unknown	unknown	black
Neva	parentage unknown	unknown	black
Vladimirskaja	parentage unknown	unknown	black
Witte Parel	parentage unknown	Belgium	white
Heinemann's RoteSpatlese	Red Duch × Aneken Lorbus	Germany	red
Zitavia	Selection of white currant	Germany	white
Werdavia	White Hollander authopollination	Germany	white
Heros	Laxtons Perfection o.p.	France	red
Junifer	Fay's Prolific authopollination	France	red
Bar le Duc	White mutant selection of red currant	France	white
White Versailles	parentage unknown	UK	white
London Market	selection of red currant	UK	red
Redpoll	Red Lake × (*R. longeracemosum* Franch.×*R. multiflorum* Kit.)	UK	red
Lopper Summer	parentage unknown	Hungaria	red
Hosszufuvtu	parentage unknown	Netherlands	red
Stanza	selection of *R.rubrum*	Netherlands	red
Rotet	Jonkheer Van Tests × Rote Spätlese	Netherlands	red
Rote Vierlander	*parentage unknown*	Netherlands	red
Rolan	Jonkheer Van Tests × Rosetta	Netherlands	red
Roodneus	Jonkheer Van Tests ×*R. multiflorum* Kit. ex Schult	Netherlands	red
Augustus	Jonkheer Van Tests × *R. multiflorum* Kit. ex Schult	Netherlands	red
Cassa	Fay's Prolific x Rondom	Netherlands	red
Rosetta	Jonkheer Van Tests × Heinemann's Rote Spatlese	Netherlands	red
Jonkeer Van Tets	Erly-ripening seedling selection of Fay's Prolific	Netherlands	red
Rovada	Fay's Prolific × Heinemann's Rote Spatlese	Netherlands	red
White Hollander	white mutant of red currant	Netherlands	white
Rosa Sport	parentage unknown	Romania	pink
Rosu Timpuriu	Rouge de Versailles × Heros	Romania	red
11_50	Rosu Timpuriu × Houghtno Castle	Romania	red
Abundent	Duch red × (Rouge de Versailles × Fay's Prolific)	Slovenia	red
Viktoria	(Heinemann's rote Spätlese x Red Lake) × Jonkheer van Teets	Slovenia	white
Morawan	Fertodi_1 × Roodknop	Slovenia	red
Koral	Klon L. × Erstling aus Vierlanden	Slovenia	red
Trent	Erstling aus Vierlanden × Kaukasishe Rote	Slovenia	red
Losan	Chenonceaux × Vierlandensky	Slovenia	red
Vitan	Erstling aus Vierlanden × Chenonceaux	Slovenia	red
Tatran	Red Lake × Goppert	Slovenia	red
Detvan	Jonkheer Van Tests × Heinemann's Rote Spatlese	Slovenia	red
Blanka	Heinemann's Rote Spatlese × red Lake	Slovenia	white
Orion	Heinemann's Rote Spatlese × red Lake	Slovenia	white
Primus	Heinemann's Rote Spatlese × red Lake	Slovenia	white
Jantar	Erstling aus Vierlanden × Heros	USA	white
Red Lake	parentage unknown	USA	red
Fay's Prolific	Victoria × Cherry	USA	red
Perfection	Fay × White Grape	Unknown	red
Erde	parentage unknown	Unknown	red
Katawskaya	parentage unknown	Unknown	red
Troitzka	parentage unknown	Unknown	red
85_6_28	parentage unknown	Germany	red

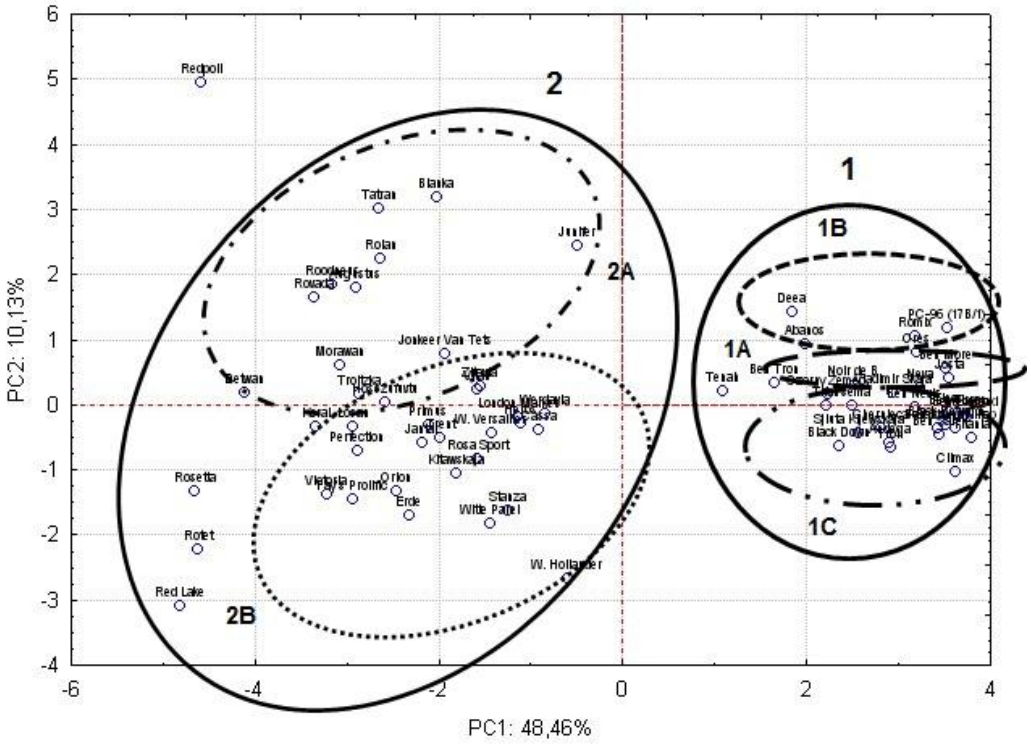

Fig 1. PCA phenotype-based relationships between accessions. A total of 75 Ribes accessions were phenotypically evaluated with 17 descriptors and were represented on the two first components (PC1 and PC2) of the principal components analysis (48,46% and 10,13% of the total variation explained by the first and the second component, respectively). Group 1 includes the *Ribes nigrum* accessions and the group 2 the *Ribes rubrum* accessions.

The STRUCTURE analysis indicated K=2 as the most likely number of clusters when the whole *Ribes* germplasm was analyzed. Both blackcurrant and jostaberry accessions were included in a first cluster, with a mean q value of 0.99; white and red and pink currants accessions were included in the second cluster with a mean q value of 0.99. The STRUCTURE analysis was therefore separately repeated on blackcurrant and red/white currant, suggesting K=1 and K=2 as the most likely number of clusters in the two datasets, respectively. K=1 (Fig 5A) indicated that all blackcurrant accessions and jostaberry varieties grouped in the same gene pool. K=2 (Fig 5B) highlighted the separation of white/pink and red currants in two gene pools. A first gene pool (A) was predominant across red accessions with a mean q of 0.87 and a second gene pool (B) represented the white and pink accessions with mean q values of 0.93 and 0.79, respectively. As the only exception, two red varieties (Red Pool and London Market) were assigned to pool B with q values higher than 0.95. The proportions of membership (q) of each individual in each gene pool are shown in Fig. 5

Phenotypic characterization

The variance explained in the PCA (Fig. 1) derived from the analysis of morphological descriptors was 58.59%. The score plot of the first and second principal components obtained by Principal Components Analyses of the seventeen different phenotypic descriptors is shown in Fig. 2: PC1 accounts for 48.46% of the variance, while the second for 10.13%. In the major black fruit group, labeled as (1) in the PCA quadrant characterized by positive values for both PCs (Fig. 1), two main clusters were resolved, the first containing all the 'Ben' varieties and two varieties from the Russian Federation together with Josta (1A), and the other containing material from Eastern Europe including accessions and one advanced selection from Poland (Ruben, Ores and PC96), along with Ronix, Deea and Abanos from Romania (1B). The quadrant characterized by positive values for PC1 and negative values for PC2 highlights a major cluster of plant material that mainly originated in the UK, the Netherlands and France (1C). In the red/white/pink fruit group (2), the separation of two subclusters was clearer and mainly corresponded to the two PCA quadrants: the first, characterized by positive values for PC2 and negative for PC1 (2A), mainly plots red fruited genetic material from the Netherlands and France, while in the quadrant characterized by negative values of both PCs (2B), most of the white fruited types and red from the USA, Slovenia and Romania cluster together. The Redpoll variety clustered separately from the other accessions.

Discussion

Molecular diversity

In this study, ten previously mapped SSR markers were used to characterize the *Ribes* spp. germplasm maintained in the soft fruit collection at FEM. All ten SSRs gave qualitatively good amplification and were polymorphic. In the whole germplasm analysis, the mean number of alleles per locus (9.9), the mean observed heterozygosity (0.75) and the H_o values, lower than the H_e values at most of the loci, were comparable to the values found in other studies carried on *Ribes* spp. (Brennan et al., 2002; Cavanna et al., 2009). The 19 unique alleles allowed the unambiguous identification of 15 single accessions (Ores, Jostabes, Ben Connan, Davison eight, Neva, Jonkeer Van Test, SelPC 96, Troll, Geo, Blanka, Roodneus, Redpoll, London Market, Stanza, Zitavia) using only one molecular marker. Considering the different

Table 2. Ten SSRs chosen for DNA analysis, their fluorescent label and the linkage group to which they were mapped in a previous study (Russel et al., 2011).

SSR Locus	Forward sequence (5'-3')	Reverse sequence (5'-3')	Florescent dye	Linkage group
g1-K04	TGTTCCCTGTTTCCTTCAAAA	GGACGTGGACGATGAGAGTT	6-FAM	1
e3-M04	CTTACCCACCCCACCACC	TGTGTTCTCATCAGAGACTTTCG	HEX	3
e4-D03	CCCAAAAGCAAATTTAGGGT	GTGAGGCATGGAACCACTTT	6-FAM	3
e1-O20	CATTGCTTGAAGTTGAACACA	CGACAGGTTCTTGCCTTAGC	HEX	1
e1-O21	TCTCTCCAACTGAGAAGGAAA A	GATTTGTTCTTGTGCAGCGA	6-FAM	4
g1-I02	TGAATATCAGACCGCCATCA	TCCAAATGAAGCTTCTCAAATC	HEX	6
g2-J08	CGCCGAGCTCTAATCACTGT	ATAGCCCATGCCCATATTCA	6-FAM	2
e3-B02	AAGACGAAGACACGACGAT	CTGATCTTTGCCGAATGGTT	6-FAM	5
gr2-J05	CAAAACTGATTAGG GATCA	TTTGAAGAAGAGATGGCGAAA	HEX	1
g2-G12	GTGACCCACCTAAACCGTCC	GGAGTGGAGGGTTGGAAAAT	HEX	7

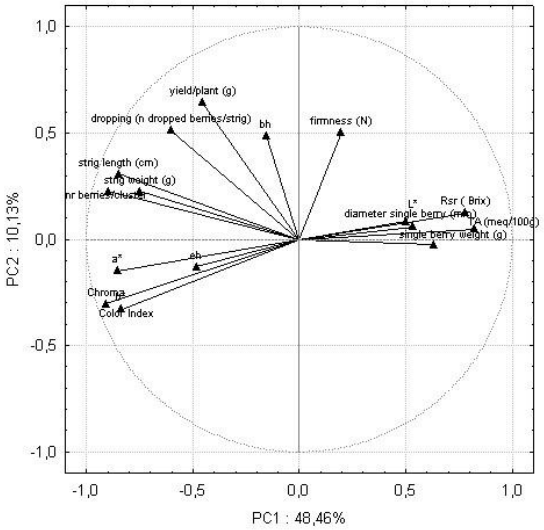

Fig 2. Phenotypic descriptors distribution according to a 2D-PCA plot illustrating their variability within the *Ribes* germplasm collection.

diversity parameters analyzed (H_e, PIC, Dj) the most informative loci were e4-D03 and e3-M04 (Table 4). The f values were negative for most of the loci analyzed in blackcurrant (8/10) and in red, white and pink currant (6/10) and indicates an excess of heterozygosity in respect to what would be expected under random mating. The same result was found in other plant species such as *Olea europaea* (Baldoni et al., 2009) and the domesticated *Vitis vinifera* (Sefc et al., 2000) where plants are derived from clonal reproduction. In fact, in this case, f values are expected to be negative since clonal reproduction enables the two alleles at each locus to independently accumulate mutations diverging within individuals (Halkett et al., 2005). The total PI value of 3.29×10^{-11} was in accordance to the PI value of 4.1×10^{-11} found in grape germplasm using nine SSR markers (Laucou et al., 2011), the PI value of 5.06×10^{-14} found in *Ribes* (Cavanna et al., 2009), and the PI value of 4.097×10^{-10} found in *Cacao* germplasm (Motilal et al., 2012). Moreover the GenClone box plot analysis showed that starting from six loci combinations it is possible to discriminate the maximum clonal diversity represented by the samples (Fig. 3). These results, combined with the PI analysis result, showed that an appropriate number of loci were used for the aims of the present work.

Accessions discrimination and underlying genetic structure in a Ribes spp. germplasm

Molecular markers have been used to characterize a germplasm including different *Ribes* spp. and a high number of accessions. Using these markers, some cases of possible mislabeling of clones considered to belong to the same variety or selection were detected. These results therefore confirmed the utility of the SSRs assayed in providing information on plant mislabeling or homonyms and synonyms (Barranco et al., 2000; Bracci et al., 2009). A clear genetic structure was observed in the *Ribes* germplasm by the UPGMA and STRUCTURE analysis. A clear separation between the species referring to blackcurrant/jostaberry and red/white currant was apparent from the data. All the accessions belonging to *R. nigrum* and crosses between *R. nigrum* and other species *(R. divaricatum, R. dikuscha* Fisch., *R. uva-crispa*) formed the largest cluster. The second largest group included all the samples belonging to *R. rubrum* and crosses of this with other species (*R. multiflorum, R. petraeum, R. longeracemosum*) or the white mutants (Cavanna et al., 2009). Within this cluster, using the UPGMA approach, it was possible to group accessions derived from the same geographical region. In sub-cluster 1, all the accessions developed in northern and middle Europe were present, with the exception of the Wellington variety that was derived from a cross between Booscop Giant (Dutch variety) and Baldwin, and other accessions that are selections of *R. nigrum* or that have unknown parentage (Baldwin Hilltop, Record, Ecm 3/5) (Cavanna et al., 2009). The Romanian accessions clustered together (sub-cluster 2) and with Blackdown, Ben Torn and Troll, all accessions sharing common ancestors (Tsema, Baldwin, *R. dikuscha*). Sub-cluster 3 grouped all the plants developed in the UK plus Climax (selection of *R. nigrum* from Canada); the latter could

Table 3. SSR allele sizes and distributions in the different species analyzed.

SSR Locus	Size (bp)	No. alleles			No. private alleles		No. unique alleles		
		b	r/w/p	all	b	r/w/p	b	r/w/p	all
g1-K04	278-300	4	4	7	3	3	1	1	2
e3-M04	294-340	9	10	14	4	5	3	2	1
e4-D03	164-242	11	10	16	6	5	5	-	4
e1-O20	192-230	4	6	11	4	6	2	2	4
e1-O21	286-306	4	6	9	2	5	2	1	2
g1-I02	120-130	3	6	6	-	3	-	-	-
g2-J08	140-178	3	7	8	1	5	-	-	-
e3-B02	145-176	7	6	10	3	3	2	3	4
gr2-J05	158-185	4	3	7	4	3	1	1	2
g2-G12	168-196	7	8	11	3	5	-	1	-
Total		56	66	99	30	43	16	10	19
Mean value		5,6	6,6	9,9	3,3	4,3	2,3	1,4	2,7

b: blackcurrant; r: red currant; w: white currant; p: pink currant.

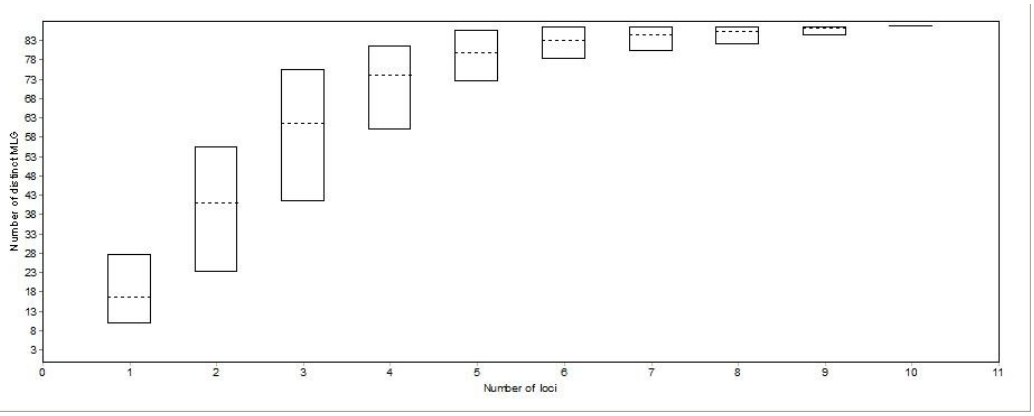

Fig 3. Box plot describing the genotypic resolution of microsatellites in a data set of a Ribes spp germplasm containing 91 accessions genotyped using 10 SSRs, analysed for each possible combination. The edges of the boxes show the minimum and maximum number of genotypes and the central line shows the average number of genotypes identified. This figure shows the improve in discriminating power gained by increasing the number of SSR markers analysed

have some parentage with the Canadian Magnus variety present in Ben Nevis pedigree that, in turn, is present in the Tines pedigree. All the varieties with Russian Federation provenance were included in sub-cluster 4. The two accessions named Josta and Jostabes are both jostaberries, but only the Jostabes variety was separated from all the others, as expected from its pedigree. The Ores variety was also separated from other accessions, being the only variety that has Ceres (Polish o.p.) in its pedigree. Within the red/white currants cluster II, all the white currant accessions plus the single pink colored variety clustered together (sub-cluster 5), according to two different clustering approaches. A concordant result was previously reported in the work by Lanham & Brennan, (1998), where Werdavia, White Pearl and Zitavia varieties were analyzed using anchored SSR markers. The last sub-cluster (6) grouped accessions with different provenance (USA, Netherlands, Slovenia, Romania, Germany, UK and France), all sharing common kinship. Two varieties, Victoria and Rote Vierlander, are the parents of Fay's Prolific, Heinemann's Rote Spatlese and Erstling aus Vierlanden that are directly present in the pedigree of most of the red currants or indirectly, such as for the varieties that are parents of Jonkeer Van Tests that is a early-ripening seedling selection of Fay's Prolific. The London Market and Redpool varieties, developed in the UK, clustered separately from the other varieties. Since the white currants are mutant selections of red currants the STRUCTURE analysis revealed a certain admixture between the red and white accessions. The inferred ancestry value for Jantar (white currant variety) was 54.6% for cluster B and 45.4% for cluster A, probably because of its parentage containing Erstling aus Vierlanden as is the case for other red varieties from Slovenia. For the two red varieties London Market and Redpoll and the 85_6_28 red selection, the inferred ancestry values were respectively 5.4 (A) and 94.6 (B), 2.7 (A) and 97.3 (B), 34.6 (A) and 65.4 (B). The London Market and Redpool varieties were both developed in the UK and are known to contain Red Lake in their parentage. Characterization of the genetic diversity present in FEM germplasm, together with the identification of mislabeling, synonymous and homonymous genotypes, were the main goals in genotyping the collection, permitting a robust classification of the plant material from which metabolomics and genetics studies have developed at FEM. To reach these goals, SSRs markers have been employed because of their reliability and reproducibility (Riaz et al,. 2008; Cheng et al., 2009; Belaj et al., 2012). The present work confirmed that these markers do represent an effective method to characterize a currant germplasm, to distinguish between different *Ribes* spp. and between different accessions (Cavanna et al., 2009; Antonius et al., 2012) on a very large number (91) of genotypes. Moreover, the present data contributed to deepen the genetic characterization of the crop through the use of SSRs, some of which were previously untested for germplasm characterisation. In the light of the present results, we were able to remove the clones that were mislabeled and to test the homogeneity of clones belonging to the same accession. In fact, admixture in varietal material is unwanted in the whole production chain both for fresh and

Table 4. Description of genetic parameters estimated for the selected SSR markers using the profile of 291 clones of 91 accessions.

SSR Locus	He			Ho			PIC			f		Dj			PI
	b	r/w/p	all	b	r/w/p	all	b	r/w/p	all	b	r/w/p	b	r/w/p	all	all
g1-K04	0,52	0,20	0,67	0,36	0,21	0,28	0,45	0,19	0,60	0,32	-0,07	0,52	0,20	0,67	0,169
e3-M04	0,70	0,74	0,85	0,33	0,67	0,51	0,66	0,69	0,83	0,53	0,09	0,70	0,73	0,85	0,039
e4-D03	0,64	0,77	0,84	0,67	0,19	0,42	0,59	0,74	0,81	-0,04	0,75	0,64	0,77	0,83	0,046
e1-O20	0,38	0,77	0,79	0,47	0,98	0,73	0,32	0,72	0,76	-0,22	-0,28	0,38	0,76	0,79	0,070
e1-O21	0,35	0,74	0,73	0,36	0,87	0,62	0,30	0,70	0,69	-0,59	-0,17	0,34	0,74	0,73	0,107
g1-I02	0,63	0,74	0,79	1,00	0,91	0,96	0,55	0,69	0,74	-0,11	-0,24	0,62	0,73	0,78	0.083
g2-J08	0,60	0,70	0,75	0,67	0,30	0,48	0,51	0,64	0,70	-0,05	0,57	0,60	0,69	0,74	0,104
e3-B02	0,67	0,40	0,61	0,70	0,32	0,50	0,60	0,36	0,57	-0,24	0,20	0,66	0,39	0,61	0.191
gr2-J05	0,58	0,45	0,75	0,72	0,57	0,64	0,49	0,35	0,71	-0,16	-0,29	0,58	0,44	0,75	0,103
g2-G12	0,77	0,69	0,77	0,89	0,79	0,84	0,72	0,63	0,73	-0,05	-0,14	0,76	0,69	0,76	0,085
Mean value	0,58	0,62	0,75	0,62	0,58	0,60	0,52	0,57	0,71	-0,05	0,06	0,58	0,61	0,75	0,1

He: expected heterozygosity; Ho: observed heterozygosity; PIC: pholymorphic information content; f: inbreeding coefficient; Dj: discrimination power;PI: probability of identity b: blackcurrant: r red currant; w: white currant; p: pink currant.

processing purposes: the high discrimination power determined and the assessment of unique alleles presence could be a powerful tool to certify and to trace the *Ribes* varieties. Moreover, nurseries could take advantage of a precise characterization to discriminate mother plants for their stocks, growers could increase the certainty of growing appropriate material, while processing dealers could have the tools to accurately choose the accessions to enrich the raw material for processing, for example, of specific bioactive compounds.

Phenotypic diversity

The results of the phenotypic evaluation supported differences found between genotypes with different geographic origin and highlighted the variability present among the species and within this particular collection. Previous studies have demonstrated similar outputs on a blackcurrant collection (Pluta et al., 2012), but this study provides a more complete representation of the species, with a focus on accessions that also have a commercial interest. The phenotypic characterization as a result of the output of the different clusters of the PCA (Fig. 1) permitted correctly classifying the plant material and plant origin with acceptable probability, and showed that the assignment of cultivars to a specific group through phenotyping is feasible, however it is not as precise as the genotyping, and is much more time consuming.

Materials and methods

Plant material and DNA extraction

All plant material was supplied from the Fondazione Edmund Mach (FEM) *Ribes* germplasm collection, located in Vigalzano (Trentino, Italy) at 500 m above sea level. The germplasm includes 85 varieties and six selections, totaling 91 accessions. The collection was started in 2003 by collecting varieties and selections from different places in Europe and from Canada, the Russian Federation and the USA (Table 1). Leaves from two to seven clones of each accession, for a total of 291 samples, were collected and stored at -20°C until later use.

The tissue from young leaves (100 mg) was lyophilized and mechanically homogenized. DNA was extracted using the NucleoSpin® 96 Plant kit (MACHEREY-NAGEL, Germany) and the Tecan Automated Workstation (Switzerland), following the manufacturer's instructions. A final dilution was done in low salt buffer (TE) provided with

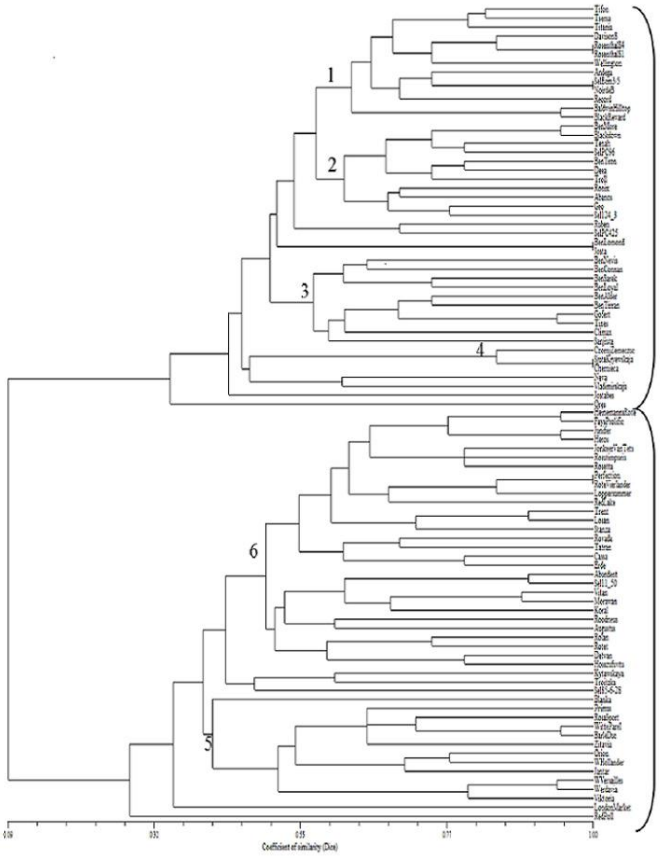

Fig 4. UPGMA dendogram of 91*Ribes* accessions based on alleles at ten SSR loci. Group I includes the blackcurrant and jostaberry varieties and is divided into four sub-clusters (1, 2, 3 and 4). Group II includes the red,white and pink currant varieties and is divided into two sub-clusters (5 and 6).

the kit and DNA was quantified with Q-bit fluorimeter (Invitrogen, Carlsbad, CA, USA).

Phenotyping

Seventeen descriptors, both subjective and objective, in accordance with those stipulated by UPOV (UPOV, 1989), were used for the evaluation and distinctness of 75 accessions within the collection. Most of the descriptors were related to fruit characteristics at maturity. The analyses were conducted

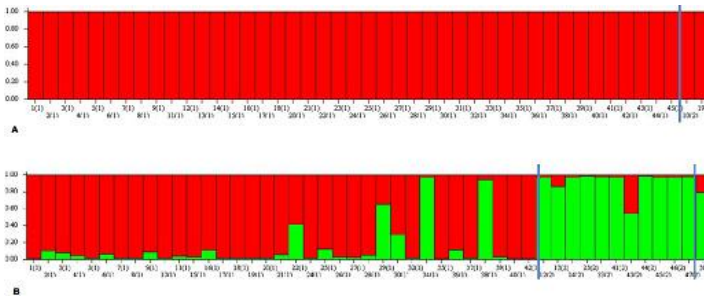

Fig 5. Results of the STRUCTURE analysis of *Ribes spp*. germplasm (A: blackcurrant. and jostaberry; B: red, white and pink currant). Each vertical bar represents one individual genotype. Individuals with multiple shades have admixed genotypes for multiple clusters. Clusters of individuals are represented by shades and population are separated by blue lines and labeled by a number between the parenthesis.

on 30 fruit clusters for 3 replicates on 3 to 5 plants per each genotype over two successive growing seasons. The descriptors used were: date of beginning and end of harvest, yield of fruit per plant (g), weight of the strig (g), strig length (cm), single berry weight (g), diameter of the single berries (mm), firmness (N) using a digital fruit firmness tester (TR Turoni srl, Forlì, Italy) with a 4 mm probe (held on a stand), number of berries per strig, attitude to fruit dropping (number of dropped berries/strig). Soluble solids content was determined with a DBR35 refractometer and expressed in °Brix content. Acidity (TA) was measured through titration with a Compact Titrator (Crison, Modena, Italy) and data were expressed in meq/100g. Colour measures were conducted by spectrophotometric means through a Minolta (CM 3600d) device for L, a, b parameters according to the Commission Internationale de l'Éclairage (CIE). Color characteristics were recorded by the use of a Minolta (L, a*, b*), Chroma $((a^*)^2+(b^*)^2)^{0.5}$ and color index $((200+(a^*/L^*))((a^*)^2+(b^*)^2)^{0.5})$ were calculated according to their respective formulas.

Amplification and allele sizing

Ten SSR loci were chosen from the list available from the Scottish Crop Institute (http://www.fruitbreeding.co.uk/RibesGenomicsSSRs.asp) and used for the analysis of *Ribes* germplasm (Table 2). Markers were selected, when possible, mapping to different chromosomes (Brennan et al., 2008; Russell et al., 2011). Information about the level of polymorphism was however available for only half the selected markers (Cavanna et al., 2009). Each of the forward primers of the set was 5' labeled with 6-FAM or HEX fluorochromes (Invitrogen). PCR was performed in a 12.5 µl reaction volume including 10-30 ng of DNA, 1.0 unit of GoldTaq DNA-polymerase (Applied Biosystems, Foster City, CA, USA), 1.2 µl of 10 X reaction buffer containing 1.5 $MgCl_2$ and supplemented with 0.32 mM dNTPs and 0.4 µM each primer. Thermal profile consisted of 7 min at 95°C; ten cycles of 45 s at 95°C, 45 s at 60°C decreasing to 55°C at 0.5°C per cycle and 30 s at 72°C, followed by 25 cycles of 45 s at 95°C, 45 s at 55°C and 90 s at 72°C, followed by a final extension of 8 min at 72°C. A total of 5 µl of each amplification product was preliminarily run on 1.5% (W/V) agarose gel in 0.5 X TBE buffer and visualized over UV light after staining with ethidium bromide. Amplicons were pooled from two different loci, marked with different fluorochromes and differing in size range (g1-IO2 (HEX) and g1-K04 (FAM), e3-M04 (HEX) and e4-D03 (FAM), e1-O21 (FAM) and e1-O20 (HEX), g2-G12 (HEX) and e3-B02 (FAM), g2-J08 (FAM) and gr2-J05 (HEX)). Samples were prepared by mixing 0.5 µl of the pooled PCRs with 9.8 µl of Hi-Di formamide™, (Applied Biosystems) and 0.2 µl of GeneScan 500 ROX size standard (Applied Biosystems). The mixture was then denaturated (95 °C for 3 min) and placed 5 min on ice prior to injection. Alleles were separated by capillary electrophoresis on the ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). Data were analyzed with GeneMapper 2.0 software (Applied Biosystems) and alleles were scored by their size in base pair (bp).

Data analysis

Genetic diversity

Different overall and locus-specific estimators of genetic diversity were recorded for the germplasm collection and within species, using the Excel microsatellites toolkit (Park, 2002). Allele-size range, total number of alleles, number of private (found in a single species but in one or more accessions) and unique (found in a single accession when a single species or the whole germplasm where analyzed) alleles are reported in Table 3. For each investigated locus, observed (H_o) and the expected (H_e) heterozygosity (Nei, 1978) were calculated, together with a number of parameters useful to estimate the markers resolution power: polymorphic information content (PIC) (Botstein et al., 1980) and the inbreeding coefficient (f) were calculated using PowerMarker software 3.25 (Liu and Muse, 2005); discriminating power of each SSR locus was calculated as follows:

$$D_j = 1 - \sum p_i\,(Np_i - 1)/(N-1)$$

where p_i was the frequency of the i-th molecular pattern revealed by locus j, and N was the number of individuals (Tessier at al., 1999).
The probabilty of identity (PI), that estimates the average probability that two unrelated individuals will have the same multilocus genotype, was calculated as:

$$PI = \left(\sum p_i^2\right)^2 - \sum p_i^4$$

using GenAlex v. 6 (Peakall and Smouse, 2006)).
GenClone v 2.0 software (Arnaud-Haond and Belkhir, 2007) was used to ensuring that the set of selected loci permits a good estimate of the real number of distinct multilocus genotypes (MLGs) (Arnaud-Haond et al., 2005). This analysis, combined with the PI estimate, allowed to ascertain the efficiency of the chosen markers combination. All the diversity parameters used are based either on a simple count (e.g. number of alleles, number of private alleles) or depend solely on allelic frequencies (H_e, H_o, PIC, Dj) and can therefore be estimated without assuming the Hardy Weinberg equilibrium.

Analysis of the genetic structure

To assess the structure of genetic diversity within and between the different *Ribes* species, shared allele frequency (SAF) between clones was determined using the PowerMarker software 3.25. The corresponding phenogram was drawn based on the UPGMA algorithm using the program Phylip 3.62 (Feselstein, 1989). All clones failing to cluster with their two or more related clones, derived from the same accession, were discarded from the following analysis due to their uncertain identity. Genetic similarity

was therefore evaluated on 91 samples (one for each selection considered) by estimating Dice coefficient (Dice, 1945). A UPGMA dendogram was generated with this coefficient using the SAHN-clustering and TREE programs of the NTSYS 2.10 software package (Rholf, 2005). A Mantel's test (Mantel, 1967) was performed to compute the correlation coefficient between the distance matrix and the cophenetic values matrix, to test the goodness of fit of the cluster analysis. The model-based clustering method implemented in STRUCTURE 2.3.3 (Falush et al., 2003, Pritchard et al., 2000) was applied to infer genetic structuring of the 91 samples and to define the most likely number of clusters (gene pools) they can be grouped in. Given a K value for the number of clusters, this method assigns individual genotypes from the entire samples to clusters in a way in which linkage disequilibrium is maximally explained. Ten independent runs of STRUCTURE were performed for each K value from 1 to 11. Each run consisted of a burn-in period of 100,000 steps, followed by 1,000,000 Monte Carlo Markov Chain replicates, assuming an admixture model and correlated allele frequencies. No prior information was used to define the clusters. The most likely K was chosen comparing 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 data between successive K values (Evanno et al., 2005). The proportion of membership (*q*) of each individual in each gene pool was estimated.

Phenotypic diversity

Multivariate statistics (principal component analysis, PCA), were carried out using the Statistica v.9 software for the analyses on the phenotypic data.

Conclusions

The characterization of genetic variability and relatedness in a species is essential for genetic improvement programmes, for marker assisted selection in breeding and for plant collection management. The present work confirmed that a small number of SSR markers are very useful for germplasm management, as demonstrated in other species (Laucou et al., 2012; Potts et al., 2012; Vilanova et al., 2012). Although a limited number, this set of markers was sufficient to effectively describe the *Ribes* germplasm variability and to discriminate between species and accessions, and thus helped increase the genetic understanding of the crop. These data, combined with the morphological and phenological characters, constitutes the necessary basis for a *Ribes* core collection available as a source of desirable traits for future uses.

Acknowledgments

The authors are very grateful to dr. Daniel James Sargent for helpful discussion and critical reading of the article.This work was supported by PAT, project INTERBERRY, Italy

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