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Genetic variation in Italian wild cherry (*Prunus avium* L.) as characterised by nSSR markers

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Abstract

The main aim of our research was to describe the level and distribution of genetic variability of wild cherry (*Prunus avium* L.) in Italy, using eight nuclear microsatellite markers. The sampled plants were grouped in 11 internally homogeneous breeding zones, defined according to their ecological and vegetational conditions. The mean observed heterozygosity (H_o) was 0.573, and the expected (H_e) 0.698. Significant departures from Hardy-Weinberg equilibrium at each locus were found for all breeding zones ($P<0.01$). The mean fixation index, calculated taking into account the estimated null allele frequencies, was 0.075, showing a slight excess of homozygotes. F_{ST} (departure of genotype frequencies within populations from Hardy-Weinberg expectations, commonly used as estimator of genetic differentiation among populations), showed a mean value of 0.046, indicating a slight, although significant, differentiation among breeding zones. However, in general, it was not possible to observe a structuring linked to the geographical location of the breeding zones. The results of the study contribute to a better understanding of our knowledge of the wild cherry genetic variation in Italy, thus making for more efficient programs aimed at the preservation of biodiversity and for more rational planning of the management of reproductive material. Since our results do not show a clear structuring of genetic variability within the Italian diffusion area of wild cherry, it is not possible to draw any indications on Regions of Provenance delimitation based only on genetic data, and the identification of the latter should be based mainly on ecological and vegetational features.

Key words

wild cherry, genetic variability, breeding zones, Regions of Provenance.

Introduction

Wild cherry (*Prunus avium* L.) is an important forest tree, usually characterised by a scattered distribution. It is used for high quality wood production, and is of great ecological and naturalistic importance, being an essential component of sensitive and threatened ecosystems, such as mixed deciduous forest. The species has hermaphroditic flowers, usually pollinated by a range of insects, mainly honeybees and bumblebees. Outcrossing is the principal mating system, with self-fertilisation usually being prevented by gametophytic incompatibility (Vaughan et al. 2008). Seeds are dispersed by gravity, and birds also play an important role in their dissemination. The species can also propagate through vegetative reproduction via root suckering, involving up to 65% of individuals in the population (Vaughan et al. 2007).

The species has a widespread distribution, extending from the Black Sea to Ireland and Spain, and from southern Scandinavia to northern Africa. In Italy it is mainly present in the Po Valley, in the bottom of Alpine valleys and along the Apennines. As we move to the south the species becomes even more and more scattered, and only a few isolated individuals can be found (Ducci 2005). The effects of the past civilisations, as well as the traditional silviculture coppice systems have played a role in determining this situation (Ducci and Proietti 1997).

Forest trees are static long-lived organisms which grow under environmental conditions that are heterogeneous in time and space. Moreover, they are exposed to many stress factors, most of which are due to human activities: pollution, climate change and habitat fragmentation. In order to survive these threats, and to persist over time, a high adaptive potential is needed, which is mainly determined by the within-species genetic diversity (Boshier and Amaral, 2004). Programmes aimed at the conservation of forest genetic resources should address the issue of maintenance of this diversity (Palmberg-Lerche 2001). To this end, knowledge of genetic variation, as well as information on mating system and pollen and seed dispersal, are of the utmost importance. These data provide important insights for preservation and restoration programmes, indicating areas of high genetic

diversity and geographic limits for seed collection, helping breeders to take decisions about crosses and germplasm management (Marchese et al. 2007), and delimiting the scale at which conservation actions should be planned (Escudero et al. 2003). Furthermore, genetic analysis can increase understanding of the historical processes that led to the present distribution of a species, while the preservation of germplasm is extremely important in order to meet future climatic, abiotic and biotic change (Marchese et al. 2007).

Molecular markers are now available which can provide us with the relevant means to acquire information on the genetic structure of populations, and to study the pattern of distribution of within-species variability. In particular, simple sequence repeats (SSRs, also known as microsatellites) are commonly used in genetic studies of plant populations. SSRs are tandem repeats of short DNA sequences (1 to 6 base pairs); they are highly polymorphic, widely distributed throughout the genome and codominant. Allelic variation can be detected quickly using the DNA-polymerase chain reaction (PCR) technique (Beckmann and Soller 1990).

Microsatellites have been widely used for genetic studies of cherry, although most studies involved the sweet cultivated forms. Research has included genetic diversity analysis (Dirlewanger et al. 2002, Wünsch and Hormaza 2002, Vaughan and Russell 2004, Ohta et al 2005, Guarino et al. 2009, Lacis et al. 2009, Ercisli et al. 2011), cultivar identification and fingerprinting (Cantini et al. 2001, Schüler et al. 2003, Gisbert et al. 2008, Clarke and Tobutt 2009, Wünsch 2009, Xuan et al. 2009, Avramidou et al. 2010, Ganopoulos et al. 2010, Gulen et al. 2010), self-incompatibility and population genetic structure evaluation and recruitment (Struss et al. 2003, De Cuyper et al. 2005, Kaçar et al. 2005, Schüler et al. 2006, Stoeckel et al. 2006, Vaughan et al. 2006, Marchese et al. 2007, Vaughan et al. 2007), and mapping genetic linkage (Olmstead et al. 2008).

In Italy the genetic diversity of wild cherry populations is also under threat due to destruction and restriction of habitat, intercross with sweet cherry, pests and diseases, low natural regeneration and competition with other species, deforestation, pollution, and climate change. Genetic diversity in wild cherries has been extensively studied because of its role as a potential gene pool for improving sweet cherry cultivars (Ducci 2005). Research has been conducted to assess genetic diversity and the level of adaptability of different clones (Ducci and Proietti 1997, Santi et al. 1998, Curnel et al. 2003).

In this study we surveyed the genetic variability of wild cherry throughout the main area of species distribution in northern and central Italy, using eight highly informative nuclear microsatellite (nSSR) markers. Our main aim was to describe the levels and distribution of genetic variability of the species in Italy, and to investigate the genetic structure of plant groups growing at the southern border of the area where the species is naturally widespread, with particular emphasis on the conservation of the species evolutionary processes and the valorisation of the adaptive potential of its breeding resources. In fact, it is well known that geographically peripheral populations are often genetically differentiated from central populations, due to smaller size, fragmentation and as response to different biotic and abiotic conditions (Lesica and Allendorf 1995). We also discuss our results in the light of the European Directive 105/1999, emphasising the preservation and restoration of wild cherry genetic resources in Italy.

Material and Methods

Plant materials

A total of 278 plants were sampled within the natural range of dispersion of the species in central and northern Italy. They were chosen at random, but in order to minimize the sampling of close relatives a minimum distance among them of about 50 m was adopted. Since in case of wild cherry, due to the low density and scattered distribution of plants, it was not considered valid to define populations *sensu stricto*, and the sampled plants were therefore grouped into 11 internally homogeneous "*breeding zones*", defined according to their ecological and vegetational conditions (Figure 1). Each breeding zone was intended to be a separate breeding population, with genetic improvement being limited to these zones to take advantage of naturally evolved adaptation (Silen and Wheat 2003). The boundaries of these breeding zones were based on physiographic, climatic and economic factors. The number of individuals per breeding zone varied from 14 to 39 (Table 2) and no substantial differences in individual density among them were present.

Molecular analysis

Young leaves or buds (100 mg of tissue) were powdered in liquid nitrogen, and genomic DNA was extracted using the QIAGEN® DNeasy plant mini kit, according to the manufacturer's protocol. A total concentration of 20 ngµl^{-1} was obtained for each sample, and DNA quality was evaluated with an Eppendorf biophotometer.

Ten simple sequence repeat markers (SSR) were selected according to the literature, and tested on our plant material (Table 1). While most of the primers were specific for wild cherry (Vaughan and Russell 2004), EMPaO04, EMPaO05 and EMPaO15 were originally designed for sweet cherry (Clarke and Tobutt 2003).

Polymerase Chain Reaction (PCR) amplifications were carried out using a DNA Engine (PTC-200) Termal Cycler, according to the protocols developed by the authors (listed in Table 1). Each amplification reaction contained 1x reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 6 µl Ready Mix Taq, 0.2 µM of each primer, 4.9 µl bidistillated water, and 2.5 ngµl^{-1} of genomic DNA. Each forward primer was labelled with 5'-fluorescence dye Cy5.

The PCR conditions varied for different primers, and were adjusted according to the literature (Vaughan and Russell 2004, Clarke and Tobutt 2003). PCR amplification products were separated in polyacrylamide gel using an ALFexpress II Sequencer (Amersham) laser sequencer. The electrophoresis was carried out, according to the instrument protocol, on 8-10 µl working solution (3 µl loading dye, 1-2 µl internal standard sizers (100 - 300), 1-2 µl external standard sizers 50-500 and 3 µl buffer) added to 2-3 µl PCR product, under the following conditions: voltage 1500 V, temperature 55°C, power 30W, running time 500 min. Fragments sizes were estimated using the Allele Locator software (Amersham Pharmacia Biotech 1999).

Data processing

At each locus alleles were characterised exclusively by the length of the DNA fragments generated by the amplification process. The percentage of polymorphic loci (P), mean number of alleles per locus (i. e. present only in one breeding zone, A), effective numbers of alleles (N_e), allele frequencies, mean number of private alleles per locus, observed heterozygosity (H_o), gene diversity (expected heterozygosity, H_e) and the inbreeding coefficient F_{IS} were calculated using EXCEL and the

GENALEX 6 software (Peakall and Smouse 2006). The same software was used to compute the population genetic structure of the overall samples for each locus with Wright's F-statistics (Wright 1946, 1951, 1965), and to compute a pairwise F_{ST} matrix among breeding zones. For the analyses of departures from Hardy–Weinberg equilibrium (HWE), and to evaluate the significance of inbreeding that occurred in each locus, in each population and in overall loci, we used ARLEQUIN 3.5 (Excoffier et al. 2005, Excoffier and Lisher 2010). The same programme was used to perform tests for genotypic linkage disequilibrium (LD).

Where significant deficiencies of heterozygotes from HWE were observed the presence of null alleles was suspected (Pemberton et al 1995). Loci with high frequencies of null alleles were identified by estimating the presence of the latter using the software MICRO-CHECKER (Van Oosterhout et al 2004). In subsequent analysis, loci with high null allele frequency were eliminated and only those with a frequency on null alleles lower than 0.19 were used. This value has been considered a threshold over which underestimation of H_e due to null alleles becomes significant (Chapuis et al. 2008). Where possible, analyses with robustness prerogative in presence of null alleles were used (Chapuis and Estoup 2007), including STRUCTURE (Pritchard et al. 2000). The inbreeding coefficients (F_{IS}) were calculated taking into account the estimated null frequencies using the programme INEST and running the individual inbreeding model (IIM) with a Gibbs sampler of 105 iterations (Chybicki and Burczyk 2009).

Unbiased genetic distances among populations were estimated according to Nei (1978), using GENALEX. This matrix constituted the base for investigating the relationships among groups, by an unweighted pair group method using the arithmetic means (UPGMA) dendrogram developed by SAHN software in the NTSYSpc package (Rohlf 2005). The cophenetic values matrix was calculated from the tree matrix using the program COPH. The cophenetic matrix was used to evaluate goodness of fit for the cluster analysis by comparing it to genetic distances matrix (MXCOMP of NTSYS).

In addition, the genetic structure was analysed using a Bayesian clustering approach with software STRUCTURE version 2.2.3 (Pritchard et al. 2000). The data was explored without considering prior classification within the sample, by performing 10 replicates of each simulation from K = 2 to K = 12, with a burn-in of 10,000 steps followed by 10,000 Markov chain Monte Carlo

(MCMC) iterations under the admixture model, and the assumption of correlated allele frequencies among populations. We used the program Structure Harvester (Earl and von Holdt 2012) that applies the Evanno's method to assess the optimal level of K (Evanno et al. 2005).

Results

All 10 primer pairs analysed produced polymorphic amplification products, which could be easily identified (Table 1). However, two markers were excluded from the analysis due to the high frequency of possible null alleles: respectively 0.23 (EMPaS11) and 0.20 (EMPaO15). The presence of null alleles was also suspected for 3 out of the remaining 8 loci, with frequencies of 0.10 (EMPaS06), 0.12 (EMPaS10) and 0.13 (EMPaS12). In total, 124 distinct alleles were scored in the 278 studied individuals. The number of alleles per locus ranged from 4 (EMPaS14) to 25 (EMPaS10), with an average value of 15.5. It was possible to detect 24 private alleles. The frequency of these alleles was always low, ranging from 0.013 to 0.048. The distribution of private alleles among microsatellites was unbiased, ranging from one (EMPaO04, EMPaS02 and EMPaS14) to six (EMPaO05). The breeding zone which displayed the highest number of such alleles was TSE (8 private alleles), while no private alleles were detected in EMR.

Sampling of individuals belonging to the same clone could be excluded since plants with identical genotypes were never detected. Statistics on the genetic diversity within breeding zones are given in Table 2. A relatively high level of intra-breeding zones variability was found, since on average more than 7 alleles per locus were observed (A = 7.53). The probability that two randomly sampled alleles in a given breeding zone were not the same was almost 70% (H_e = 0.698), whereas the observed heterozygosity was lower than expected (mean H_o = 0.573). Since the difference, that determines a significant positive value for mean inbreeding coefficient, could be due to the presence of null alleles the inbreeding coefficients were recalculated. Taking into account the frequencies of null alleles, we found that deviations from the Hardy-Weinberg equilibrium were low (F_{IS} ranging from 0.031 in FON to 0.220 in HPD with a mean of 0.075). Significant per locus departure from Hardy-Weinberg equilibrium were however found in all breeding zones ($P<0.01$).

The genetic divergence between breeding zones was investigated by computing a Nei's genetic distance matrix (Nei 1978), and by a pairwise F_{ST} matrix (Table 3). Most of the diversity was found within breeding zones, with only a small amount among breeding zones: the average F_{ST} value was 0.046 (SE = 0.003) and ranged from 0.034 (EMPaS14) to 0.059 (EMPaS12). Almost all pairwise F_{ST} values were significantly greater than zero. The Nei's distances values varied between 0.053 (TSE and TSW) and 0.223 (VNE and FVG). The UPGMA dendrogram confirmed the presence of differentiation between breeding zones, although only in rare cases it was possible to observe a structuring linked to their geographical location. The cophenetic correlation indicated a fair fit of the cluster analysis to data ($r = 0.605$, $P<0.01$). In particular, VNE and FVG, which are geographically close, were identical; another group consisted of FON and LOM, which are located in the same Region, while PDA showed the highest amount of genetic differentiation (Figure 2).

Following the method of Evanno et al. (2005), the Bayesian clustering results obtained with STRUCTURE indicate that K=11 clusters represents the most likely representation of the overall genetic structure that we analysed (Figure 3). Breeding zones showed a high level of admixture and no general trends were detected.

Discussion

The main aim of our research was to describe the level and distribution of genetic variability of wild cherry in northern and central Italy, which, together with the southern part of the country (where the presence of the specie is extremely scattered) represents the southern border of the species diffusion area.

In general, our values are higher than those found in studies of sweet cherry: this most likely reflects the fact that the wild cherry conserves a wider basis of genetic diversity compared to the cultivated forms. The number of alleles in this study (range 4-25 per locus) is high, when compared with data reported for other wild cherry populations: Vaughan et al. (2007) detected a range of 4–14 alleles ($N_a = 7.77$) in 551 plants, Stoeckel et al. (2006) detected 4–21 alleles in 350 plants (among which, 247 were from the same population), while Guarino et al. (2009) observed a number of alleles ranging between 2 and 14 in 50 plants from three different countries. Our results are consistent with

the fact that Italy was probably a centre of dispersion of wild cherry in Europe after the last glacial period, where a higher number of alleles were conserved than in other countries. The size ranges of alleles overlapped with reported values, when compared with results of previous works (Clarke and Tobutt 2003, Vaughan and Russell 2004, Guarino et al. 2009).

We found neither low differences between H_e and H_o, nor the excess of heterozygotes that was reported in previous research (for sweet cherry see Dirlewanger et al. 2002, Wünsch and Hormaza 2002, Marchese et al. 2007, Ganopoulos et al. 2010; for wild and sweet cherry see Guarino et al.2009; for wild cherry see Clarke and Tobutt 2003, Schüler et al. 2003, Vaughan and Russell 2004, Stoeckel et al. 2006, Avramidou et al. 2010). On the contrary, our F_{IS} always gave positive values, indicating an excess of homozygotes. This could be due to the presence of null alleles for some of SSRs markers that increase the number of homozygotes. For instance, in analysis of a pool of 50 wild cherry trees, compared with near 80 sweet cherry cultivars, Guarino et al. (2009) found different frequencies of null alleles, ranging from 0.011 (EMPaS01) to 0.327 (EMPaS02). However, our F_{IS} values remained positive notwithstanding the correction applied by MICRO-CHECKER for null alleles and the consequent exclusion from the analysis of two markers where the presence of null alleles exceeded the threshold commonly adopted in literature.

To explain the positive value of inbreeding coefficient, it is also possible to assume the presence of a Wahlund effect, that is the structuring of the populations in subunits within which mating is more probable. This does not seem to be the case of our sampling, since we collected material from plants not closer than 50 m from each other and sampling density was homogeneous within each breeding zone.

Nevertheless, to limit the impact of the number of homozygotes on the interpretation of inbreeding indices, it is important to notice that an excess of homozygotes was also found for Italian populations with isozymes (Ducci 2005), where H_o and H_e values were lower than those found in the present study, but F_{IS} values were relatively similar in size. In our work, the higher values of F_{IS} were found in HPD, LOM, TSW and VNW. Some of these areas are producers of sweet cherry in Northern Italy, and it is possible that some of our samples consisted of individuals derived from seed produced by cultivated trees widespread in the wild or as results of intercross between wild and sweet forms.

This level of inbreeding contrasts with the fact that cherry is a species characterised by a well-known self-incompatibility system that prevents self-fertilisation (Sonnenveld et al. 2003, Granger 2004, Schüler et al. 2006, Sonnenveld et al. 2006, Marchese et al. 2007, Vaughan et al. 2008). The system is based upon a single multiallelic locus with gametophytic action that controls the ribonucleases in the style of flowers and arriving pollen grains. When the allele of the haploid genotype of the pollen is identical to one of the diploid genotype of the style, fertilisation does not occur, while crosses between individuals sharing only one S-allele can occur, although seed production is limited. Many studies have paid attention to these kind of alleles, that are particularly important in cultivation of sweet cherry (Wiersma et al. 2001, Sonnenveld et al. 2006), but also for the maintenance of wild cherry in the wild (De Cuyper et al. 2005, Vaughan et al. 2006). Since individual trees are often widely scattered, the number and proximity of compatible pollen donors have a large impact on seed set and the genetic diversity of the progeny.

However, self-incompatibility can be overcome under particular conditions, for instance when a population goes through a bottleneck (Reinartz and Les 1994, Gigord et al. 1998).

Another explanation is suggested by the evidence that in natural populations of *Prunus avium* individuals with a high suckering ability can produce more gametes, thereby having a higher probability of undergoing somatic mutations at the gametophytic incompatibility S-locus, as already observed by Lewis and Crowe (1954).

Self-incompatibility could be bypassed by other circumstances. In *Sorbus domestica* L., a predominantly self-incompatible species belonging to the *Rosaceae* family, Kamm et al. (2011) found that self-fertilisation is probably due to a break-down of the incompatibility system occurring as flowers grow old without being pollinated, or under cold weather conditions, while there was no selfing occurred with controlled pollinations of newly opened flowers. Holderegger et al. (2008), studying 15 populations in Switzerland of another self-incompatible Rosaceae (*Pyrus pyraster* Burgsd.), with both S-alleles and nuclear SSRs, found positive values of F_{IS} (0.021-0.164). They explained this fact by the spatially restricted pollen dispersal that can cause the production of seeds from pollination by a limited number of compatible trees in the neighbourhood. The genetically related offspring could therefore produce a progeny with elevate F_{IS} index. The questions posed by our

samples can only be answered by S-allele analysis in the future. Reduced heterozygosity at nuclear microsatellite loci, representative of whole-genome inbreeding, is likely to be associated with reduced individual fitness and impaired potential of the population to adapt to a changing environment, and it is therefore important to better identify the way in which our populations share genetic variability.

Population structure in wild cherry has been characterised by relatively few studies: many researches having focused on the cultivated sweet cherry, employing many different markers (Granger et al. 1993, Gerlach and Stösser 1997, Struss et al. 2001, Wünsch and Hormaza 2002, Xhou et al. 2002, Struss et al. 2003). Studies with chloroplast DNA (Mohanty et al. 2001a, 2001b) found a low level of differentiation among populations spread in different European regions, and Panda et al. (2003) found a higher cpDNA diversity in wild cherry with respect to a group of common sweet cultivars.

In Italy, genetic differentiation measured among the eleven zones was relatively high, and only a small number of alleles per locus are in common among populations (only 1 allele for EMPaS12; 2 alleles for EMPaS14; 3 alleles for EMPaS01, S06, S10, O04, O05; 4 alleles for EMPaS02). We found a F_{ST} = 0.046, comparable with other studies developed in France (Frascaria et al. 1993, Mariette et al. 1997), where G_{ST} values among populations were 0.05 and 0.06 respectively, or in Georgia (F_{ST} = 0.057) (Santi and Dufour 2010).

The value of differentiation is compatible with the reproductive system. Wild cherry plants are insect pollinated, especially by bees, which use the nectar and pollen as an important nutrient source. Pollen can be transferred by various modes, resulting in largely variable dispersal distances. "*Short pollen dispersal*" is generally the most common, due to insect-mediated "*tree-to-tree*" transfer, that tends to cause a differentiation in plants populations. Larger distances of transfer could potentially be realised by other processes, such as "*insect-to-insect*" transfer within the colonies of social insects. When a colony uses different nutrient sources it can accumulate a vast number of pollen mixtures, from wide surrounding areas, in its hive (Free and Williams 1972, De Grandi-Hoffman et al. 1984, 1986). However, at lower frequencies, long distance foraging has also been observed (Zurbuchen et al. 2010). As a typical pioneer tree species wild cherry colonises early forest successional stages as a result of forest disturbances, but it is generally replaced by climax tree species during subsequent

306 succession of the local disturbance, so it is difficult to identify pure, extensive and continuous
307 populations, where insects could share pollen exchange. In the Mediterranean environment we find
308 small groups, and their suckers, or isolated trees, so pollen transfer among distant groups become more
309 difficult, since it is known that smaller or less dense populations attract fewer pollinators.

310 From UPGMA we observe a general lack of geographic pattern. This could also be due to
311 different management strategies of wild cherry within the considered area. For instance, in the eastern
312 regions wild cherry is traditionally cultivated for wood production, and populations are larger and
313 widespread in larger areas, but there is also an extensive cultivation of sweet cherry for fruit
314 production. “Bosco Fontana”, in Lombardy, is a particular situation. It’s a wood situated in a
315 biogenetic reserve of 235 ha, established during the 70’s of the 20th century on an ancient hunting
316 reserve, which preserves a relict of the ancient plain forest in the Po Valley, rich in woody species and
317 managed by the local “National Forest Service”, and where coppicing has been avoided for 60 years.
318 In the Apennines there are small populations, isolated in the valley bottoms, with few individuals, very
319 distant from each other. Furthermore, the silvicultural practices that privilege the vegetative
320 propagation contributes to shaping the genetic variation in these zones, and generally wild cherry has
321 been managed as other species, with coppicing.

322 At the individual level, STRUCTURE analysis using Evanno’s method suggests that wild
323 cherry is constituted from such a high number of groups, that the entire studied gene pool can be
324 considered as an unstructured population. This result is not so far from that obtained by Mariette et al.
325 (2010) in a collection of 211 wild cherry trees sampled in France, and also to those revealed in Italian
326 populations of walnut (*Juglans regia* L.) (Ferrazzini et al. 2007b), that share with wild cherry the
327 anthropic effect due to cultivation for wood and fruit production.

328 Jordano et al. (2007) found that in *Prunus mahaleb* L. seed dispersal due to different types of
329 frugivores resulted in distinct contributions of different distance classes and microhabitats, with only a
330 few species responsible for long-distance dispersal events. Small-sized birds accounted for most short-
331 distance dispersal, and larger frugivores (both birds and mammals) accounted for most long-distance
332 dispersal. They pointed out that three components are particularly important when we want to
333 understand genetic distribution by seed dispersal: the frugivore abundance, their feeding and post-

feeding behaviour and the structure of landscape. While these factors could partially explain the lower genetic distance among VNE and FVG as well as among FON and LOM, that are relatively close from the geographic point of view (with distances compatible with the animal impact on seed dispersal), the human influence on wild cherry distribution seems to be one of the most important causes of the lack of a clear pattern in general distribution of the genetic diversity. As for most European forests, the studied areas have experienced human activities and management for generations. The traditional management method (coppice-with-standards) has probably favoured the maintenance of clonal lineages through time, and due to the edible flesh of fruits, long distance seed dispersal by humans appears as a source of gene flow among populations.

Foresters began to use wild cherry for reforestation and afforestation in the 1970s, usually with material of unknown origin. Frequently, this material originated not only from wild cherry, but also from sweet cherry or even from sour cherry. For this reason, it is not so unlikely that many individuals originated from the introduction of domesticated trees into the wild woods, or from the intercross among wild and sweet forms. In Italy, since 1988, reproductive material for plantations has been collected from physiographic seed areas (Ducci et al. 1988).

Conclusions

The results of the study contribute to a better understanding of our knowledge of wild cherry genetic variation in Italy, thus making for more efficient programs aimed at the preservation of the biodiversity. Furthermore, our results give useful indications on how to act for more rational planning of the management of reproductive material. In the light of the European Council Directive 105/1999, forest reproductive material falling into the "*source-identified*" and "*selected*" categories should be used only within the Region of Provenance where it originated. The transfer of material from one Region to another should be avoided, due to possible problems associated with adaptability. The identification of Regions of Provenance is therefore a basic aspect for a rational management of activities linked with forest tree propagation, including afforestation and *in situ* genetic preservation. In the case of wild cherry, in Italy, we cannot describe populations *sensu stricto*, but use breeding zones, where trees grow with a scattered dispersion. Since our results do not show a clear structuring

of genetic variability within the Italian diffusion area of wild cherry, it is not possible to draw any indications on Regions of Provenance delimitation based only on genetic data, contrary to the results of similar studies carried on in Italy on different species (common ash and Scots pine), where genetic data proved to be useful for Regions of Provenance identification (Ferrazzini et al. 2007a, Belletti et al. 2012). Therefore, the identification of Regions of Provenance for wild cherry in Italy should be based mainly on ecological and vegetation features. Results similar to ours were obtained also in France, where it has been suggested to consider a single Region of Provenance (Anonymous 2003). Furthermore, the development of seed orchards where it should be possible to collect clones from different sites should be encouraged, since they would allow the preservation of as much as possible of the scattered genetic diversity of wild cherry, and to simplify the operation of finding reproductive material for forest breeders.

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Figure legends

- Figure 1. Geographical location of the breeding zones where individuals of wild cherry analysed in this study were sampled.
- Figure 2. Dendrogram constructed from UPGMA cluster analysis of 11 breeding zones of wild *Prunus avium* in northern and central Italy, based on Nei's genetic distance for SSRs produced by eight primers.
- Figure 3. Probability of assignment of 278 plants of wild cherry from Italy to the three genetic clusters identified by hierarchical STRUCTURE analysis. Each vertical bar corresponds with a distinct genotype and different colours indicate the part of its genome assigned to each cluster.

Table titles

- Table 1. Allelic diversity of the ten microsatellite loci considered for the study.
- Table 2. Statistics of genetic variation within Italian wild cherry at eight microsatellite loci. N, sample size; A, mean number of alleles per locus; N_e, effective number of alleles per locus; P_a mean number of private alleles per locus; H_O, average observed heterozygosity; H_e, average gene diversity or expected heterozygosity; F_{IS}, average inbreeding coefficient calculated taking into account the estimated null allele frequencies. Values in parenthesis are standard errors.
- Table 3. Pairwise F_{ST} (above the diagonal) and genetic distances (according to Nei (1978), below the diagonal) among the 11 breeding zones considered in the study.

600 Table 1

Locus	Reference	Number of alleles	Molecular weight range (bp)
EMPaS01	Vaughan and Russell 2004	8	225-254
EMPaS02	Vaughan and Russell 2004	16	133-148
EMPaS06	Vaughan and Russell 2004	24	200-230
EMPaS10	Vaughan and Russell 2004	25	151-185
EMPaS11*	Vaughan and Russell 2004	11	81-109
EMPaS12	Vaughan and Russell 2004	23	121-152
EMPaS14	Vaughan and Russell 2004	4	197-213
EMPaO04	Clarke and Tobutt, 2003	9	177-195
EMPaO05	Clarke and Tobutt, 2003	15	230-262
EMPaO15*	Clarke and Tobutt, 2003	24	202-300

601

602 * markers excluded from the analysis due to the high frequency of possible null alleles.

603 Table 2

Breeding zone	**Code**	**N**	**A**	**N_e**	**P_a**	**H_o**	**H_e**	**F_{IS}**
Inner hills of Piedmont	HPD	27	8.5	5.2	0.8	0.490	0.734	0.229
Western Alps of Piedmont	PDA	22	7.1	4.6	0.4	0.523	0.687	0.075
Fontana Forest	FON	23	6.4	3.8	0.1	0.596	0.666	0.031
Prealps of Lombardy	LOM	30	9.1	5.1	0.9	0.540	0.687	0.111
Eastern Veneto	VNE	22	7.1	4.4	0.1	0.585	0.730	0.033
Western Veneto	VNW	37	8.4	4.7	0.3	0.560	0.718	0.085
Friuli Venetia Giulia	FVG	14	6.3	3.9	0.4	0.644	0.660	0.042
Emilia Romagna	EMR	16	6.3	4.2	0.0	0.617	0.683	0.034
Western Tuscany	TSW	39	8.8	4.7	0.8	0.583	0.731	0.103
Eastern Tuscany	TSE	31	9.1	5.0	1.0	0.602	0.733	0.041
Marche	MAR	15	5.9	3.8	0.3	0.558	0.648	0.041
Overall mean		**24.9**	**7.53 (0.410)**	**4.48 (0.259)**	**0.44**	**0.573 (0.019)**	**0.698 (0.019)**	**0.075 (0.047)**

604 Table 3

	HPD	**PDA**	**FON**	**LOM**	**VNE**	**VNW**	**FVG**	**EMR**	**TSW**	**TSE**	**MAR**
HPD	**0.000**	0.019	0.028	0.023	0.022	0.025	0.025	0.020	0.021	0.029	0.035
PDA	0.105	**0.000**	0.023	0.021	0.025	0.021	0.031	0.020	0.013	0.015	0.018
FON	0.136	0.108	**0.000**	0.039	0.026	0.025	0.035	0.038	0.026	0.024	0.027
LOM	0.125	0.110	0.187	**0.000**	0.032	0.023	0.035	0.019	0.024	0.028	0.030
VNE	0.136	0.132	0.127	0.174	**0.000**	0.021	0.042	0.019	0.018	0.022	0.035
VNW	0.140	0.113	0.128	0.118	0.120	**0.000**	0.036	0.020	0.020	0.016	0.024
FVG	0.126	0.166	0.170	0.176	0.223	0.187	**0.000**	0.036	0.039	0.040	0.039
EMR	0.117	0.104	0.182	0.098	0.088	0.097	0.192	**0.000**	0.021	0.029	0.031
TSW	0.124	0.065	0.124	0.135	0.109	0.115	0.205	0.104	**0.000**	0.010	0.020
TSE	0.162	0.067	0.113	0.148	0.129	0.092	0.202	0.136	0.053	**0.000**	0.022
MAR	0.180	0.084	0.126	0.151	0.168	0.116	0.194	0.142	0.088	0.094	**0.000**

605