Genetic relationship among wild, landraces and cultivars of hazelnut (*Corylus avellana*) from Portugal revealed through ISSR and AFLP markers

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Abstract The genus *Corylus*, a member of the birch family Betulaceae, includes several species that are widely distributed throughout temperate regions of the Northern Hemisphere. This study assesses the genetic diversity in 26 international cultivars and 32 accessions of *Corylus avellana* L. from Portugal: 13 wild genotypes and 19 landraces. The genetic relationships among the 58 hazelnuts (*Corylus avellana* L.) were analyzed using inter simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) markers. Eighteen ISSR primers and seven AFLP primer pairs generated a total of 570 unambiguous and repeatable bands, respectively, from which 541 (95.03 %) were polymorphic for both markers. Genetic similarity index values ranged from 0.239 for wild types and cultivars to 0.143 for landraces and wild types. The genetic relationships were presented as a Neighbor-Joining method dendrogram and a two-dimensional principal coordinate analysis (PCoA) plot. The Neighbor-Joining dendrogram

showed three main clusters, and the PCoA analysis has shown to be congruent with the hierarchical analysis. Bayesian analysis clustered all individuals into three groups showing a good separation among wild genotypes, landraces and cultivars. The genetic diversity found on wild genotypes and Portuguese landraces may provide relevant information for the diversity conservation and it will be useful in breeding programs and to identify local selections for preservation.

Keywords Hazelnuts · Genetic diversity · Molecular markers · Bayesian analysis

Introduction

Hazelnuts, a member of the *Corylus* botanical genus, have been cultivated in China for more than 5,000 years. They grow in temperate regions of the Northern hemisphere and in Europe they are absent only in Iceland, some Mediterranean islands (Cyprus, Malta and Baleari) and in northernmost and southernmost parts of the continent (Kasapligil 1972). Turkey is the major world hazelnut producer (430,000 tons of dry, in-shell nuts) followed by Italy (128,940 tons), USA (34,927 tons), Azerbaijan (32,922 tons) and Georgia (31,100 tons) (FAO Production Yearbook 2011). In Portugal, hazelnut production has suffered a sharp decline since the 1990s, due to a significant abandonment of its cultivation in favor of agronomic crops leading to the loss of local landrace. Human impact on hazelnut is assumed to have occurred since the Mesolithic era (10,000–6,000 years BP). It is known to have been cultivated by the Romans, but the history of the domestication of *Corylus avellana* is still under debate (Tallantire 2002; Boccacci and Botta 2009; Kuster 2000). The strong demand

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and intensive trade in hazelnuts suggests a strong human impact on the genetic structure of hazel, concerning the distribution of hazel genotypes all over Europe. Thus, extant genetic structures are considered to be a result from both natural processes such as postglacial remigration and local adaptation and human impact due to domestication and transfer of germplasm (Leinemann et al. 2013). As a key requirement for plant breeding and evolutionary studies, the loss of wild and landrace genotypes is potentially dramatic since it contributes for a strong genetic diversity reduction. Some reports suggested that some local cultivars were selected from local wild populations (Lagerstedt 1975; Tasiaas Valls 1975; Thompson et al. 1996; Gökirmak et al. 2009). However, Boccacci et al. (2013), Campa et al. (2011) and Ferreira et al. (2010) did not find evidences of wild ancestry in local cultivars. Crossings of economically important *C. avellana* cultivars with wild genotypes were reported by Mehlenbacher (1991) aiming specific traits. Efforts to preserve wild and local hazelnut genetic resources are of great interest in improvement programs as plant genetic erosion is continuously occurring, leading to the loss of genes and alleles (Erfatpour et al. 2011). The traditional methods to characterize and identify cultivars and rootstocks in fruit tree species are based on phenotypic observations, but this approach is slow and subject to environmental influences mainly due to the long-time generation for trees. In hazelnut, molecular markers as random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) were used in the cultivar fingerprint (Galderisi et al. 1999; Miaja et al. 2001; Ferrari et al. 2004), identification of self-incompatibility alleles (Pomper et al. 1998; Mehlenbacher 1997) resistance to eastern filbert blight (Mehlenbacher et al. 2004; Chen et al. 2005) and to analyze genetic relationships among cultivars (Boccacci and Rovira Botta 2006, 2008; Gökirmak et al. 2009). Amplified fragment length polymorphisms (AFLP) have also been used as a DNA fingerprinting technique to assess genome-wide diversity for individuals, populations and species, cultivars identification (Ferrari et al. 2004; Kafkas et al. 2009). ISSR was also used with the same purpose by Ferreira et al. 2010 and Gürçan and Mehlenbacher 2010. In other woody species, AFLP and ISSR have also been used in analysis of genetic diversity in *Citrus* spp. (Fang and Roose 1997; Biswas et al. 2011), *Vitis vinifera* L. (Moreno et al. 1998; Ergül et al. 2006) or *Olea europea* L. (Essadki et al. 2006; Albertini et al. 2011). In Portugal, the number of landraces has decreased in the last decades and the few ones actually identified have been poorly characterized. The main goal of this work was to assess the polymorphism level detected by AFLP and ISSR markers in hazelnut landraces, wild and cultivars) and to identify the genetic relationships between these three groups.

Table 1 List of *Corylus avellana* accessions used in this study

Accessions	Groups	Name	Local	Origin
Ca 1	Landraces		Viseu	Portuguese
Ca 2	Landraces		Viseu	Portuguese
Ca 3	Landraces		Viseu	Portuguese
Ca 4	Landraces		Viseu	Portuguese
Ca 5	Landraces	Grada de Viseu	Viseu	Portuguese
Ca 6	Landraces		Viseu	Portuguese
Ca 7	Landraces	Veiga	Felgueiras	Portuguese
Ca 8	Landraces	Comum	Felgueiras	Portuguese
Ca 9	Landraces		Castelo de Paiva	Portuguese
Ca 10	Landraces		Castelo de Paiva	Portuguese
Ca 11	Landraces	Tubulosa	Moimenta da Beira	Portuguese
Ca12	Landraces	Rubra	Moimenta da Beira	Portuguese
Ca 17	Landraces		Tondela	Portuguese
Ca 18	Landraces		Tondela	Portuguese
Ca 19	Landraces		Vila Real	Portuguese
Ca 20	Landraces		Vila Real	Portuguese
Ca 21	Landraces		Vila Real	Portuguese
Ca 22	Landraces		Vila Real	Portuguese
Ca 25	Landraces	Molar	Viseu	Portuguese
Ca 23	Wild		Ponte de Lima	Portuguese
Ca 24	Wild		PNDI	Portuguese
Ca 28	Wild		PNPG	Portuguese
Ca 29	Wild		PNPG	Portuguese
Ca 30	Wild		PNPG	Portuguese
Ca 31	Wild		PNPG	Portuguese
Ca 32	Wild		PNPG	Portuguese
Ca 33	Wild		PNPG	Portuguese
Ca 34	Wild		Ponte de Lima	Portuguese
Ca 35	Wild		Ponte de Lima	Portuguese
Ca 36	Wild		PNA	Portuguese
Ca 37	Wild		PNA	Portuguese
Ca 38	Wild		PNA	Portuguese
Ca 13	Cultivars	Butler	UTAD	USA
Ca 14	Cultivars	L. d'Espagne	UTAD	UK
Ca 16	Cultivars	Merveille de Bollwiller	UTAD	Germany
Ca 26	Cultivars	Ran Trapezunsi	Felgueiras	Bulgaria
Ca 27	Cultivars	Rimski	Felgueiras	Bulgaria
Ca43	Cultivars	Nottingham	IRTA-Mas de Bover	UK
Ca44	Cultivars	Sivri	IRTA-Mas de Bover	Turkey

Table 1 continued

Accessions	Groups	Name	Local	Origin
Ca45	Cultivars	Garibaldi	IRTA-Mas de Bover	Germany
Ca46	Cultivars	Marxant-1	IRTA-Mas de Bover	Spain
Ca47	Cultivars	San Giovanni	IRTA-Mas de Bover	Italy
Ca48	Cultivars	Martorella	IRTA-Mas de Bover	Spain
Ca49	Cultivars	Karidaty	IRTA-Mas de Bover	Turkey
Ca50	Cultivars	Común Alava	IRTA-Mas de Bover	Spain
Ca51	Cultivars	Ennis	IRTA-Mas de Bover	USA
Ca52	Cultivars	Bard	IRTA-Mas de Bover	UK
Ca53	Cultivars	Tonda Bianca	IRTA-Mas de Bover	Italy
Ca54	Cultivars	Gunslebert	IRTA-Mas de Bover	Germany
Ca55	Cultivars	Ince Kara	IRTA-Mas de Bover	Turkey
Ca56	Cultivars	Jardinera	IRTA-Mas de Bover	Spain
Ca57	Cultivars	Rote Zellernuss	IRTA-Mas de Bover	Germany
Ca58	Cultivars	Jemstegaard-5	IRTA-Mas de Bover	USA
Ca59	Cultivars	Butler	IRTA-Mas de Bover	USA
Ca60	Cultivars	Mortarella	IRTA-Mas de Bover	Italy
Ca61	Cultivars	Nocchione	IRTA-Mas de Bover	Italy
Ca62	Cultivars	Pere Mas	IRTA-Mas de Bover	Spain
Ca63	Cultivars	<i>C. avellana</i> var contorta	IRTA-Mas de Bover	UK

PNDI Douro international natural park, *PNPG* Peneda-Gerês national park, *PNA* Alvão natural park, *UTAD* University of Trás-os-Montes and Alto Douro (collection field)

Materials and methods

Plant material

A total of 58 trees were analyzed in this study (Table 1): (1) 19 Portuguese landraces (local forms cultivated by farmers), collected from North and central Portugal; (2) 13 wild accessions collected with the collaboration of forest keepers in natural (Alvão and Douro International) and national (Peneda-Gerês) parks located in Northern Portugal where hazelnut wild populations are present, and (3) 26

well-known reference cultivars from several European countries (England, USA, Spain, Italy, Germany, Bulgaria and Turkey). The 58 genotypes were divided into three groups: landraces, wild and cultivars.

DNA isolation

Immature catkins were collected in the field, kept cool during transport (about 2 h) and stored at -80°C . Total genomic DNA was extracted using the DNeasy kit (Qia-gen) (Hilden, Germany), according to the manufacturer's instructions. DNA concentration was determined by both spectrophotometries at 260 nm and gel electrophoresis, using a known quantity of *HindIII*-digested Lambda DNA.

AFLP analysis

PCRs were performed as described by Vos et al. (1995) with minor modifications. The procedures were as follows: 200 ng DNA from each sample was digested with ten units of *EcoRI* (recognition sequence: 5'GAATTC-3') and 20 units of *Mse I* (5'TTAA 3') and 10 % BSA to a final volume of 40 μl using 10X Ligase-buffer as reaction buffer. This mixture was incubated at 37°C for 2 h and 70°C for 15 min. To the double-digested DNA sample, a total volume of 20 μl of ligation reaction mixture was added including 75 pmol of *EcoRI* adaptor and 75 pmol of *Mse I* adaptor, three unit of T4-DNA ligase, and the same reaction buffer as above. The reaction mixture was incubated overnight at 37°C . Digested/ligated DNA samples were diluted tenfold with H_2O . A 40 μl pre-selective PCR mixture contained 10 μl of diluted template mixture, 40 pmol of both *MseI* primer (*MseI* adaptor sequence) and *EcoRI* primer (Table 2), 10 μM dNTPs, 10X Taq Buffer with $[(\text{NH}_4)_2\text{SO}_4]$, 10 mM MgCl_2 and one unit Taq polymerase. The cycle profile used was: 95°C 1 min, 95°C 30 s, 56°C 30 s, 72°C 1 min (20 cycles) and a final extension at 72°C 2 min. The pre-amplification product was diluted tenfold with H_2O , and then used as a template for selective amplification to generate AFLPs. The selective amplification reaction was conducted in a final volume of 8 μl containing 1.5 μl of diluted pre-amplification reaction, 2.5 μM of each *MseI* + 3 and *EcoRI* + 3 primers (Table 2), 150 μM dNTPs, 10X Taq Buffer with $[(\text{NH}_4)_2\text{SO}_4]$, 1.5 mM MgCl_2 , and one unit Taq polymerase. Amplifications were performed using a thermal cycler Biometra (UNO II-thermoblock) using the following cycling conditions: 95°C for 60 s, 95°C for 30 s, 65°C for 30 s, 72°C for 60 s (cycle 1), the annealing temperature was then lowered by 0.7°C in each cycle during the following nine cycles and then 95°C for 30 s, 56°C for 30 s and 72°C for 60 s from cycles 11–35. A total of 8 μl of the AFLP selective amplification product was mixed

Table 2 ISSR and AFLP primers sequences

Marker systems	Primers	Sequences
ISSR	808	(AG)8C
	809	(AG)8G
	810	(GA)8T
	811	(GA)8C
	814	(CT)8A
	817	(CA)8A
	818	(CA)8G
	820	(GT)8C
	823	(TC)8C
	825	(AC)8T
	826	(AC)8C
	830	(TG)8G
	841	(GA)8YC
	847	(CA)8RC
	850	(GT)8YC
	855	(AC)8YT
	888	BDB(CA)7
	889	DBD(AC)7
AFLP	Adaptor EcoRI F	CTCGTAGACTGCGTACC
	Adaptor EcoRI R	AATTGGTACGCAGTC
	Adaptor MseI F	GACGATGAGTCCTGAG
	Adaptor MseI R	TACTCAGGACTCAT
	EcoRI + 1	GAC TGC GTAC C AATTC A
	Mse + 1	GACGATGAGTCCTGAGTAAC
	E32 + M32	GACTGCGTACCAATTCACG + GACGATGAGTCCTGAGTAACAC
	E38 + M32	GACTGCGTACCAATTCAGG + GACGATGAGTCCTGAGTAACAC
	E38 + M36	GACTGCGTACCAATTCAGG + GACGATGAGTCCTGAGTAACTC
	E38 + M37	GACTGCGTACCAATTCAGG + GACGATGAGTCCTGAGTAACTT
	E39 + M31	GACTGCGTACCAATTC + GACGATGAGTCCTGAGTAACAA
	E39 + M32	GACTGCGTACCAATTC + GACGATGAGTCCTGAGTAACAC
	E39 + M34	GACTGCGTACCAATTC + GACGATGAGTCCTGAGTAAAT

with 2.0 µl of loading buffer. Electrophoresis was performed on an SEA 2000[®] Electrophoresis Apparatus (Elchrom Scientific). 10 µl of mixture was loaded onto Poly(NAT) 9 % from Elchrom Scientific gel with 0.75 X TAE buffer after a pre-run electrophoresis at 120 Volts for 10 min, and then run at 120 Volts for 1.5 h. After electrophoresis, the gel was stained with ethidium bromide. AFLP bands were visually scored with the aid of Biocapt software.

ISSR analysis

The 18 ISSR primers (UBC808, UBC809, UBC810, UBC811, UBC814, UBC817, UBC818, UBC820, UBC823, UBC825, UBC826, UBC830, UBC841, UBC847, UBC850, UBC855, UBC888 and UBC889), that combined higher polymorphism with good reproducibility

of the generated fragments were selected. ISSR amplifications were performed in a volume of 20 µl containing 20 ng total DNA, 10 µl Master mix (Taq PCR Master Mix KIT-QIAGEN) and 7.5 µM primer. Following the initial denaturation at 94 °C for 5 min, the following 44 cycles amplification program was used: nine cycles of 15 s at 94 °C, 45 s annealing step at two degrees less than the optimum annealing temperature, and 75 s at 72 °C; followed by 35 cycles of 15 s at 94 °C, 45 s annealing step at the optimum annealing temperature, 75 s at 72 °C, and a final extension reaction step of 7 min at 72 °C. At least two PCR amplifications were performed for each sample with ISSR primers to evaluate the reproducibility of the bands. DNA amplification fragments were separated in a 1.8 % agarose gel using TBE buffer, and stained with ethidium bromide. Gels were analyzed with Biocapt software.

Data analysis

Only clear and distinct bands were scored for ISSR and AFLP analysis. Binary matrices based on this two molecular markers dataset were then constructed based on the presence or absence of a band. ISSR and AFLP data for 58 hazelnuts accessions were analyzed using Popgene 1.32 software (Yeh et al. 1999), for dominant character of the markers and diploid character of the plant material. This setting enables us to calculate the following parameters: number of alleles (N_a); effective number of alleles (N_e); Shannon's information index (I), which measures gene diversity (Shannon and Weaver 1949); Nei's gene diversity index ($h = 1 - \sum p_i^2$, where p_i is the frequency of the i th allele of the locus; Nei 1973); total genetic diversity (H_T); genetic diversity within groups (H_S); relative magnitude of differentiation among groups [$G_{ST} = (H_T - H_S)/H_T$] and an estimate of gene flow (N_m) from G_{ST} (Slatkin and Barton 1989), $N_m = 0.5(1 - G_{ST})/G_{ST}$ (McDermott and McDonald 1993). The information content of each marker system was determined according to the indices of Powell et al. (1996): effective multiplex ratio (EMR) [number of polymorphic products from a single amplification reaction $EMR = n_p (n_p/n)$] and marker index (MI) (the product of effective multiplex ratio $MI = PIC \times n^\circ$ polymorphic loci). The ability of the markers to differentiate the genotypes was assessed by calculating their resolving power (Rp) according to Prevost and Wilkinson (1999) using the formula $R_p = Plb$, where $l_b = 1 - (2 \cdot 10.5 - pl)$, where p is the proportion of the 58 genotypes containing the I band. PIC (Polymorphic Index Content) was calculated using the following formula:

$$PIC = 1 - \sum_{i=1}^i p_i^2 - \sum_{i=1}^{i-1} \sum_{j=i+1}^i 2p_i^2 p_j^2$$

where p_i and p_j are the frequencies of the i th and j th alleles.

The PIC value of a locus ranges from 0 (monomorphic) to 1 (highly informative) and indicates the level of informativeness for linkage studies (Guo and Elston 1999), and it was calculated using the software PICcalc (Nagy et al. 2012). An analysis of molecular variance (AMOVA) was performed to partition the genetic variation among populations and within populations (Schneider et al. 2000). The significance of each variance component was tested with permutation tests (Excoffier and Slatkin 1992). Genetic distances were estimated according to Nei (1978) and a principal coordinate analysis (PCA) (Gower 1966) was performed. Wright's F_{ST} was used to estimate population differentiation. These indices were calculated using the GenAlEx 6 software (Peakall and Smouse 2006). Genetic similarity matrices based on Jaccard and Neighbor-joining cluster analysis were used to construct genetic trees by

Table 3 Number of bands, percentage of polymorphic bands, polymorphism information content (PIC), resolving power (Rp), effective multiple ratio (EMR) and marker index (MI) of ISSR, AFLP and ISSR + AFLP markers used to fingerprint 58 hazelnut

	ISSR	AFLP	ISSR + AFLP
Primer number	18	7	25
Total number of bands	378	192	570
Mean number of bands	21	27.430	20.400
No. of polymorphic bands	372	169	541
Mean no. of polymorphic bands	20.67	24.400	21.640
Polymorphic rate (%)	98.400	87.328	95.030
Total resolving power	254.620	158.480	413.107
Average resolving power	14.330	22.640	16.524
Average polymorphic information content	0.350	0.363	0.354
Average effective multiplex ratio	21.860	21.480	20.671
Average marker index	7.490	7.840	7.286

Darwin software (Perrier and Jacquemoud-Collet 2006). STRUCTURE ver. 2.3 software (Pritchard et al. 2000) was used to investigate the genetic population structure. This method uses a Markov Chain Monte Carlo (MCMC) algorithm to cluster individuals into populations on the basis of multilocus genotype data (Pritchard et al. 2000; Falush et al. 2003). The number of populations (K) was estimated by performing ten independent runs for each K (from 1 to 8), using 1,000,000 MCMC repetitions and 50,000 burn-in periods. Any prior information about the population of origin was used, and correlated allele frequencies and admixture were assumed. The average of the log-likelihood estimates for each K was calculated. The ad hoc statistic DK (Evanno et al. 2005) was used to set the number of populations (K).

Results

AFLP

The AFLP fragments ranged from 50 to 950 bp, the majority being distributed from 50 up to 500 bp. Each primer set produced 20–34 scorable fragments. A total of 192 AFLP fragments were detected, of which 169 (88 %) were polymorphic (Table 3). Thus, each primer set generated an average of 27.43 fragments, 24.4 being polymorphic. Thirty-one of the 192 AFLP fragments were unique. Twenty-four unique fragments having been detected in cultivars, six private fragments were found in landraces and only one in wild genotypes. The average of PIC was 0.363 and the primer pair E38 + M32, showed the

Table 4 Genetic variation among Portuguese landraces, wild and cultivars of hazelnuts

	na	ne	h	I	Ht	Hs	Gst	Nm
AFLP	1.882	1.550	0.314	0.465	0.302	0.188	0.379	0.820
ISSR	1.984	1.465	0.286	0.442	0.274	0.186	0.322	1.052
AFLP + ISSR	1.949	1.494	0.296	0.450	0.283	0.186	0.342	0.960

na number of alleles, ne effective number of alleles, h Nei's (1973) gene diversity index, I Shannon's information index, Ht total genetic diversity, Hs intra-population genetic diversity, Gst relative magnitude of differentiation among groups, Nm estimate of gene flow from Gst

highest PIC value (0.375). The average of Rp, EMR and MI for AFLP markers was 22.64, 21.48 and 7.84, respectively (Table 3). The E32 + M32 primer pair had the highest Rp, EMR and MI (30.21, 30.12 and 11.29, respectively), while E39 + M32 had the lowest Rp and EMR (13.79 and 12.) (data not shown). For all the 58 hazelnuts accessions, the number of alleles, Ne, h, I was 1.882, 1.550, 0.314, and 0.465, respectively. The value for total genotype diversity among groups (Ht) was 0.302; while within groups (Hs) was 0.188. Mean Gst value 0.379 indicated that 63 % of the genetic diversity lies within the group. Estimate of Nm in the group was 0.820 (Table 4).

ISSR

The eighteen primers produced a total of 378 scorable markers among the genotypes. The size of amplified products ranged from 225 to 1,800 bp. The total number of polymorphic markers and the percentage of polymorphism were 372 and 98.41, respectively (Table 3). Ten out of the 372 polymorphic fragments were considered specific for landraces and four specific for cultivars. These results indicate that only 14 out of the 372 (3.8 %) bands could be considered genotype-specific. The PIC average was 0.350 and the highest PIC was 0.370 (primer 823) and the lowest was 0.300 (primer 847). The average of Rp, EMR, and MI was 14.33, 21.86, and 7.49, respectively (Table 3). For all the 58 hazelnuts accessions, the number of alleles, Ne, h, I was 1.984, 1.465, 0.286, and 0.442, respectively. The value for total genotype diversity among groups (Ht) was 0.274 while within groups (Hs) was 0.186. Mean Gst value 0.322 indicated that 68 % of the genetic diversity resided within the group. Estimate Nm in the group was 1.052 (Table 4).

AFLP and ISSR combined data

The integration of data obtained from the two molecular markers, using the 25 selected primers, yielded a total of 570 reliable fragments, 541 of which were polymorphic (95.03 %). Thirteen unique fragments were found in the landraces genotypes, one in the wild genotypes and four in the cultivars. These 18 fragments, representing 3.7 % of the polymorphic fragments, are putative genotype-specific

markers. Genetic similarity was calculated from the Jacard coefficient considering AFLP and ISSR approaches both individually and combined. Based on AFLP marker, the similarity index values ranged from 0.042 to 0.733, while for ISSR markers ranged from 0.043 to 0.877. The similarity index values based on both markers together ranged from 0.060 to 0.798. A hierarchical analysis was performed for each marker separately (data not shown) and the result was consistent with the analysis for the two markers combined. The hierarchical analysis generated by the method of Neighbor Joining based on Jacard coefficient, separated the 58 genotypes in three groups (Fig. 1). The first group was composed only by cultivars. The second group was divided in two subgroups. The first subgroup consisted of eleven wild genotypes, two cultivars from Bulgaria ('Ran Trapezunsi' and 'Rimski') and one landrace (Ca25). Four landraces (Ca19, Ca20, Ca21 and Ca22) and two wild genotypes formed the second subgroup. The third group was divided into two subgroups. The first subgroup consisted only of landraces and the second subgroup contains two landraces (Ca17 and Ca18) and three cultivars, one from Germany ('Merveille de Bollwiller'), one British ('Longue d'Espagne') and another from USA ('Butler'). The principal coordinates analysis (PCoA) included both AFLP and ISSR approaches individually and combined. Based on AFLP markers, the three most informative principal coordinates (PC) explained 92.09 % of the total variation, while for ISSR markers the three most informative principal coordinates (PC) explained 91.89 % of the total variation. For both markers combined, the three most informative principal coordinates (PC) explained 92.51 % of the total variation. The results of the analysis for each marker separately (data not shown) were consistent with the analysis for the two markers combined. The PCoA divided the 58 genotypes into three groups. Group I comprised all the wild genotypes, two cultivars ('Ran Trapezunsi' and 'Rimski') and five landraces (Ca19, Ca20, Ca21, Ca22 and Ca25). Group II was formed by fourteen landraces and three cultivars ('Butler', 'L. d' Espagne' and 'M. Bollwiller') and group III was consisted only of cultivars (Fig. 2). The results of PCoA analysis were in accordance with the cluster analysis. The Mantel test among the Nei's genetic diversity indices showed high correlation between

Fig. 1 NJ cluster analysis of hazelnut genotypes based on the Jaccard distance using 25 primers for ISSRs + AFLPs

AFLP and ISSR ($r = 0.897$) based on similarity. The correlation found between the AFLP and AFLP + ISSR was $r = 0.958$ and this correlation is higher than the correlation found in AFLP and ISSR. For ISSR and AFLP + ISSR, the correlation (r) was 0.986 indicating that ISSR data are closer to AFLP + ISSR combined data (Table 5; Fig. 3). The F_{ST} values found for AFLP and ISSR separately were similar to the values for F_{ST} of the two markers combined (data not shown). Genetic differentiation using the F_{ST} parameter showed that the pairwise values for F_{ST} ($P < 0.01$) ranged from 0.407 (wild and landraces) to 0.503 (cultivars and wild) (Table 6). Data indicate that the rate of gene flow between wild and landraces was low but insufficient to find homogenization in the Portuguese hazelnut gene pools. Nevertheless, the genetic differentiation between the landraces and cultivar group ($F_{ST} = 0.484$) was slightly lower than the genetic differentiation between cultivars and wild. AMOVA analysis was performed separately for AFLP, ISSR and combined analysis of the 58 hazelnuts. F-statistic was estimated using a Bayesian approach with an analogue of the F-statistic, the Φ -statistic. The hierarchical distribution of genetic variation within and among populations was different between AFLP and ISSR or AFLP + ISSR markers. ISSR and AFLP + ISSR markers revealed a genetic

variation between-population with Φ_{PT} values of 0.454 ($P < 0.0001$) and 0.481 ($P < 0.0001$), respectively, whereas among-populations differentiation was high for AFLP with a Φ_{PT} value of 0.536 ($P < 0.0001$). Molecular variance for both markers combined was 48 % among groups, while within the groups was 52 % (Table 7) indicating that there is more variation within the groups than that among groups. The organization of genetic diversity was further analyzed with STRUCTURE program in a stepwise fashion from 2 to 8 populations. The estimation of the statistic ΔK revealed the highest value for $K = 3$ ($\Delta K = -12,613.68$) indicating the existence of three main clusters (Fig. 4). The same analysis was performed for the AFLP and ISSR data set separately (data not shown) and the results obtained were in agreement with the result obtained for the two markers combined. The F_{ST} values were 0.742 for landraces versus wild genotypes, 0.714 for landraces versus cultivars and 0.719 for cultivars versus wild genotypes. In the data set, at $K = 3$, the hazelnut accessions split into three groups (Fig. 4). Group I consists only of cultivars. Group II is formed by fourteen landraces and three cultivars ('Butler', 'Longe d'Espagne' and 'Merveille de Bollwiller'). Group III is formed by all wild accessions, five landraces (Ca19, Ca20, Ca21, Ca22 and Ca25) and two cultivars ('Ran Trapezunsi' and 'Rimski').

Fig. 2 Principal coordinates analysis of 58 unique hazelnut genotypes shown by groups for the AFLPs + ISSRs

Comparing these results with the Neighbor-joining dendrogram and the PCA scatterplot, there was a general agreement concerning the clustering of wild, landraces and cultivars.

Discussion

Several powerful marker techniques are currently available for genetic analysis of plant species. The choice of the most appropriate technique for a specific study is not obvious and depends on the aims of the research and the biology and genetic structure of the species. In this study, two of the most widely adopted marker techniques, AFLP and ISSR were used. The results of this work clearly demonstrate that both AFLP and ISSR markers can be used for the identification of hazelnuts accessions. In fact, all of the analyzed genotypes were uniquely identified both by their AFLP fingerprints and ISSR profiles. Notably, each genotype produced its own unique AFLP and ISSR fingerprinting profile using any of the ISSR and AFLP primers. Nybom (2004) with three dominant molecular markers (RAPDs, AFLP and ISSR) concluded that genetic diversity revealed by these molecular markers is usually similar and generally directly comparable. Although, our results for AFLP and ISSR differed in the genetic diversity values, they are very similar in population structure. The genetic diversity estimation was higher for the AFLP than for the ISSR markers, but the intra-population genetic diversity is quite similar for both markers with 0.187 for AFLP and 0.186 for ISSR. Estimates of genetic similarity using

genetic fingerprinting data are a useful tool in plant breeding, allowing breeders to make informed decisions regarding the selection of germplasm to be used in crossing schemes. Fingerprints themselves are also useful to breeders per se for protection of their own cultivars and for seed producers, growers and end users for checking the identity and purity of their products. ISSR markers were more efficient than the AFLPs assay for polymorphism detection, as they detected 98.040 % as compared to 87.328 % for AFLPs markers. Ferreira et al. (2010) found an average of six polymorphic bands per primer for the ISSRs, a lower result than the one now found for ISSRs. For AFLP, Kafkas et al. (2009) found in hazelnuts a much higher mean number of polymorphic bands (29.88) than for ISSR (3.96) or RAPD (3.84). The mean number of polymorphic bands found for AFLP was higher than for the ISSR, which is in agreement with Kafkas et al. (2009). However, in our study, we did not find such a large discrepancy between the values of the mean number of polymorphic bands for AFLP and ISSR. Patzak (2001); Talhinhas et al. (2003) and Kafkas et al. (2006) also reported a higher mean number of polymorphic bands for AFLPs than for ISSRs and RAPDs. The values for Rp and MI with ISSR, were lower (14.330 and 7.490, respectively) than for AFLPs (22.640 and 7.840, respectively). The index EMR estimated using ISSR (21.860) was slightly higher than the one using AFLPs (21.480). Those results showed that both markers systems—primer/marker were effective for the separation of 58 hazelnuts into groups. PIC was slightly higher for AFLP than for ISSR, which corroborates the results reported by Kafkas et al. (2009). The relative

magnitude of differentiation among cultivars (G_{ST}) was slightly higher for AFLP (0.368) than for ISSR (0.322). Among the three groups of genotypes (wild, landraces and

Table 5 Regression analysis between Nei's genetic diversity obtained from AFLP, ISSR and AFLP + ISSR combined data

Marker combination	r	P value
AFLP and ISSR	0.897	<0.0001
AFLP and AFLP + ISSR	0.958	<0.0001
ISSR and AFLP + ISSR	0.986	<0.0001

Fig. 3 Relationship between ISSR and AFLP + ISSR data sets for the genetic distances

Table 6 Pairwise values for F_{ST} of hazelnuts groups studied

	Landraces	Wild
Wild	0.407	
Cultivars	0.484	0.503

Table 7 Population differentiation analysis

	Among groups (%)	Within groups (%)	Φ_{PT}	P
AFLP	54	46	0.536	<0.0001
ISSR	45	55	0.454	<0.0001
AFLP + ISSR	48	52	0.481	<0.0001

cultivars), the highest genetic diversity was found in the well-known cultivars and the landraces. The lower values for G_{ST} were, in general, observed in the group of wild genotypes. These results are consistent with the different origin of each group of genotypes. The group of cultivars is a diverse set representing the world's most productive geographical areas (Spain, Italy, Turkey and USA), while the Portuguese landraces and wild genotypes included in this analysis were collected in a limited area from northern and central Portugal. The sexual or asexual reproduction also influences the genetic diversity. Sexual reproduction generates higher levels of diversity than the propagation using vegetative methods. At the beginning of this work, it was postulated that Portuguese wild genotypes may originate mainly from seeds derived from cross-pollination in a very strict and isolated area and that Portuguese landraces had been essentially obtained by grafting or rooted suckers' propagation, causing a decrease in the genetic diversity. However, this work reveals that the Portuguese landraces showed a higher genetic diversity than the wild genotypes, in contrast with our expectations based on the reproduction system. This fact is probably due to the low number of wild individuals identified in Portugal in a very limited area surrounded by high mountains hindering pollen gene flow. This fact can also be considered an alert for the need for short- and long-term diversity conservation. Genetic similarity was calculated from the Jacard coefficient considering ISSR and AFLP approaches individually as well as combined. The hierarchical analysis has shown a clear separation of the landraces, wild and cultivar groups, for the two systems of markers and for the two markers combined. In the hierarchical analysis (ISSR, AFLP and AFLP + ISSR), in the group of the wild hazelnuts, we always found the same two cultivars, 'Ran Trapezunsi' and 'Rimski' from Bulgaria, and five Portuguese landrace (Ca19, Ca20, Ca21, Ca22 and Ca25). This fact allows us to speculate, that those landraces and the two cultivars may have a former common origin with wild genotypes. Martins et al. (2012), based on chloroplast microsatellite loci,

Fig. 4 Assignment of 58 *Corylus* genotypes to three groups by Structure for AFLP + ISSR system. Each individual bar represents an accession. Numbers 1–19 = Landraces, 20–32 = Wild and 33–58 = Cultivars

observed that the wild genotypes had unique haplotypes (H, I, J and L) suggesting that this group constitutes an independent source of genetic variability (hotspot of diversity) and a valuable resource of genetic traits for hazelnut. Also, for the group of Portuguese landraces three cultivars were always grouped (one from Germany ('Merveille de Bollwiller'), one from British Islands ('Longe d'Espagne') and one from USA ('Butler'), we also postulate a common origin. A highlight in this group of the Portuguese landraces was the clear separation of the Portuguese landraces from all other genotypes of different geographical origins. The twenty-one of the twenty-six cultivars clustered together in a separated group. These findings raise an interesting question on the origin of five Portuguese landraces and two cultivars ('Ran Trapezunsi' and 'Rimski' from Bulgaria). Differentiation between Portuguese wild genotypes and landraces, and the strong relationship among the Portuguese landraces, suggest that the Portuguese landraces Ca19, Ca20, Ca21, Ca22 and Ca25 may have been derived from the introduction of a few primitive cultivars, followed by a relatively local evolution that could include crosses among them and with local wild hazelnut. The differentiation between Portuguese landraces and the group of cultivars is in agreement with the differentiation found by Ferreira et al. (2010) with ISSR, Campa et al. (2011) and Boccacci et al. (2013) with SSR among locally cultivated accessions and a group of reference cultivars (diverse set representing the world's most important production areas). There is an agreement in the diversity analyses between the PCoAs and the hierarchical analysis, despite the relatively low proportion of total data captured in the PCoA plot. Genetic differentiation can be studied using the F_{ST} parameter which evaluates gene flow among populations (Slatkin and Barton 1989). Wright (1978) suggested that values between 0.05 and 0.15 indicated moderate genetic differentiation, while values above 0.25 indicated high differentiation. The F_{ST} results obtained, revealed that identical allele and genotype distribution among gene pools does not happen equally in all pairwise comparisons between groups. The Portuguese landraces were only subjected to empirical selection made by farmers, mainly based on quality and fruit production factors. Improvement methods over cultivars were performed by breeders since public breeding programs were initiated in Italy and the US in the 1960s, Spain and France in the 1970s, and Turkey in the 1980s (Thompson et al. 1996). This fact helps to explain the F_{ST} value found between the Portuguese landraces and the cultivars ($F_{ST} = 0.484$). Data indicated that the rate of gene flow caused by dissemination of hazelnut plants was not sufficient to genetically homogenize the landraces that grew in different geographical areas. The high genetic differentiation between the wild and landraces groups ($F_{ST} = 0.407$)

was expected. These two groups are separated by Peneda-Gerês and Alvão Mountains that provide the climate conditions for hazelnuts thrive, but are also possible barriers to plant migration. Nevertheless, the genetic differentiation between cultivars and wild group ($F_{ST} = 0.503$) was higher than the genetic differentiation between the landraces and cultivars. Thus, a significant genetic differentiation among gene pools has occurred, and each group constitutes an independent source of genetic variability and a valuable resource of genetic traits for hazelnut breeders. The Bayesian clustering approach that probabilistically assigns individuals to populations based on genotypes differentiated the individuals into three groups. Assignment of some individuals like the three cultivars in the group of the landraces and the seven accessions (five Portuguese landrace and two cultivars) in the group of the wild genotypes was already explained in the hierarchical analysis. Knowledge of genetic variation within and among populations provides essential information for the formulation of appropriate management strategies for conservation (Milligan et al. 1994; Francisco-Ortega et al. 2000). The knowledge of genetic variation and the genetic relationship between genotypes can be an important consideration for efficient rationalization and utilization of hazelnuts germplasm resources. We strongly believe that genetic diversity data from Portuguese wild and landraces accessions may provide relevant information for diversity conservation, identification of local selections for preservation in collections and for future genetic and morphological-pathological studies. The low number of wild individuals found must be considered a warning sign for the urgent need for short- and long-term diversity conservation.

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