Research Article

Agronomic, chemical and genetic profiles of hot peppers (*Capsicum annuum* ssp.)

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A study on morphology, productive yield, main quality parameters and genetic variability of eight landraces of hot pepper (*Capsicum annuum* ssp.) from Southern Italy has been performed. Morphological characters of berries and productivity values were evaluated by agronomic analyses. Chemical and genetic investigations were performed by HPLC and random amplified polymorphic DNA (RAPD)-PCR, respectively. In particular, carotenoid and capsaicinoid (pungency) contents were considered as main quality parameters of hot pepper. For the eight selected samples, genetic similarity values were calculated from the generated RAPD fragments and a dendrogram of genetic similarity was constructed. All the eight landraces exhibited characteristic RAPD patterns that allowed their characterization. Agro-morphological and chemical determinations were found to be adequate for selection, but they resulted useful only for plants grown in the same environmental conditions. RAPD application may provide a more reliable way based on DNA identification. The results of our study led to the identification of three noteworthy populations, suitable for processing, which fitted into different clusters of the dendrogram.

Keywords: Agronomic characteristics / Capsaicinoid / Carotenoid / DNA polymorphism / Hot pepper

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1 Introduction

The genus *Capsicum*, which originates from tropical and humid zone of Central and Southern America, belongs to the *Solanaceae* family and comprises peppers of important economical value. Several *Capsicum* species exist, but three are widely spread due to a hot or pungent berry: *C. frutescens* L., *C. chinense* Jacq. and *C. annuum* L. This last one, originating from Mexico, was the first to be introduced worldwide, and nowadays it is the most widely spread from the point of view of the familiar consumption and industrial processing [1].

Hot pepper is a well-known spice. It is used dried or fresh in various pharmacological preparations and has large application in cookery to enrich foods with its unique flavor. It has a long history as a source of healthy and biologically active compounds, such as flavonoids, phenols, carotenoids, capsaicinoids and vitamins [1, 2]. Carotenoids confer the characteristic color to the fruit and are synthesized mostly during the ripening stage. These natural pigments are antioxidant, free-radical scavenger, vitamin A precursor, stimulating immune cell activity and cancer preventive [3, 4]. Carotenoids exist as non-oxygenated (carotenes) and oxygenated (xanthophylls) hydrocarbons, consisting of eight isoprenoid units (C40), characterized by chromophores from yellow to red. The carotenogenesis, associated with the transformation of chloroplast to chromoplast, begins with the sequential production of carotenes (β-carotene, β-cryptoxanthin and zeaxanthin). These are the biosynthetic precursors of the red fraction that is mainly represented by the capsanthin, considered characteristic of the *Capsicum* genus [5].

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Abbreviations: **CTAB**, cetyltrimethylammonium bromide; **NLc**, Nei and Li's coefficient; **RAPD**, random amplified polymorphic DNA; **TCC**, total carotenoid content; **VR1**, vanilloid receptor 1; **UPGMA**, unweighted pair group method using arithmetic average

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Pepper hotness is due to the presence of lipophylic alkaloids at different concentrations, collectively named capsaicinoids [6–9]. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin (8-methyl-N-vanillyl-6-nonanamide) account for about 90% of total pungency. At genetic level, it has been demonstrated that capsaicinoid production is due to the complete dominance of the *C* allele, mapped on chromosome 2. Consequently, the homozygous recessive condition (*cc*) results in the lack of the capsaicinoid biosynthesis [10]. From a physiological point of view, capsaicinoids elicit the sensation of burning pain by selective activation of the vanilloid receptor 1 (VR1) on sensory neurons towards the central nervous system [11]. VR1 is a heat-activated cation channel of the nociceptive pain pathway and functions as a transducer of thermal stimuli *in vivo*. The white placental tissue of the pepper produces other three chemically related compounds of minor importance: nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin. Capsaicin and its related analogues have been shown to promote the programmed cell death (apoptosis) of prostate cancer lines *in vitro* and significantly reduce the growth of the cancer mass *in vivo* [12].

The physico-chemical parameters of hot pepper are conditioned by several factors [13]: (i) endogenous (genotype, plant growth and fruit maturation state); (ii) ecological (geographical origin, pedoclimatic and cultivation conditions); (iii) technological (processing and storage of raw material). Therefore, not all cultivars can be used directly, but they must preliminarily satisfy a series of right agronomic and industrial requirements since industries need uniform and stable berries suitable for the transformation. Mostly appreciated is a high content in carotenoids, which directly depends on pigment asset. Other aspects of interest are thin pericarp of the mature berry, in order to reduce the drying step during the industrial processing and consequently the costs; the grouped ripened berries useful for mechanical collecting; the high agronomic yield; the resistance to climatic and environmental factors.

The Calabria region in Southern Italy, is a representative place of growth and diffusion of this important spice because of its exceptionally favorable climatic conditions. Here, the growers have preferred to select traditional populations of *C. annuum* ssp. This fact has led, over many years, to the production of commercial cultivars and hybrids with higher yields and more uniform morphology, preserving the local germplasm. In order to successfully achieve the biodiversity protection, the various landraces have to be characterized. However, the discrimination of each variety from the others is difficult because it is based only on morphological characters, whose expression can be influenced by developmental and environmental factors [13].

Nowadays, unequivocal identification of plant cultivars, resolution of taxonomical controversies and monitoring of genetic diversity within and between cultivars have been performed by PCR-based molecular markers, founded on DNA analysis, in a wide number of important crops [14–17]. Random amplified polymorphic DNA (RAPD) analysis represents a variation of the conventional PCR, as it simply employs an arbitrary oligodeoxynucleotide as primer that is designed without any knowledge of the base sequence of the genomic DNA template [14, 18, 19]. The development of RAPD markers is increased in recent years and has proved to be useful for initial biodiversity screening in studies of niche plants, in order to detect genetic variation in wild and domesticated populations. Recently, RAPD markers have also been used with success in the study of medicinal plants including *Allium cepa* and *A. kermesinum* [20], *Ocimum* spp. [17, 21], *Rosmarinus officinalis* [22] and *R. tomentosus* [23], *Thymus vulgaris* [24]. At present, no correlation has been revealed between the agro-morphological and pungency characteristics of pepper and its genetic fingerprint, although enzyme and protein polymorphism have been investigated in pepper [25, 26], as well as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and RAPD profiles of closely related genotypes [27–32].

The aim of the present work was the characterization of hot *Capsicum* populations located in the Calabrian region by using a multidisciplinary approach. The RAPD-PCR procedure analyzed the genetic distances among populations in study and was carried out together with agronomic trials and quali-quantitative analysis in order to select the best landraces for quality and benefit production.

2 Materials and methods

2.1 Plant material

During the year 2002, the "Accademia Italiana del Peperoncino" in Diamante (CS), Italy, collected and certified seeds of 16 Calabrian populations of *Capsicum annuum* ssp. from Cosenza province (Belvedere Marittimo, Bisignano, Buonvicino, Diamante, Santa Maria del Cedro, Verbicaro and Maierà), while other three populations were from Reggio Calabria province (Terreti). In the year 2003, these landraces were sowed in the same conditions at the experimental field of the Stazione Sperimentale per le Industrie delle Essenze e dei Derivati dagli Agrumi (SSEA), Reggio Cala-

Table 1. The selected Calabrian populations of hot pepper in study

Population number	Place of origin (Province)
1	Verbicaro (CS)
3	S. Maria del Cedro (CS)
5	Bisignano (CS)
10	Diamante (CS)
13	Belvedere M. (CS)
14	Maierà (CS)
17	Terreti (RC)
18	Terreti (RC)

bria, Italy. Successively, 8 landraces were selected from the 19 initial samples and propagated in the year 2004 (Table 1). Plants and fruits were harvested at the identical growth stage for agro-morphological and chemical analysis. All the samples for the genetic analysis were young leaves stored at −80°C until DNA isolation. Voucher specimens of the landraces in study were dried and stored under vacuum at Laboratory of Biochemical and Microbiological Technologies of SSEA.

2.2 Agronomic and morphological analysis

Hot pepper were raised up during 2003 and 2004 years in the SSEA experimental field (20 m asl) at Reggio Calabria, Italy. For each action, the ground had a scheme consisting of 30 plants for population (150 cm between rows and 20 cm in the row) with a 156.6 m^2 area (27 × 5.8 m). Sorghum was implanted among the rows of seedlings, in order to maintain purity of each landrace by preventing occasional cross-pollination determined by the presence of insects. At the beginning of March 2003, the seeds of each population were sown in alveolar containers consisting of 160 cells and set in a cold greenhouse at Nazareno Cooperative Society, Reggio Calabria. At the end of May, all plants with four leaves were transplanted at SSEA experimental field after soil treatment by glyphosate herbicide and fertilization with nitrogen, phosphorus and potassium. After a base enrichment, only nitrogen was applied two times for coverage fertilization. Plants were irrigated by flush and furrow irrigation method, once a day until harvest. After the first year, 8 of the 19 hot pepper populations were included in the subsequent trial of year 2004 based on uniformity of the population phenotype (Table 1). Therefore, these eight crops were propagated to confirm their agro-morphological stability. In both examination years, mature fruits were harvested twice, at the beginning of September and in mid of November; immature crop was collected only in November. Then, the productivity values and the main parameters of the fresh fruits (weight, length, diameter at shoulder and thickness of pulp) were determined on 30 plants and 50 fruits for each landrace, respectively.

2.3 Capsaicinoid analysis

Capsaicinoids were extracted and analyzed according to the American Spice Trade Association (ASTA) method 21.3 (1998) with some modifications [33]. Briefly, samples weighing from 2 to 10 g of fresh, dried and seed tissues were separately homogenized in a blender and mixed with 50 mL of dichloromethane:methanol (2:1). After high-speed mixing for 30 min, the organic layer was filtered through Whatman filter paper. This procedure was repeated three times until the starting material was colorless. The pooled organic layers were evaporated at room temperature and the dried materials were suspended in 25 mL ethanol. Then, each extract was further filtered through a 0.45-μm nylon filter before the HPLC analysis. Standards of capsaicin and dihydrocapsaicin were purchased from Sigma-Aldrich (Italy). Standard curves were prepared using suitable linear working range of capsaicin and dihydrocapsaicin serial dilutions from 25 to 1000 mg/kg. A Surveyor HPLC instrument (ThermoFinnigan, Italy) equipped with photodiode array (PDA) detector, thermostated column compartment, autosampler, LC pump and connected with a computer was used for chromatographic analyses. The separation was carried out using a Luna (Phenomenex, USA) RP C18 column (250 × 3.0 mm id; 5-μm average particle size) thermostated at 25°C. The injection volume was 20 μL and the elution was performed in 60 min with a mobile phase made of ACN:water (3:2), containing 1% of acetic acid, at a flow rate of 1.5 mL/min. The chromatograms were recorded by measuring UV absorption at 280 nm via Excalibur software (ThermoFinnigan). Three runs were performed for each sample and the result was the average value of determinations.

2.4 Carotenoid analysis

Carotenoid content was determined according to the method of De Sio *et al*. [34]. Briefly, 35 mL of dichloromethane:methanol (2:1) was added to 5.0 g of grounded fresh fruits. After shaking, the organic layer was separated. This procedure was repeated until the starting material was colorless. The pooled organic layer was evaporated to dryness at temperature not higher than 35°C. For carotenoid saponification, the residue was suspended in 6 mL of ethyl ether, made up with 6 mL of 10% KOH in methanol and allowed to stand overnight in the dark at room temperature. Afterward, the mixture was transferred into a 250 mL separation funnel with 20 mL of ethyl ether and then with 100 mL of 10% w/v aqueous NaCl. After shaking, the aqueous layer was discarded and the ether layer was washed with water until became neutral to phenolphthalein. Finally, the ethyl ether layer was desiccated over anhydrous sodium sulfate and evaporated to dryness. The dried samples obtained from the extraction were dissolved in different volumes of chloroform containing 0.1% w/v butylated hydroxytoluene (BHT) and filtered through 0.45-μm nylon filter immediately before chromatographic analysis. Carotenoid standards were prepared in chloroform containing 0.1% BHT using serial dilutions from 5 to 20 mg/L of each standard principle: capsanthin, zeaxanthin, β-cryptoxanthin, β-carotene (Extrasynthese, France). The solutions were stored under nitrogen at −20°C and were found stable at least 15 days. The HPLC analyses were performed by a Surveyor instrument (ThermoFinnigan) connected with photodiode array (PDA) detector on an YMC (USA) RP C30 column (250 × 3.0 mm id; 3-μm average particle size), thermostated at 30°C. The injection volume was 10 μL and the elution was performed at flow rate of 1 mL/min, with a linear

Table 2. Details of the 12 random primers used in this study and their results in RAPD analysis of pepper genome

Primer code	Nucleotide sequence (5′ to 3′)	% GC	Total DNA fragments		Polymorphic DNA fragments (%)
AN10	CTGTGTGCTC	60	8	6	(75.0)
AX08	AGTATGGCGG	60	6	4	(66.7)
AX20	ACACTCGGCA	60	9	0	(0.0)
G02	GGCACTGAGG	70	5	0	(0.0)
G19	GTCAGGGCAA	60	9	7	(77.8)
Q05	CCGCGTCTTG	70	7	4	(57.1)
R19	CCTCCTCATC	60	6	1	(16.7)
S07	TCCGATGCTG	60	8	3	(37.5)
U4	GACAGACAGG	60	7	4	(57.1)
U5	CGACAGACAG	60	5	1	(20.0)
U13	CCAGTGCTCT	60	7	2	(28.6)
V17	ACCGGCTTGT	60	5	1	(20.0)

gradient from 5% B in A to 70% B in A in 35 min, where solvent A was methanol:water (95:5), containing 0.1% BHT and 0.05% triethylamine (TEA) and solvent B was dichloromethane, containing 0.1% BHT and 0.05% TEA. The data were collected at 450 nm and processed by Excalibur software (ThermoFinnigan). The results were the average values of three runs for each sample.

2.5 Color estimation

Before color measurement, the pepper pulps without seeds were desiccated by circulating air at room temperature in the dark for 20 days. Then, the starting material was homogenized in a blender to obtain the sample to analyze. The color determination on dried pulp was performed by HunterLab – D25 Tristimulus Colorimeter (USA), calibrated with the C2-26772 color reference material. The results were expressed by parameters "L", "a", "b" and "a/b", where "L" indicates luminosity, ranging from white (0) to black (100), "a" indicates the color from green to red, and "b" the color from blue to yellow. The result was the average value of three determinations for each sample.

2.6 DNA isolation

Genomic DNA was isolated from fresh and young pepper leaves through the protocol reported by De Masi *et al.* [17]. In brief, 0.15 g of leaves was collected from ten individual plants of each selected population and bulked to constitute a unique homogeneous sample of 1.5 g. The obtained sample was ground to small particles in a mortar in presence of liquid nitrogen. Then, the powder was suspended in preheated (60°C) lysis buffer consisting of 1.4 M NaCl, 2% w/v cetyltrimethylammonium bromide (CTAB), 200 mM Tris-HCl pH 8.0, 20 mM EDTA, 2% v/v 2-mercaptoethanol, 5 mM ascorbic acid. The sample was kept at 60°C for 30 min and then treated by chloroform:butanol (24:1). A volume of cold isopropanol was added to the water phase with the purpose to precipitate the nucleic acids. The collected precipitate was washed in 70% ethanol and dissolved in double-distilled sterile water. The co-extracted RNA was eliminated by incubation with RNase A (5 μg/mL) at 37°C for 60 min. After a further purification with 5 M ammonium acetate (1/10 volumes) and cold ethanol (3 volumes), the DNA was suspended in double-distilled sterile water. After quantification by reading absorbance at 260 nm, working solutions of 1 ng/μL were prepared. Integrity and size of genomic DNA were evaluated by gel electrophoresis analysis, purity was checked by 260/280 absorbance ratio.

2.7 RAPD analysis

The eight DNA samples were analyzed by 12 arbitrary oligodeoxynucleotide primers (10-mer) selected among those have had reproducible amplification patterns in RAPD-PCR test (Table 2). The optimized PCR assay [17] was done in a 50 μL reaction volume containing 3 mM $MgCl_2$, 200 μM of each dNTP, 20 pmol of single primer, 10 ng of pepper genomic DNA as template and 2.5 U of Stoffel Fragment *Taq* DNA polymerase in reaction buffer provided by manufacturer (Applied Biosystems, USA). Each reaction mixture was assembled in ice (cold start) to prevent aspecific annealing of primers to DNA template prior to PCR. The PTC-100 thermal cycler (MJ Research, USA) with heated lid was programmed with an initial incubation at 94°C for 3 min (initial DNA template denaturation), followed by 45 amplification cycles made of three steps: denaturation at 94°C for 1 min, annealing at 40°C for 1 min and elongation at 72°C for 1 min. The cycles were concluded with a final elongation at 72°C for 7 min. The reaction products were maintained at 4°C for short time before electrophoresis analysis. Positive and negative controls were included and each experiment was performed in triplicate to verify the reproducibility of the procedure. The RAPD amplicons (25 μL) were separated by 1X TBE buffered electrophoresis on 2% w/v agarose gel, containing ethidium bromide (0.5 μg/mL), for 90 min at 10 V/cm. The resultant bands in the gel were visualized under UV light and digital-

Table 3. Productivity values for the hot pepper populations in the year 2004

Population (number and origin)	Marketable (g)		Total (g)	Precocity (%)	Immature (g)	Potential (g)	Immature (%)
	I harvest	II harvest					
1 – Verbicaro	2520	940	3460	73	3000	6460	46
3 – S. M. del Cedro	1580	845	2425	65	750	3175	24
5 – Bisignano	1348	725	2073	65	820	2893	28
10 – Diamante	616	470	1086	57	400	1486	27
13 – Belvedere	1528	358	1886	81	98	1984	5
14 – Maierà	1127	1129	2256	50	105	2361	4
17 – Terreti	1173	191	1364	86	258	1622	16
18 – Terreti	1790	345	2135	84	300	2435	12

ized by Electrophoresis Documentation and Analysis 120 System (Kodak ds, USA). Approximate size of amplicons was estimated using 100-bp DNA ladder as molecular weight standard (Amersham Pharmacia Biotech, USA). Reproducible and bright RAPD fragments were scored in each genotype as "1" if present or as "0" if absent. These data were used to calculate pair wise similarity matrix according to Nei and Li's genetic similarity coefficient (NLc) = $2N_{xy}$ $(2N_{xy}+N_x+N_y)^{-1}$, where N_{xy} is the number of bands common to samples *x* and *y*, N_x and N_y are the number of bands unique to sample *x* and *y*, respectively. NLc represents the fraction of shared DNA fragments between genotypes. The Multi-Variate Statistical Package (MVSP) ver. 3.1 clustering program was utilized to construct a dendrogram calculated through the unweighted pair group method using arithmetic average (UPGMA) [35].

3 Results and discussion

3.1 Agronomic and morphological analysis

The morphological features weight, length, diameter at shoulder and thickness of the pulp were determined on 50 mature berries (first and second harvest). The immature fruits were weighed and the plant heights were measured in the last harvest.

In Table 3 the values of the crop production of 2004, corresponding to the two harvests, are reported. The most productive population, on the base of total commercial fruits, resulted from Verbicaro n. 1 with 3460 g of mature production (average 115 g per plant), followed by S. M. del Cedro n. 3 with 2425 g (average 81 g per plant). The greatest amount of mature fruits was of the two populations coming from Terreti n. 17 (86%) and n. 18 (84%), with the higher productivity in the first harvest respect to the second (precocity), owing to their strong vocation to be cultivated in vase for ornamental purpose. The landraces suitable for industrial transformation resulted from Belvedere n. 13 with 81%, followed by the populations Verbicaro n. 1 with 73%, S. M. del Cedro n. 3 with 65% and Bisignano n. 5 with 65%. Verbicaro n. 1 (Fig. 1A) was also the population

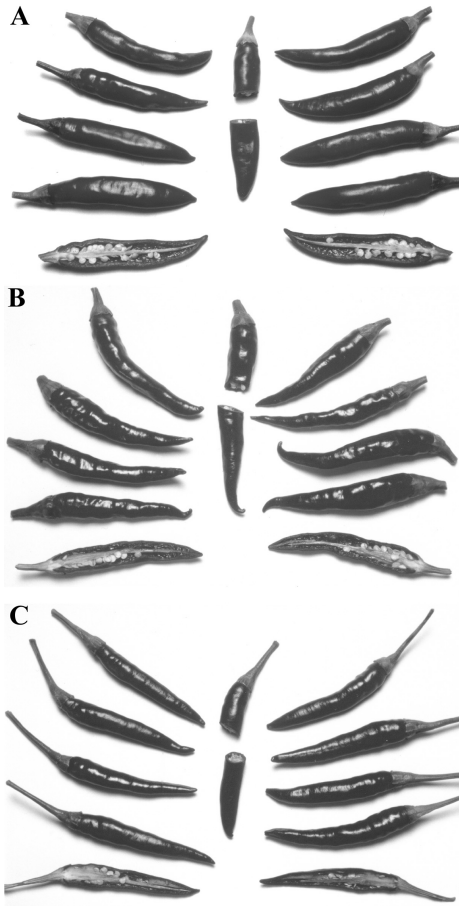

Figure 1. The fruits of the three hot pepper populations from Verbicaro (A), S. M. del Cedro (B) and Bisignano (C) selected in this study.

that showed the greatest productive potential with 6460 g, reached thanks to the presence of 3000 g of green fruits at the end of the vegetative cycle of the plant, followed by S. M. del Cedro n. 3 with 3175 g (Fig. 1B) and Bisignano n. 5 with 2893 g (Fig. 1C). The population Verbicaro n. 1, for the extent of its productive cycle, merits particular attention regard to future planning, because it should be bedded out very early.

Table 4. Morphological traits for hot pepper in the year 2004

Population (number and origin)	Plant height (cm)	Fresh fruit			
		Weight (g)	Length (cm)	Diameter (cm)	Pulp thickness (mm)
1 – Verbicaro	125.8	4.3	8.0	1.39	1.83
3 – S. M. del Cedro	98.8	3.3	6.8	1.30	1.41
5 – Bisignano	81.5	4.2	7.9	1.37	2.00
10 – Diamante	72.7	3.1	6.9	1.14	1.48
13 – Belvedere	96.3	6.6	9.6	1.33	1.79
14 – Maierà	90.6	2.9	6.3	1.39	1.40
17 – Terreti	64.8	14.5	12.5	1.97	2.37
18 – Terreti	57.3	6.2	2.6	2.61	3.01

Table 5. Capsaicin and dihydrocapsaicin contents in seeds, fresh and dried fruits over the years 2003-04[a)]

Population (number and origin)	Sample	Capsaicin (mg/kg)	Dihydrocapsaicin (mg/kg)	Total (mg/kg)
1 – Verbicaro	Fresh fruit	258	123	381
	Dried fruit	1156	478	1634
	Seed	53	29	82
3 – S. M. del Cedro	Fresh fruit	1062	398	1460
	Dried fruit	1996	744	2740
	Seed	383	138	521
5 – Bisignano	Fresh fruit	607	274	881
	Dried fruit	2537	1152	3689
	Seed	1895	828	2723
10 – Diamante	Fresh fruit	731	358	1089
	Dried fruit	1303	614	1917
	Seed	213	131	344
13 – Belvedere	Fresh fruit	293	162	455
	Dried fruit	1019	616	1635
	Seed	310	202	512
14 – Maierà	Fresh fruit	729	266	995
	Dried fruit	1550	636	2186
	Seed	250	124	374
17 – Terreti	Fresh fruit	223	199	422
	Dried fruit	374	288	662
	Seed	85	74	159
18 – Terreti	Fresh fruit	231	115	346
	Dried fruit	902	498	1400
	Seed	387	182	569

a) The data represent the means of three determinations.

In Table 4, the results of the morphological evaluations on 30 plants of each population and on 50 marketable fruits for population in 2004 are depicted. The plants subjected to the comparison showed good health conditions, development uniformity and homogeneous conformation, with exception of the two from Terreti (n. 17 and 18) with 6 and 11% of extraneous plants, respectively, and for the fruit pendent trait of the population n. 17, characteristic that is common with Verbicaro n. 1. In addition, this last population was 125.8 cm high, particularly higher than others, and therefore it can partly justify its elevated productive values. It must be underlined that the agronomic peculiarities of the two populations from Terreti set them apart from others. The berry weight is influenced by length, pulp thickness and, partially, by diameter at the shoulder. In particular, Belvedere n. 13, with 6.6 g weight and 9.6 cm length of berry, was distinguished from the others. The most elevated values of pulp thickness are distinctive of Bisignano n. 5 (2.00 mm) and Verbicaro n. 1 (1.83 mm). At last, Verbicaro n. 1 and Maierà n. 14 presented the greatest diameter at the shoulder with 1.39 cm. The population from Verbicaro n. 1 showed the best results in the morphological and agronomic characteristics, thus it can be considered the most suitable landrace for industrial processing (Fig. 1A).

Table 6. Carotenoid content in fresh hot pepper in the year 2004[a)]

Population[b)]	Carotenoids (mg/kg)				TCC	Caps/Zeax
	Capsanthin	Zeaxanthin	β-cryptoxanthin	β-carotene		
1 – Verbicaro	8.4	1.2	1.1	2.1	12.8	7.0
3 – S. M. Cedro	14.7	4.6	0.4	0.7	20.4	3.2
5 – Bisignano	7.9	1.1	1.2	1.9	12.1	7.2
10 – Diamante	19.9	3.3	1.1	1.7	26.0	6.0
13 – Belvedere	15.7	4.9	1.5	1.7	23.8	3.2
14 – Maierà	18.8	2.5	1.0	2.0	24.3	7.5
17 – Terreti	16.2	1.6	0.5	1.0	19.3	10.1
18 – Terreti	12.2	3.1	1.3	1.9	18.5	3.9

a) The data represent the means of three replicates.
b) Number and origin.

3.2 Capsaicinoid analysis

Capsaicinoid content was estimated by HPLC technique. In a typical chromatogram of pepper extract three peaks corresponding to nordihydrocapsaicin, capsaicin and dihydrocapsaicin were present. Total capsaicinoid content (sum of capsaicin and dihydrocapsaicin) represents the pungency power in seeds and fruits, fresh or dried.

The results of capsaicin and dihydrocapsaicin content in seeds, fruits (fresh or dried) in the eight Calabrian populations of hot pepper are illustrated in Table 5. The values of total capsaicinoid content on the fresh berries of the eight populations varied from a minimum of 346 mg/kg for Terreti n. 18 (with round fruit), to a maximum of 1460 mg/kg for S. M. del Cedro n. 3. The same value on dried berries ranged from a minimum of 662 mg/kg of Terreti n. 17 (with long berry), to a maximum of 3689 mg/kg for Bisignano n. 5. The oscillations among the total values of capsaicinoid in fresh and dried berries are due to different contents of water (in percentage). The distribution of capsaicinoids in the different populations and in diverse tissues (seeds, fresh and dried fruits) showed a ratio of around 1:1 (capsaicin:dihydrocapsaicin) for Terreti n. 17, increasing to 3:1 for S.M. del Cedro n. 3 (Table 5).

The pungency in mg/kg for fresh fruits showed that the spiciest population was S. M. del Cedro n. 3 with 1460 mg/kg (Fig. 1B), followed by Diamante, Maierà and Bisignano. Instead, the poorer populations resulted from Belvedere, Verbicaro and Terreti n. 17 and 18. Bisignano n. 5 showed the best pungency degree for dried fruit with 3689 mg/kg (Fig. 1C), followed by those native of S. M. del Cedro with 2740 mg/kg and Maierà with 2186 mg/kg. Finally, the hottest seed population came from Bisignano with 2723 mg/kg.

The agro-morphological characters together with pungency values allowed us to discriminate the landraces in study. The observed differences among the eight populations were undoubtedly imputable to genetic factors, in fact, the populations were grown in the same field with identical pedoclimatic conditions. In addition, all fruits were harvested for analysis at the same age and developmental stage. However, these parameters are known to have great variability depending on environmental factors, so they can provide an uncertain tool for classification, but, on the other hand, they are very important for the food industry and the final consumers.

3.3 Carotenoid analysis

Quantitative analysis of carotenoids in fresh fruits indicated that the dominant component was capsanthin, according to the carotenogenic pathway characteristic of the *Capsicum* genus. The data in Table 6 showed a particularly high value for capsanthin (19.9 mg/kg) in Diamante n. 10 with 26.0 mg/kg of total carotenoid content (TCC). Populations of Maierà n. 14, Belvedere n. 13 and S. M. del Cedro n. 3 followed with 24.3, 23.8 and 20.4 mg/kg, respectively, the last two being particularly rich in zeaxanthin with 4.9 and 4.6 mg/kg, correspondingly. Bisignano n. 5 and Verbicaro n. 1 presented the lowest quantity of total carotenoids, even though Verbicaro had the maximum value in β-carotene (2.1 mg/kg). Finally, Terreti n. 17 and 18 had an adequate amount of TCC with 19.3 and 18.5 mg/kg, respectively.

The preference of pepper processing industries has stimulated attention in more reliable cultivars for pigment richness and, in recent times, Hornero-Méndez *et al*. [5] have proposed the application of capsanthin-to-zeaxanthin ratio (Caps/Zeax) along with TCC, as useful indexes for establishing high-producing carotenoid varieties of pepper. Furthermore, they proved the relationships between Caps/Zeax ratio and color evaluation in 12 pepper cultivars suitable for industrial paprika production.

The landraces characterized in our study showed, in general, high values of both TCC and Caps/Zeax ratio, demonstrating that they were valuable for breeding purpose. Exceptions were Belvedere and S. M. del Cedro that presented high TCC and the lowest Caps/Zeax ratios, thus achieving their maximum carotenogenic capacity [5].

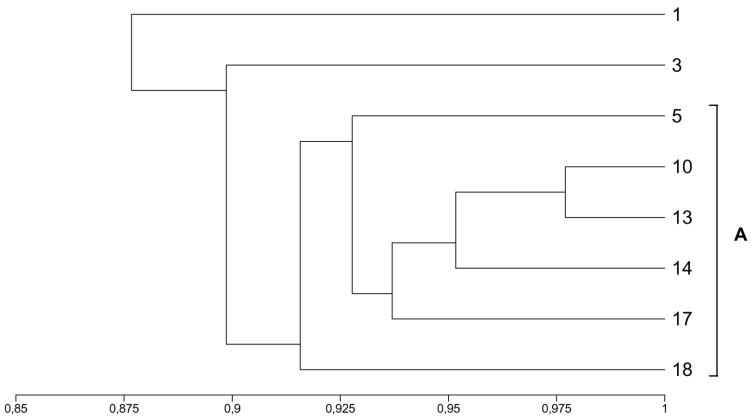

Figure 2. Dendrogram of eight populations, each indicated by its code name, of Calabrian hot pepper generated by UPGMA clustering analysis using 82 RAPD markers. Six samples fit into cluster A. The numerical scale indicates Nei and Li's coefficient of genetic similarity.

Table 7. Color determination in dried hot pepper pulp in the year 2004[a)]

Population (number and origin)	Color (Hunter)			
	L	a	b	a/b
1 – Verbicaro	26.1	20.3	12.2	1.66
3 – S. M. del Cedro	26.6	22.5	12.2	1.84
5 – Bisignano	28.7	24.3	14.3	1.70
10 – Diamante	26.5	21.0	12.4	1.69
13 – Belvedere	29.0	24.0	14.0	1.71
14 – Maierà	29.3	21.9	13.9	1.58
17 – Terreti	26.2	20.2	12.2	1.66
18 – Terreti	26.3	22.5	12.2	1.84

a) The data represent the means for three replicates.

3.4 Color estimation

Color value results (a/b ratios) showed an intense red color for each population (Table 7). In particular, a/b ratio was high in S. M. del Cedro n. 3 and Terreti n. 18 (a/b = 1.84); while, Bisignano n. 5, Diamante n. 10 and Belvedere n. 13 followed with about a/b = 1.70. Verbicaro n. 1 and Terreti n. 17 had both 1.66, whilst Maierà n. 14 showed the lowest value with 1.58. Nevertheless, these data were not at all correlated with carotenoid contents due to different extent of water.

The Hunter color analysis remains particularly useful, together with Caps/Zeax index, in industrial processing controls. In fact, we found correlation between color features and Caps/Zeax ratios for all landraces considered.

3.5 Genetic molecular analysis

In our previous work, we established the procedure of extraction and purification of genomic DNA from leaves of hot pepper and the corresponding genotyping through RAPD-PCR [36]. The performed experimental optimization has represented the preliminary part of the study, which, therefore, has continued with the DNA amplifications by RAPD-PCR of the eight populations of hot pepper employing 12 unique and arbitrary primers. In such a way, it has been possible to get detailed information on the existing genetic variations and to identify the populations of hot pepper in study. Particular attention has been turned to DNA isolation, because the secondary metabolites (alcaloids, tannins, flavonoids, polyphenols etc.) inhibit the enzymatic activity of the *Taq* DNA polymerase during the PCR reaction. To such aim, CTAB cationic detergent revealed particularly efficient for the purification of the DNA. In fact, a double treatment with CTAB buffer allowed the obtainment of highly purified DNA useful in the RAPD-PCR analysis. The next step was to determine the optimal conditions for the amplification of the DNA through RAPD-PCR. In particular, four different quantities of DNA target (1, 10, 100 and 200 ng) and three different *Taq* DNA polymerases were tested. The Stoffel Fragment of AmpliTaq DNA polymerase (Applied Biosystems) resulted to be the most suitable enzyme for the efficiency, the sensibility and the reproducibility of the RAPD analysis.

RAPD-PCR was performed on the pepper populations by using 12 primers. Among them, 10 random primers revealed polymorphic amplified DNA fragments, while 2 primers (AX20 and G02) showed no polymorphism (Table 2). Except these, the percentage of polymorphic bands for each primer ranged from 16.7 (R19) to 77.8 (G19). Genetic similarities among *C. annuum* populations, estimated through Nei and Li's coefficient (NLc), were based on both shared and unique amplification products, transforming band patterns into a binary matrix. NLc values, across eight populations, ranged from a minimum of 0.85, among Verbicaro n. 1 and S. M. del Cedro n. 3, to a maximum of 0.98, among Diamante n. 10 and Belvedere n. 13 (Table 8). The highest similarity was detected among the populations n. 5, 10, 13, 14, 17 and 18.

The primer U4 gave the best information among the 12 primers selected for RAPD analysis, showing genetic polymorphism for all the populations in study, with the exception of Verbicaro and Terreti n. 17 populations. A complete differentiation for these last two populations was obtained by primer AN10.

Table 8. Genetic similarity matrix based on Nei and Li's coefficient

Population number	18	17	14	13	10	5	3	1
18	1.00							
17	0.92	1.00						
14	0.92	0.91	1.00					
13	0.92	0.95	0.94	1.00				
10	0.92	0.95	0.96	0.98	1.00			
5	0.90	0.91	0.94	0.93	0.93	1.00		
3	0.87	0.88	0.91	0.91	0.92	0.91	1.00	
1	0.87	0.86	0.88	0.88	0.89	0.90	0.85	1.00

The landraces were analyzed through UPGMA algorithm to assess their relatedness. The obtained dendrogram contained a major group, cluster A, of closely related samples that included populations number 5, 10, 13, 14, 17 and 18 (Fig. 2). It was likely that these six landraces have part of their genomes in common, representing the 75% of tested samples. This finding was expected because normally, in Southern Italy, the various kinds of hot peppers share the same cultivation area and therefore seeds could be easily spread on the identical area. Instead, the remaining two populations (number 1 and 3) were diversified, because cross-pollination might have occurred in remote times. In fact, these two genotypes fell each in separate and one – represented cluster, showing much lower values of similarity coefficient. So, the population number 1 represented the most genetically distant sample, whereas the populations of cluster A were genetically very close and showed high similarity coefficients with NLc > 0.90 (Table 8).

Our results provided evidence that the landraces were clearly distinct among them, though they grew in the same area. Moreover, the molecular analysis allowed us to estimate the genetic variability. The dendrogram revealed all the eight populations were differentiated in a sure and univocal way. This points out that, at a local level, a notable degree of biodiversity exists, thus each population presents its own genetic identity.

4 Concluding remarks

Natural pepper populations usually are heterogeneous, composed by plants different in morphology and capsaicinoid contents, because they propagate by seeds and the pollination is mainly entomophilous, leading to a high level of polymorphism. Our results showed that the populations were genetically different, each with genetic uniformity to guarantee quality and yield stability. Three landraces were selected based on the best results obtained. The population from Verbicaro showed the greatest productivity, followed by S. M. del Cedro and Bisignano. The spiciest fresh fruit was from S. M. del Cedro, while the population from Verbicaro resulted very poor in capsaicinoids. That from Bisignano demonstrated the best pungency degree for dried fruit and seeds, followed by S. M. del Cedro.

Morphology and chemistry are adequate tools for selection only for plants developed in the same environmental conditions, while RAPD analysis may represent a more general instrument in selection, genetic preservation, commercial trade and origin control of typical Italian products.

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5 References

[1] Siviero, P., Il peperoncino piccante, *Essenze Derivati Agrumari* 2002, *72*, 53 – 72.

[2] Rosa, A., Deiana, M., Casu, V., Paccagnini, S., *et al*., Antioxidant activity of capsinoids. *J. Agric. Food Chem.* 2002, *50*, 7396 – 7401.

[3] Maoka, T., Mochida, K., Kozuka, M., Ito, Y., *et al*., Cancer chemopreventive activity of carotenoids in the fruits of red paprika *Capsicum annuum* L. *Cancer Lett.* 2001, *172*, 103 – 109.

[4] Collera-Zuniga, O., Jiménez, F. G., Gordillo, R. M., Comparative study of carotenoid composition in three Mexican varieties of *Capsicum annuum* L. *Food Chem.* 2005, *90*, 109 – 114.

[5] Hornero-Méndez, D., Costa-Garcia, J., Minguez-Mosquera, M. I., Characterization of carotenoid high-producing *Capsicum annuum* cultivars selected for paprika production. *J. Agric. Food Chem.* 2002, *50*, 5711 – 5716.

[6] Hoffman, P. G., Lego, M. C., Galetto, W. G., Separation and quantitation of red pepper major heat principles by reverse-phase high-pressure liquid chromatography. *J. Agric. Food Chem*. 1983, *31*, 1326 – 1330.

[7] Choi, S.-H., Suh, B.-S., Kozukue, E., Kozukue, N., *et al*., Analysis of the contents of pungent compounds in fresh Korean red peppers and in pepper-containing foods. *J. Agric. Food Chem.* 2006, *54*, 9024 – 9031.

[8] Kirschbaum-Titze, P., Hiepler, C., Mueller-Seitz, E., Petz, M., Pungency in Paprika (*Capsicum annuum*). 1. Decrease of capsaicinoid content following cellular disruption. *J. Agric. Food Chem.* 2002, *50*, 1260 – 1263.

[9] Kirschbaum-Titze, P., Mueller-Seitz, E., Petz, M., Pungency in Paprika (*Capsicum annuum*). 2. Heterogeneity of capsaicinoid content in individual fruits from one plant. *J. Agric. Food Chem.* 2002, *50*, 1264 – 1266.

[10] Blum, E., Liu, K., Mazourek, M., Yoo, E. Y., *et al*., Molecular mapping of the *C locus* for presence of pungency in *Capsicum. Genome* 2002, *45*, 702 – 705.

[11] Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., *et al*., The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 1997, *389*, 816 – 824.

[12] Mori, A., Lehmann, S., O'Kelly, J., Kumagai, T., *et al*., Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. *Cancer Res.* 2006, *66*, 3222 – 3229.

[13] Estrada, B., Bernal, M. A., Diaz, J., Pomar, F., Merino, F., Capsaicinoids in vegetative organs of *Capsicum annuum* L. in relation to fruiting. *J. Agr. Food Chem.* 2002, *50*, 118 – 1191.

[14] Karp, A., Seberg, O., Buiatti, M., Molecular techniques in the assessment of botanical diversity. *Annals Botany* 1996, *78*, 143 – 149.

[15] Henry, R. J., *Identification of Plants Using Molecular Techniques in Practical Application of Plant Molecular Biology.* Chapman & Hall, London 1997.

[16] De Masi, L., Castaldo, D., Galano, G., Minasi, P., Laratta, B., Genotyping of fig (*Ficus carica* L.) via RAPD markers. *J. Sci. Food Agric*. 2005, *85*, 2235 – 2242.

[17] De Masi, L., Siviero, P., Esposito, C., Castaldo, D., *et al*., Assessment of agronomic, chemical and genetic variability in common basil (*Ocimum basilicum* L.). *Eur. Food Res. and Technol.* 2006, *223*, 273 – 281.

[18] Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., Tingey, S. V., DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 1990, *18*, 6531 – 6535.

[19] Welsh, J., McClelland, M., Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res*. 1990, *18*, 7213 – 7218.

[20] Storsberg, J., Schulz, H., Keusgen, M., Tannous, F., *et al*., Chemical characterization of interspecific hybrids between *Allium cepa* L. and *Allium kermesinum* Rchb. *J. Agr. Food Chem.* 2004, *52*, 5499 – 5505.

[21] Vieira, R. F., Grayer, R. J., Paton, A., Simon, J. E., Genetic diversity of *Ocimum gratissimum* L. based on volatile oil constituents, flavonoids and RAPD markers. *Biochem. Syst. Ecol.* 2001, *29*, 287 – 304.

[22] Angioni, A., Barra, A., Cereti, E., Barile, D., *et al*., Chemical composition, plant genetic differences, antimicrobial and antifungal activity investigation of the essential oil of *Rosmarinus officinalis* L. *J. Agr. Food Chem*. 2004, *52*, 3530 – 3535.

[23] Martin, J. P., Bermejo, J. E. H., Genetic variation in the endemic and endangered *Rosmarinus tomentosus* Huber-Morath & Maire (Labiatae) using RAPD markers, *Heredity* 2000, *85*, 434 – 443.

[24] Echeverrigaray, S., Agostini, G., Atti-Serfini, L., Paroul, N. *et al*., Correlation between the chemical and genetic relationships among commercial thyme cultivars. *J. Agr. Food Chem.* 2001, *49*, 4220 – 4223.

[25] Belletti, P., Lanteri, S., Saracco, F., Allozyme variability in *Capsicum*, in: *Proceedings of the Eighth Meeting on Genetics and Breeding on Capsicum and Eggplant*, Roma, Italy, 7-10 Sept 1992, pp. 221 – 226.

[26] Odeigah, P. G. C., Oboh, B., Ahhalokpe, I. O., The characterization of Nigerian varieties of pepper, *Capsicum annuum*, and *Capsicum fructescens* by SDS-polyacrylamide gel electrophoresis of seed proteins. *Genet. Res. Crop Evol*. 1999, *46*, 127 – 131.

[27] Prince, J. P., Lackney, V. K., Angeles, C., Blauth, J. R., Kyle, M. M., A survey of DNA polymorphism within the genus *Capsicum* and the fingerprinting of pepper cultivars. *Genome* 1995, *38*, 224 – 231.

[28] Paran, I., Aftergoot, E., Shifriss, C., Variation in *Capsicum annuum* revealed by RAPD and AFLP markers. *Euphytica* 1998, *99*, 167 – 173.

[29] Lefebvre, V., Goffinet, B., Chauvet, J. C., Caromel, B., *et al.*, Evaluation of genetic distances between pepper inbred lines for cultivar protection purposes: comparison of AFLP, RAPD and phenotypic data. *Theor. Appl. Genet.* 2001, *102*, 741 – 750.

[30] Arnedo-Andres, S., Gil-Ortega, R., Luis-Arteaga, M., Hormaza, I., Development of RAPD and SCAR markers linked to the Pvr4 locus for resistance to PVY in pepper (*Capsicum annuum* L.). *Theor. Appl. Genet.* 2002, *105*, 1067 – 1074.

[31] Ilbi, H., RAPD markers assisted varietal identification and genetic purity test in pepper, *Capsicum annuum*. *Scientia Horticulturae* 2003, *97*, 211 – 218.

[32] Lanteri, S., Acquadro, A., Quagliotti, L., Portis, E., RAPD and AFLP assessment of genetic variation in a landrace of pepper (*Capsicum annuum* L.), grown in North-West Italy. *Genet. Resour. Crop Evol.* 2003, *50*, 723 – 735.

[33] Official Analytical Methods of the American Spice Trade Association 1997, 4th ed., ASTA Inc., Englewood Cliffs, New Jersey 1997.

[34] De Sio, F., Servillo, L., Loiudice, R., Laratta, B.,Castaldo, D., A chromatographic procedure for the determination of carotenoids and chlorophylls in vegetable products. *Acta Alimentaria* 2001, *30*, 395 – 405.

[35] Kovach, W. L., *MVSP – A MultiVariate Statistical Package for Windows, ver 3.1*. Kovach Computing Services, Pentraeth, Wales, UK 1999.

[36] Galtieri, V., Laratta, B., Minasi, P., De Masi, L., Indagine genetico-molecolare RAPD-PCR su popolazioni calabresi di peperoncino piccante (*Capsicum annuum* ssp.). *Essenze Derivati Agrumari* 2004, *74*, 63 – 66.

