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Chemical and Genomic Combined Approach Applied to the Characterization and Identification of Italian *Allium sativum* L.

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Chemotype analyses and random amplified polymorphic DNA (RAPD) genomic analyses have been applied to the characterization of *Allium sativum* variety from Voghiera (Ferrara, Italy), a typical Italian product actually demanding the Protected Designation of Origin (PDO). The garlic from Voghiera is characterized by peculiar morphological and composition characteristics. The proximate composition and atomic absorbance spectrometry elemental pattern of this garlic suggested as the chemical composition did not depend on the intrinsic pedologic soil features only, but it was probably connected to some peculiar genetic characters. Amplification of genomic DNA using random primers highlighted a good clustering differentiating of Voghiera *Allium sativum* from five commercial reference samples used in this study (Piacentino, Serena, France, China, and Adriano varieties), confirming the existence of intervarietal genetic difference. The intravarietal polymorphisms of Voghiera samples were low.

KEYWORDS: Garlic; chemotype; RAPD; PCA; variety identification

INTRODUCTION

Authentication and identification of selected plant varieties is a critical point for the protection of typical, PDO, and/or PGI foods. The chemotype/genotype combined analyses represent a powerful tool in establishing the authenticity of typical products, as well as in protecting them.

Among many plant products, the bulbs or cloves of common garlic (*Allium sativum* L., Liliaceae) are widespread used as food as well as dressing for foods. The use of garlic in some countries such as Egypt and China has been established and documented for 5000 years (*1*). Since ancient times, it has been proposed as a traditional remedy for a variety of diseases (*2*); recent studies have validated many of the medicinal properties attributed to garlic and its powerful potential to lower the risk of disease. Cancer preventive actions of garlic extracts and its components have been demonstrated in animals (*3*). Epidemiological studies show an inverse correlation between garlic consumption and reduced risk of gastric and colon cancer. Garlic has been shown to have antithrombotic activity, to lower blood lipids, and to have a cardioprotective effect (*4*, *5*). The mechanisms of garlic have been ascribed to its potent antioxidant property, to its ability to stimulate immunological responsiveness, as well as to its ability to modulate prostanoid synthesis (*6*). The worldwide production of garlic in 2002 was 11.5 million tons, with the leadership of China (8.6 million tons), followed by India and the Korean Republic (0.5 and 0.4 million tons, respectively). The European production of garlic represents only 6% of the world amounts. Italian production is around 50 000 tons (major regional production: Campania, Sicilia, Veneto, and Emilia-Romagna; *7*).

Garlic is a monocot sexually sterile plant, usually not able to produce vital seed. The classic propagation happens only by vegetative way, through the burial of the cloves. This particular type of reproduction has favored the spread of local ecotypes, that adapt themselves well to their environmental situations.

These ecotypes, commonly called "variety", are distinguished from others by these characteristics: the color of the cloves' external tunics, the dimension of the bulbs, the number/dimension of the cloves, and the precocity.

The cultivated ecotypes in Italy mainly belong to two types: (i) white garlic, characterized by white-silvered tunics, regular bulbs with 14−15 cloves, proper for autumn seeding; and (ii) pink garlic, characterized by pink external tunics, and more than 20 cloves for each bulb.

Some ecotypes, interesting from a biological and agronomic point of view and therefore long-time qualified on the national and foreign markets, have been enrolled in the Italian Register of the varieties of garlic. This book was created to protect ecotypes of long tradition, often present on the market with different denominations, and to ensure sanitary seed material (*8*).

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Currently, the Italian market officially has four enrolled varieties: Piacentino, Serena, Cristop (white garlic), and Sulmona (pink garlic).

Three of them (Piacentino, Serena, and Sulmona) are national ecotypes, while Cristop has a French origin. Garlic from Voghiera (Ferrara, Italy) is a typical Italian product actually demanding the Protected Designation of Origin (PDO).

Chemical composition of plant foods can be affected by nutrients present in the ground as well as by the fertilization methods: these effects may be studied by means of instrumental classic analyses (atomic absorbance spectrometry, HPLC, electrophoretic techniques).

The distinguishment of "varietal groups" of *Allium sativum* is currently obtained via morphophysiological characters (*9*). Other alternative approaches for the classification and clustering are isoenzymes analysis (*10*), in some cases coupled with DNA-related methods (*11*, *12*). The use of PCR-based methods such as random amplification of polymorphic DNA (RAPD; *13*, *14*) or amplified fragment length polymorphism (AFLP; *12*, *15*) is a powerful tool for assessing genetic relationships in plant variety.

The RAPD technique involves the amplification of genomic DNA using short primers, called "random primers" (*16*, *17*). Different electrophoretic complex patterns can be obtained, and the genetic pathway reveals polymorphism among DNA samples from different individual organisms. Using the RAPD approach, a single mutation point could be clearly identified by means of the passing of one single band by the complex fingerprint. Moreover, by means of appropriate statistical methods, RAPD analysis has been effective in detecting population diversity among various types of organisms (*18*, *19*).

Principal component analysis (PCA) is a powerful multivariate statistical technique commonly used for the linear mapping of multidimensional data onto lower dimensions, with minimal loss of information (*20*). PCA is useful to extract the information from large databases and to make sense of large bodies of interrelated data. The first principal component is that linear combination of the columns of X (an *m*-dimensional data set) that describes the greatest amount of variability (*21*). As is well-known, the use of PCA allows a more objective interpretation of the RAPD fingerprint, reducing the descriptor parameters to two or three only (*22*).

The aims of this work are as follows: (i) to analyze the proximate and chemical composition of Voghiera garlic samples and to verify the possible influence of the ground and/or different fertilization on the quality of the product, (ii) to assess the genetic differences between some ecotypes of *Allium sativum* by means of the PCA both on the chemical and on the genomic markers, and finally (iii) to evaluate a typical product of long tradition and enroll it in the Italian Register of the garlic varieties.

MATERIALS AND METHODS

Plant Material. DNA samples were obtained from garlic bulbs grown in three experimental fields. The fields were divided into nine randomized blocks (6 × 8 m) with three levels of fertilization and three replications: 150 kg/ha P_2O_5, 150 kg/ha K_2O, and 0 kg/ha N; 150 kg/ha P_2O_5, 150 kg/ha K_2O, and 125 kg/ha N; and 150 kg/ha P_2O_5, 150 kg/ha K_2O, and 250 kg/ha N.

Between the blocks, a strip of uncultivated ground of about 0.5 m was maintained to facilitate seeding.

Twenty-seven samples of *Allium sativum* present in the three most central files were withdrawn to exclude possible interferences among the various blocks.

All reference samples (Piacentino, Serena, garlic from France and from China) were purchased from different import−export firms; Adriano garlic variety was purchased from a local supermarket.

Proximate Composition. *Moisture.* The samples were carefully cleaned, separating the tunics from the cloves. The cloves were thinly ground and then dried in an oven at 110 °C, until a constant weight was achieved. Moisture was expressed by % (w/w).

Total Nitrogen Compounds. Total nitrogen compounds were determined on 1 g of sample, according to the Kjeldahl method (International Dairy Federation. Determination of nitrogen content. Kjeldahl method, Norma FIL-IDF, No 20B, Brussels, Belgium, 1993).

Lipids Extraction. About 10 g of dried product was put in the thimble and introduced into the extraction unit (Velp Scientifica, Usmate, Milan, Italy). Extraction phase: 50 mL of diethyl ether (Carlo Erba, Rodano, Milan, Italy). Boiling stones: 30 min with thimble immersed in boiling solvent, 30 min of reflux washing. The extraction vessel was put in an oven at 100 °C for 30 min, in a desiccator, and then weighed to obtain the total lipids content.

Chemical Analysis. *Atomic Absorption Spectrometry.* Na, K, Mg, Zn, Ca, Fe, Cu, Ni, Mn, Cr, Pb, and Cd were determined according to described methods.

The garlic samples were thinly ground, accurately homogenized, and dried at 110 °C in a laboratory oven. One gram of each sample was mineralized in CEM's digestion vessels (PTFE mod. SV140, FKV) with $HNO_3-H_2O_2$ in a microwave digester (Millestone MLS 1200, FKV) coupled with a module for steam extraction (EM 5, FKV). Triplicate extractions were done on each sample. A Perkin-Elmer graphite furnace mounted on a Perkin-Elmer (mod. 1100B) atomic absorption spectrometer (AAS) was used with an autosampler. The spectrometer was equipped with deuterium background corrector and single-element Intensitron (Perkin-Elmer). Hollow cathode lamps were used for the measurements of copper at 324.8 nm.

The accuracy of the measurement was evaluated by means of recovery tests, and the precision, expressed as coefficient of variation (CV%), was in the range of 0.6−2.4. Standard solutions of each element were prepared by diluting reference standard solutions for AAS (BDH certified atomic absorption reference solutions). All reagents and chemicals were of "pro-analysis" grade, and the water used was obtained by means of a Milli-Q-system (Millipore, Bedford, MA). The samples were checked against reference standards and measured for their absorbance, after instrument calibration. The average of five readings of absorbance was taken in all samples.

Genetic Analysis. *DNA Reagents and Instrumentation.* Taq-polymerase, $MgCl_2$ solution, and buffer 10X were purchased from Genenco (Milan, Italy); dNTPs were obtained from M-Medical (Milan, Italy). Primers were synthesized by M-Medical. Gels were prepared with agarose LE from Euroclone. All other chemicals were from Fluka (Buchs, Switzerland).

Amplification was carried out using a PE Applied Biosystem PCR system 9700 (Monza, Italy). The electrophoretic analyses were performed using a Power Pack 300 power supply equipped with a Subcell agarose gel electrophoresis system (Bio-Rad, Segrate Milan, Italy).

Genomic DNA Extraction. The extraction of the genomic DNA has been carried out with a Nucleon Phytopure plant and fungal extraction kit (Amersham-Pharmacia Biotech), specific for vegetable material. DNA concentration was quantified using Smart-Spec 3000 spectrophotometer (Bio-Rad, Segrate Milan, Italy). The quality of the DNA samples was evaluated by means of ratio of absorbance at 260 and 280. The quality of genomic DNA was further checked by subjecting it to agarose gel electrophoresis.

DNA Amplification and Detection. The optimized PCR amplification solution (50 μL) contained 11.9 μL of ultrapure H_2O, 2.5 μL of 10X buffer [100 mM Tris-HCl, 500 mM KCl, 500 mM $(NH_4)_2SO_4$, 15 mM $MgCl_2$, pH 8.7, at 20 °C], 2 μL of dNTPs (dATP, dCTP, dGTP, and dTTP, 2.5 mM cad.), 1 μL of 10 μM of each primer, 2.5 μL of 25 μM $MgCl_2$, 0.5 unit of Taq-polymerase, and 5 μL of genomic DNA (0.1 ng/μL). Each reaction solution was overlaid with 50 μL of mineral oil to prevent evaporation and cross contamination.

The thermal cycler was programmed for RAPD analyses as follows: initial denaturation at 94 °C for 5 min, 45 amplification cycles

Table 1. Details of the Random Primers (10-mer) Used in This Study

primer (operon series)	sequence (5′ to 3′)	melting temp °C (at 400 nM)	% of G + C
A1	CAGGCCCTTC	31.5	70
A2	TGCCGAGCTG	35.3	70
A5	AGGGGTCTTG	30.5	60
A7	GAAACGGGTG	27.6	60
A8	GTGACGTAGG	15.8	60
A9	GGGTAACGCC	35.9	70
A10	GTGATCGCAG	22.4	60
A11	CAATCGCCGT	34.1	60
B1	GTTTCGCTCC	35.8	60
B2	TGATCCCTGG	26.7	60
B4	GGACTGGAGT	17.4	60
B5	TGCGCCCTTC	39	70
B6	TGCTCTGCCC	33.2	70
B7	GGTGACGCAG	28	70
B10	CTGCTGGGAC	26.5	70
B18	CCACAGCAGT	21.3	60

at 94 °C for 40 s (denaturation), 32 °C for 40 s (annealing), 72 °C for 90 s (extension), and a final step of extension at 72 °C for 7 min; 16 primers (10 mer) were used (**Table 1**).

Aliquots of 12 μL of amplified sample were loaded in agarose gels 2% and analyzed by electrophoresis for 100 min at 80 V, in TBE buffer 1X (8.8 mM Tris-HCl, 8.8 mM boric acid, and 0.2 mM EDTA). The samples were added to loading buffer (1X) and then stained with 0.5 μg/μL ethidium bromide. All RAPD profiles were analyzed using the Fluor-S MultImager detector, equipped with the Quantity One software from Bio-Rad (Segrate, Milan, Italy).

Statistical Analysis. The analysis of variance (ANOVA) was carried out on all data by StatMost program. Principal component analysis (PCA) was performed by using Mathematica software (Wolfram Research Inc., Champaign, IL).

RESULTS AND DISCUSSION

Chemical Analysis. To characterize all samples of Voghiera garlic (sowed in three different randomized fields each with three types of fertilization), we first study the proximate composition (lipids, proteins as total nitrogen compounds, and minerals content).

The fat content did not show any substantial differences among the three different kinds of fertilization or among the randomized fields.

To evaluate the differences between Voghiera garlic and other varieties, five commercial garlic varieties were bought and analyzed: Piacentino and Serena (Italian garlic already enrolled in the Italian garlic register of variety), one sample of Chinese garlic (commercial), one sample of French garlic (commercial), and a local Italian garlic called "Adriano". Fat percentages shown by these five referenced garlic varieties were included between 0.12% and 0.20%. The comparison of Voghiera garlic with reference standards exhibited high homogeneity in fat content (**Figure 1**).

The garlic analyses proceeded with total nitrogen content determination (Kjeldhal method). The comparison of different randomized blocks of Voghiera garlic showed the same differences in nitrogen value. Voghiera garlic fertilized using P_2O_5 and K_2O only exhibited a smaller ratio of total nitrogen (TN) than blocks also fertilized with 125 and 250 kg/ha of nitrogen; these results were confirmed by three different experimental fields (**Figure 2**). The difference between 125 and 250 kg/ha of N (a very high quantity, about 2 times the nitrogen fertilization admitted in garlic production disciplinary by Emilia Romagna Italian Region, which provides a maximum of 150 kg/ha of nitrogen fertilization) seemed to be not statistically significant.

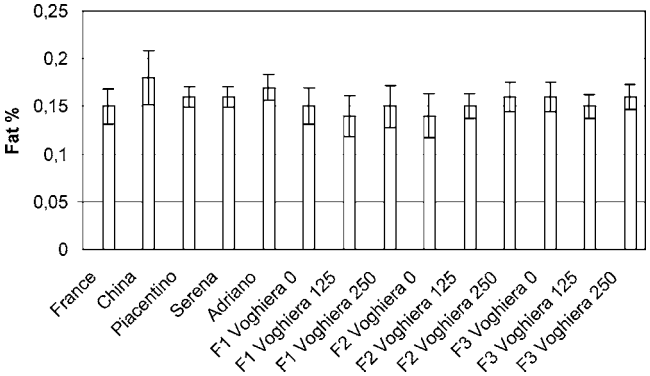

Figure 1. Fat content (% w/w, dried weight) of commercial references as compared to Voghiera garlic sowed in three different fields (F1, F2, F3) with three fertilization levels (0 kg/ha of nitrogen, 125 kg/ha of nitrogen, 250 kg/ha of nitrogen). All data are reported as the average of three values from three repeated experimental blocks.

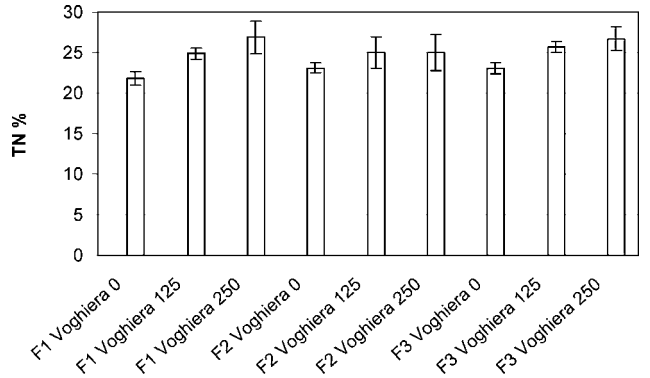

Figure 2. Total nitrogen content (TN, % w/w, dried weight) of Voghiera varieties sowed in three different fields (F1, F2, F3) with three fertilization levels (0 kg/ha of nitrogen, 125 kg/ha of nitrogen, 250 kg/ha of nitrogen). All data are reported as the average of three values from three repeated experimental blocks.

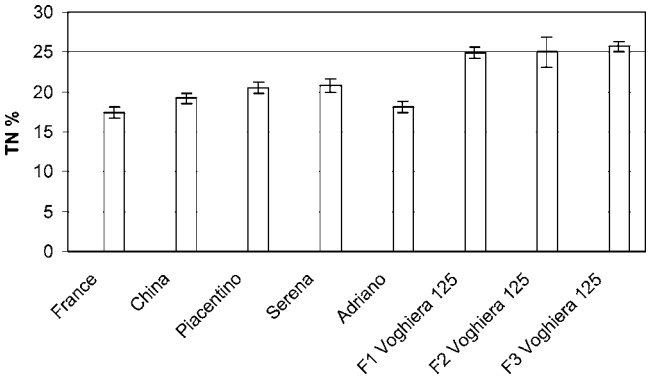

Figure 3. Total nitrogen content (TN, % w/w, dried weight) of Voghiera variety sowed in three different fields (F1, F2, F3) and fertilized with 125 kg/ha of nitrogen and in reference samples. All data are reported as the average of three values from three repeated experimental blocks.

It seemed interesting to focus attention on Voghiera 125 kg/ha (quantity of nitrogen usually employed for garlic production, as described in the disciplinary) and compare these data with the TN content of reference samples (**Figure 3**).

The total nitrogen content of Voghiera garlic was higher then those of reference samples compared in this study, showing that nitrogen content in Voghiera samples could be related to the variety characters. The fertilization approach does not seem to influence nitrogen content.

Table 2. Mineral Content of Commercial Garlic References As Compared to Voghiera Garlic Sowed in Three Different Fields (F1, F2, F3) with Three Fertilization Levels (0, 125, and 250 kg/ha of Nitrogen)[a]

	μg/g								
garlic	Mg	Zn	Ca	Fe	Cu	Ni	Mn	K	Na
France	408.6	17.7	258.8	12.1	3.5	0.4	6.4	12 122	47.7
China	676.4	12.2	316.5	22.9	2	0.5	10	15 015.2	649.3
Piacentino	550.7	23.4	302.9	13.1	4.8	0.8	5.5	12 942.3	23.4
Serena	804.4	24.1	221.9	12.2	6.1	1.9	7.4	13 784	27.5
Adriano	542.5	14.9	212.9	14.4	3.9	N.R.	7.5	13 425.5	32.6
				Field 1					
Voghiera 0	695.7	26.4	298.5	22.1	6.6	1.4	7.2	17 163.3	175.9
Voghiera 125	740.1	28.2	316.6	23.8	6.4	1.3	7.3	16 656.0	217.3
Voghiera 250	743.5	29.3	321.4	23.6	6.6	1	7.5	15 944.7	291.1
				Field 2					
Voghiera 0	683.4	28.2	325.4	22.9	6.4	0.3	6.6	13 576.5	473.6
Voghiera 125	769.6	27.6	300.5	21.7	6.7	0.2	6.3	13 771.9	420.7
Voghiera 250	728.6	28.7	300.5	22.8	5.9	0.3	5.9	15 799.4	473.6
				Field 3					
Voghiera 0	643.8	30.8	333.3	21.9	7.9	1	7.4	14 572.3	340.1
Voghiera 125	642.7	32.6	322.9	23.8	8.3	1.3	7.2	13 177.8	330.6
Voghiera 250	725	34	322.5	22.7	8.2	0.8	7.2	14 724.2	417

[a] The data are reported as the average of three values (CV% ≤ 2).

Table 3. Component Loading for the Extraction of Five Principal Components on the Elemental Composition (Nine Variables)[a]

component	variance %	cumulative % of variance
1	34.2	34.2
2	18.8	53.0
3	15.8	68.8
4	11.5	80.3
5	5.4	85.7

[a] Variance and cumulative variance are expressed as percentages.

Mineral Content. The analysis of the elemental composition has been developed using atomic absorption spectroscopy methodology, as described in the Material and Methods section.

Concentrations of some potentially toxic heavy metals (Cd, Cr, Pb), which have hard adverse effects on different living species, were below the limit of detection (0.01 ppm for all metals).

Na, K, Ca, Mg, Mn, Cu, Fe, Zn, and Ni detected in different garlic varieties are showed in **Table 2**.

It was interesting to compare the K concentration toward other elements. The data from the analysis revealed that the concentration of K was significantly higher, according to literature references (*23*). This result was not surprising, due to the nature and role of this fundamental element for garlic, as well as other plants which grew from an underground bulb. Moreover, the presence of Na was significantly different among the analyzed varieties. The concentration of sodium in Voghiera variety was about 5 times higher than that in Piacentino, Serena, Adriano, and France samples; China samples contained the highest concentration of sodium ion.

To perform a rational and statistical analysis of all of the elements, principal component analysis was used. **Table 3** reports the amount of variance for PCA analysis on the elemental composition. However, the concentration of heavy metals was not considered for statistical analysis. This yielded the design of a 9 × 32 (elements/samples) data matrix.

The cluster analysis of all samples leads to the identification of three clusters inside the Voghiera variety, corresponding to the three different experimental fields.

Other garlic samples (references) did not form a cluster; in fact, they were located in different areas of space, defined by the first three principal components (**Figure 4**). Voghiera garlic samples were all located in the left part of the figure; Piacentino, Serena, Adriano, China, and France samples were located in the opposite side.

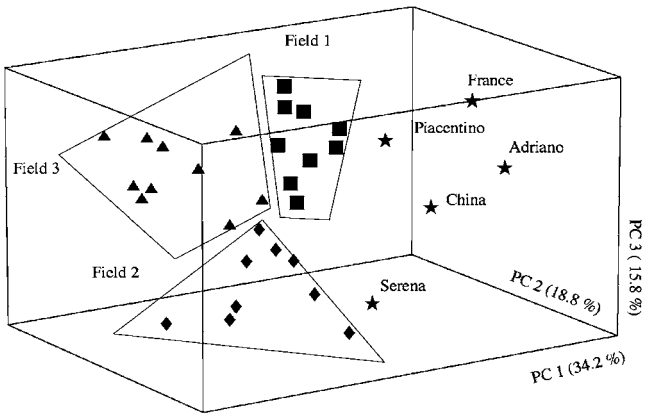

Figure 4. Principal component analysis of the 32 samples of garlic analyzed, based on the elemental composition (nine variables) where the three first principal components explain 68.8% of the total variation.

This result highlighted a good clustering among Voghiera *Allium sativum* and a clear separation from the commercial reference samples, using PCA on elemental composition. Moreover, the pattern of micro- and macroelements did not seem to depend on only intrinsic pedologic soil features, but it was also probably related to genetic characteristics.

Genetic Analysis. Genomic DNA extracted from different *Allium sativum* samples was amplified using a RAPD protocol as described previously. A total of 16 primers (10-mer) were used, allowing a total of 131 bands (32 garlic samples). The study of polymorphisms among different plant varieties is classically obtained by the analysis of complex fingerprints generated by RAPD (common bands toward total generated band number; *13*, *14*, *24*). This kind of data processing is currently expressed by "% homology degree".

In our study, some "defined" values were arbitrarily assigned to all bands generated, using the same approach described in a previous work (*25*). Briefly, RAPD bands were recorded in a binary form as a series of "0" and "1", where a "1" denotes the

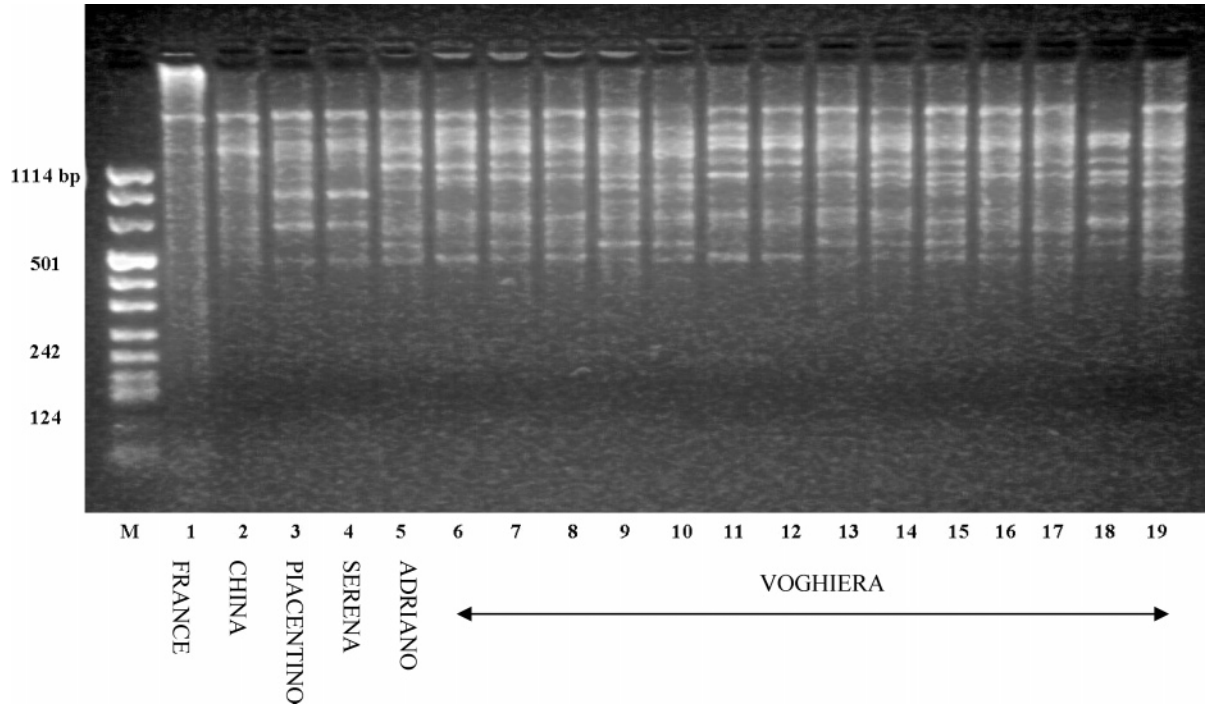

Figure 5. Typical RAPD complex fingerprint generated with B1 primer and separated by agarose gel electrophoresis (2% w/V). Lane M, marker; lane 1, garlic from France; lane 2, garlic from China; lane 3, garlic Piacentino; lane 4, garlic Serena; lane 5, garlic Adriano; lanes 6–19, garlic Voghiera.

Table 4. Component Loading for the Extraction of Five Principal Components on the Complete Data Set (131 RAPD Bands)[a]

component	variance %	cumulative % of variance
1	94.4	94.4
2	1.4	95.8
3	0.8	96.6
4	0.6	97.2
5	0.5	97.7

[a] Variance and cumulative variance are expressed as percentages.

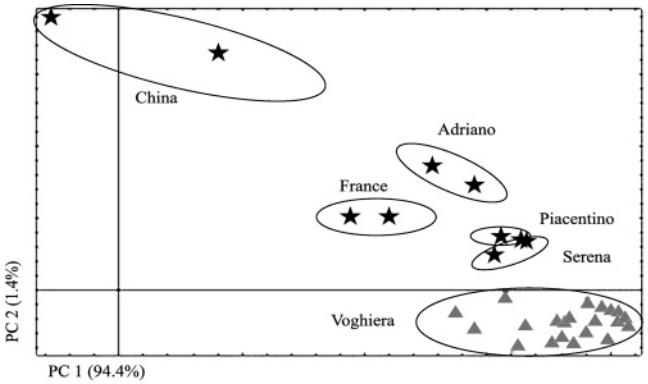

Figure 6. Principal component analysis of the 32 samples of garlic analyzed obtained translating the information contained on 89 selected RAPD-bands (11 selected random primers), where the two first principal components explain 95.8% of the total variation.

presence of a band and a "0" its absence. A variable value based on the total mean bands indicates the "nonamplified" lanes. (In fact, the total lack of a band pattern could be caused by an unsuccessful amplification.)

Out of the 16 RAPD primers used for the amplification of the 32 garlic accessions, 15 have lead to polymorphic products. An example of the amplification pattern obtained with one RAPD primer is shown in **Figure 5**. A total of 131 amplification products were scored, and 100 (76.3%) were polymorphic. After the amplification step, all of the profiles were statistically treated as described in the Materials and Methods section.

The genetic diversity between the garlic of Voghiera and some reference samples was analyzed using the principal component analysis approach; the clusters were defined on the Euclidean distance. PCA was done on a covariance matrix (*26*) to express the information obtained by RAPD fingerprints as well as to understand the relationships existing among the various genotypes. A study of intervarietal polymorphisms was also considered.

Studying all of the 131 RAPD bands, a clustering with a good separation was obtained; the reference samples were clearly separated from the Voghiera garlic. **Table 4** reports the amounts of variance for this statistical analysis. Higher variance extracted values represent a greater degree of representation; in this case, it is evident that the two first new variables, created as replacement for the original variables (components 1 and 2), still retain their original character, while the other variables (components 3, 4, and 5) contain a very low percentage of information and can be neglected.

To optimize the clustering of Voghiera garlic as well as to improve and simplify the possibility to their recognition, the study was completed by a selection of the random primers used. The primers were selected considering all possible combinations. This approach leads us to conclude that the minimum number of RAPD bands providing a constant clustering with a high variance value has been 89 characters, corresponding to the amplification of 11 selected RAPD primers (A1, A2, A5, A7, A8, A9, A10, B1, B5, B6, B7). The graphical representation of PCA on the selected characters is presented in **Figure 6**.

The samples were located in different areas of the plane defined by the first two principal components (**Figure 6**), so it can be assumed that a genetic difference between the samples of common garlic and the Voghiera garlic could exist.

It is also interesting that other sample clusters correlate their geographic origin and their variety. Particularly, the samples

coming from China were in an isolated location regarding the remaining referenced samples. This indicates that beyond a geographic distance exists "a real" genetic distance.

This result is in accord with the hypothesis that from the original habitat reported as the Tien Shan Mountains located in Central Asia, four subtypes of garlic were spread to the rest of the world (Asiatic, Continental, Mediterranean-European, and Mediterranean-African; *15*).

In conclusion, the PCA processing of chemical data (chemotype) showed significant differences in micro- and macroelements content and total nitrogen compounds between Voghiera ecotype and other commercial samples from different geographic origin. The RAPD technique was used to assess the polymorphism of Voghiera garlic among the "*Allium sativum* L." group. The analysis confirmed this typical Italian garlic as a potential novel variety to add to the register of Italian varieties. The object definition of this suggestion will be achieved by analyzing more samples of Voghiera garlic, using the same combined approach.

The results obtained also confirm that a relationship between garlic features and soil exists, revealing the extremely great importance of agricultural production disciplinary and Protected Designation of Origin (PDO), actually proposed to the European Union Commission.

Finally, we suggest the use of combined chemical and genetic analyses to link the product to the typical production area of this variety of garlic.

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