The Americas Journal of Plant Science and Biotechnology ©2010 Global Science Books

Molecular Markers: Are They Really Useful to Detect Genetic Variability in Local Garlic Collections?

Sandra García Lampasona[1,2*] • José Luis Burba[1] • Philipp W. Simon[3]

[1] EEA La Consulta INTA, CC 8 (5567) La Consulta, Mendoza, Argentina
[2] Laboratorio de Biología Molecular Facultad de Ciencias Agrarias, UNCuyo, Almirante Brown 500, 5505, Chacras de Coria, Mendoza, Argentina
[3] Vegetable Crops Research Unit, United States Department of Agriculture-Agricultural Research Service, Department of Horticulture, University of Wisconsin, Madison, WI 53706, USA

Corresponding author: * sgarcia@fca.uncu.edu.ar

ABSTRACT

The cultivation of garlic (*Allium sativum* L.) is an important activity in economic and social terms in Argentina. The traditional techniques of improvement have allowed us to obtain monoclones with proven superiority, compared with to the populations of origin. Currently these materials have been registered in the INASE (Argentine National Institute of Seeds) as new cultivars. The aims of our studies have been to characterize the genetic diversity of selected garlic clones, to verify their identity and to propose a new tool to facilitate the legal protection of germplasm. To achieve these goals we have evaluated molecular markers such as RAPDs, AFLP and fAFLP. Their usefulness is discussed in this paper.

Keywords: *Allium sativum* L., garlic, fingerprint, molecular markers
Abbreviations: AFLP, amplified fragment length polymorphism; **fAFLP**, fluorescent amplified fragment length polymorphism; **PCR**, polymerase chain reaction; **RAPD**, random amplified polymorphic DNA; **T**, ton

CONTENTS

INTRODUCTION

The common garlic (*Allium sativum* L.) is one of the oldest crops, cultivated for more than 5000 years, and it is used for food as well as medicinal applications. The cultivation of garlic is an important activity in economic and social terms, due to the great manpower demand that represents, for the great number of family companies which produce it and for the possibilities that exist for its commercialization. Garlic has a high potential profitability among the vegetables, but it is necessary to improve the technology transfer to the productive sector so that its production is more efficient. A crucial aspect in this sense is to have genetically pure, disease-free propagules for cultivation available to growers, which may generate an increase in productivity from 30 to 100% per hectare of cultivation.

Garlic is a widely cultivated crop with current world-wide production of 16 million tons cultivated in 1.22 million ha (FAOSTAT data 2008). Asia accounts for much of the world area (80.7%) and production (85.6%), being the second in yield with 11.4 T/ha (**Table 1**). The countries which make up this block are China, India and the Republic of Korea. Europe, represented by Spain, France and Italy accounts for 11.1% of the harvested surface and 6.4% of the world production, with relatively low yields of 6.2 T/ha. Brazil, Argentina and Chile account for most of the South American production, with averages of 7.9 T/ha, contributing 4% of the harvested surface and 2.9% of the world production. North America (Mexico and United States) have yields of 6.5 T/ha that contribute 2.5% of the world production just on 1.6% of the harvested surface.

Argentina produces 120,000 tons of garlic of which

Table 1 Details of the Centers of World Production (*).

Region	Surface harvested production		Production		
	Hectares	%	Tons	%	Yield (T/ha)
Asia	908,668	80.7	10,367,531	85.6	11.4
Europe	125,050	11.1	779,154	6.4	6.2
South America	44,546	4.0	351,553	2.9	7.9
North America	18,062	1.6	297,763	2.5	16.5
Rest	29,287	2.6	311,076	2.6	10.6
Total	1,125,613	100	12,10,077	100,0	

*Source: FAO databases.
%: percentage, T: tons

Received: 14 August, 2009. Accepted: 10 December, 2010.

Invited Review

65% is dedicated to the commercialization in the international markets, 25% destined to the domestic market and the rest left for seeds. Argentina is the second largest world exporter and it is the main supplier of garlic in the Mercosur. Mendoza, the main producer region, has 2,000 farmers who dedicate to this activity. Annual income for garlic export is between 50 and 100 million dollars -Brazil being the main importer-, and another 33 million goes to the national market. About 95% of garlic cultivation in Argentina is concentrated in Mendoza and San Juan provinces, with an approximate area of 15,600 ha.

The principal advances realized for garlic production in Argentina in the last 20 years are:

• The area under cultivation doubled.
• Yields increased 50 - 60%.
• International markets have expanded including longer export season and higher value.
• Management of crop production is more efficient (reduced fertilizer and water use, better seed quality, etc.).
• The system for postharvest management was adjusted and maintenance of the product packaging.
• Exports are now made 12 months of the year.
• Postharvest storage quality and product packaging has improved.

Germplasm of a cultivated crop and its wild species relatives is necessary to realize the full potential of a crop (as resistance to pathogen, salinity, drought, etc.), and this need is even more urgent in the last few years due to anticipated climatic change. With limited economic resources for the collection and appropriate conservation of germplasm, it is necessary to determine the number and quality of the samples to maintain the diversity of a species of agricultural interest, and their potentially useful wild related species. The development of appropriate technologies for the conservation of seeds, bulbs and other propagules *ex-situ* preservation in germplasm banks should include technology advances to fingerprint germplasm. In the recent years the origin and evolution of cultivated plants has stimulated great interest and new studies have been initiated to analyze the distribution, ecological behavior and genetic interaction of wild relatives and cultivated crops (Harlan 1992). Studies of this type are of interest for garlic.

In 1926, Vavilov suggested that maximum genetic diversity occurs in the origin of the cultivated plants in his essay "On the origin of the cultivated plants". This is especially true if most of the variation is controlled by dominant genes and if the center of origin also contains wild races of the cultivated crop in question. With this, Vavilov described eight centers of diversity. According to the laws of homologous series of variation that Vavilov proposed, the pattern of variation observed in a cultivated species follows the same pattern in related, wild species.

From this, the concept of centers of origin has evolved. In this way Harlan in 1971 defined centers of diversity but he disassociated them with centers of origin. He mentioned three primary centers of domestication, each one with no associated center of origin. This center of genetic diversity of garlic is located in Central Asia, where it expanded towards Egypt, China and the Mediterranean area. Garlic was probably introduced to the American continent by the Spanish conquerors to Mexico, where it was disseminated southward to Chile and later it was introduced further to the Northern hemisphere. It has been suggested that the wild species that gave origin to the cultivated garlic is *A. longicuspis*. Some researchers suggest that *A. longicuspis* is not a separate species but rather to subgroup or subspecies of *Allium sativum* (Etoh and Simon 2002). Nonetheless, *A. longicuspis* is considered either the closest wild relative of garlic or its wild ancestor.

The lack of identification of garlic commercial types and cultivars results in misleading names and multiple names for the some clone. It is for this reason that germplasm of Asian origin known in Latin America as 'Vietnam', 'Taiwan', 'Chinese' all may be the same variety, and in fact in the country of origin of these clones may not be Asia. The same thing happens with garlic coming from the European Union which is known by its country of origin as 'Russian' or 'Polish'. These idiomatic customs, and the subjective use of colors to name garlic clones does not make the panorama clearer. For example, cultivars of the Ecophysiological Group IV are named "red" in Argentina, "pink" in Chile, "roxos" in Brazil (that means violet in Portuguese) and "violet" in Spain, however these names are also used to name varieties of other groups like Pink Paraguayan (Ecophysiological Group II) or Violeta of Cadours (Ecophysiological Group III). The phonetic translation of the varieties of Chinese origin, for example, can generate even more confusion since different names are used for the same material such it is the case of "Cangshan" (Changshan) or "Jiaxiang", ("Jingxian") or "Jiading" ("Jaiding"). On the other hand those clones coexist and there are also clonal populations with great variability, to further confuse the identification of the garlic clones (Burba 1997).

The genetic improvement of garlic is limited by the absence of sexual reproduction in many clones. Because the garlic has spread vegetatively, genetic variation might have its origin in the accumulation of chromosome aberrations and mutations over time (Matus *et al*. 1999).

Most cultivated garlic clones do not flower and those flowering are often nearly or completely sterile, since very few fully mature flowers are produced (Simon and Jenderek 2003). The lack of sexual reproduction in garlic has restricted the improvement of the crop through conventional breeding. In the last decades, researchers have made progress towards understanding the factors involved in garlic sterility and fertility. However, within the last 15 years, true seed production via sexual reproduction in garlic has been demonstrated, and it is now thought that variation created by genetic recombination is the basis for differences observed among existing garlic clones. These results indicate that garlic breeding has the potential to be a significant tool for future garlic improvement (Etoh and Simon 2002; Ipek *et al*. 2005; Simon and Jenderek 2003; Zewdie *et al*. 2005).

Recently, systems for garlic seed production have been developed (Etoh and Simon 2002; Simon and Jenderek 2003).With routine seed production underway, inheritance studies and the construction of linkage maps for this crop, are now possible. Ipek *et al.* (2005) an Zewdie *et al*. (2005) published the first garlic genetic maps, using different marker systems on F2 families with different genetic background. Ipek *et al.* (2005) used two garlic populations generated by self-pollination of relatively unrelated plants to construct two low-density linkage maps, based on AFLP markers.

Morpho-physiologic characters have been employed by numerous authors (Messiaen *et al*. 1993; Burba 1997) to define varietal groups in garlic, so that the characterization of genetic variation is an essential activity to carry out conservation programs and improvement of garlic, as in other plants.

Since 1989 the Argentine Agricultural Experimental Station La Consulta INTA (National Institute of Agricultural Technology) has had a program to improve garlic. Clonal selection was used because sterility limits the possibility of improvement through sexual reproduction. This program has resulted in monoclones with proven superiority, compared to original populations. These materials have been registered in the INASE (Argentine National Institute of Seeds) as new cultivars.

Although the morphological characterization of garlic germplasm has clarified ambiguous denominations, it presents a series of disadvantages. The available descriptions are based on anatomical and morphological characteristics and consequently they are limited in number, incomplete, and affected by the environmental factors.

In spite of the fact that the use of molecular markers has played an important role in germplasm evaluation the last 10 years, there have been few reports about this topic for garlic. This is made more complicate by the fact that this species has a large genome (2C 32.7 pg) (Evans *et al.* 1983)

which has made molecular analysis more laborious. Most *Allium* species are diploid 2n=2x=14, 16 or 18. The number of chromosomes of garlic and most (> 90%) *Allium* species native to the Mediterranean basin is eight (Ved Brat 1965). This is in contrast to 95% of the North American *Allium* species that have a basic chromosome number of seven, and a few *Allium* species from Eurasia that possess a basic chromosome number of nine (Ved Brat 1965).

WHY MOLECULAR MARKERS?

To generate enough molecular markers with smaller amounts of less pure DNA, polymerase chain reaction PCR-based methods are used including random amplified polymorphic DNA (RAPDs) (Pooler and Simon 1993; Maaβ and Klaas 1995; Bradley *et al*. 1996; Al-Zahim *et al*. 1997; Lallemand *et al.* 1997; García-Lampasona and Burba 1999; Mota *et al.* 2004; Buso *et al.* 2008) and amplified fragment length polymorphism (AFLP) (Garcia-Lampasona *et al*. 2003; Ipek *et al*. 2003; Volk *et al*. 2004; Ipek *et al*. 2005; Rosales-Longo and Molina-Monterroso 2007).

The Amplified Fragment Length Polymorphism (AFLP) has provided a powerful and reliable DNA fingerprinting tool for genomes of any origin or complexity (Zabeau and Vos 1993; Vos *et al*. 1995). This technique is advantageous because variability can be assessed at a large number of independent loci, and data is obtained quickly and reproducibly. The resulting DNA fingerprint provides a large number of genetic markers and the multiplex ratio, defined as the number of information points analyzed per experiment, is much higher than for other types of markers (Powell *et al*. 1996). AFLP is reported to be more informative than RAPD, isoenzymes or nuclear RFLP. AFLPs has been applied to characterize the genetic diversity in soybean (Maughan *et al*. 1996), lentil (Sharma *et al*. 1996), wild bean (Tohme *et al*. 1996), tea (Paul *et al*. 1997), rice (Zhu *et al*. 1998), azuki (Yee *et al*. 1999), cassava (Sánchez *et al*. 1999) and carrot (Shim and Jorgensen 2000).

The potential use of AFLP for cultivar identification has been pointed out by the Molecular Markers Group of the International Union for Plant Varieties Protection, UPOV (Working Group on Biochemical and Molecular Techniques and DNA-Profiling in Particular, 1995). They recommend AFLP for germplasm identification, legal protection of cultivars, genetic resources conservation and breeding programs (Fischer and Bachmann 2000).

The aims of this study were (i) characterize the genetic diversity of the selected garlic clones, (ii) verify the identity of the material, avoiding duplications in a germplasm bank collection, and (iii) propose a new tool to facilitate the legal protection of the materials.

HOW HAVE RAPDs AND ISOZYMES BEEN USED IN GARLIC?

The World

Allium sativum is a predominantly sterile species. Nevertheless, there is great variability in morphological and physiological features including varying degrees of bolting and flower formation, which led to the proposition of three botanical varieties. While *Allium longicuspis* is referred to as the wild progenitor, its status as a separate species has been disputed. Pooler and Simon (1993) investigated a collection of 110 garlic clones with morphological and isozyme methods for an infraspecific classification. Thirteen isozyme systems were tested, although, because inconsistent staining or lack of variability, only four were useful, and 17 different enzyme groups were detected.

While flower characteristics correlated well with isozyme data, bulb-related traits or geographical origin had little predictive value for the genetic relationship of accessions. Maaβ and Klaas (1995) tested 300 clones with isozymes, and 48 of this were tested with RAPDs as well, to compare the two marker systems. There is a gene pool with many accessions from areas close to the center of origin, in Central Asia, and this was suitable for investigating the genetic relationship between cultivated clones with primitive features, derived strains and a feral accession of *A. longicuspis*. Twelve isozyme systems were tested which identified 22 loci, ten of which were polymorphic and defined 16 isozyme groups. Predictably, the 125 RAPD markers allowed a more detailed distinction, but generally both markers gave a good delimitation of varieties *sativum* (bolting and non-bolting type could be separated) and *ophioscorodon*. The third variety *pekinense* was not dintinguishable by either marker from *longicuspis*-type plants, nor was an accession determined as *A. longicuspis* separated from more primitive (i.e. partially fertile) garlics based on molecular markers.

Maaβ and Klaas (1995) determined four garlic groups based on dendrograms of isozyme and RAPD data. Ipek and Simon (1998) showed that AFLP was a useful technique for assessing the genetics relationship in garlic clones and identified ten groups in 9 diverse collection of garlic (Ipek *et al.* 2003).

A similar range of accessions was investigated by Al-Zahim *et al*. (1997). Their results differed in some important aspects. Twenty-seven named garlic cultivars were structured with 63 polymorphic RAPD bands generated from 26 primers. Eleven accessions were assigned to variety *ophioscorodon*, 11 to variety *sativum* and five to *A. longicuspis*. In agreement with Maaβ and Klaas (1995), the accessions of var. *sativum* (only non-bolting accessions were included) grouped together: however, these workers found genetic differentiation within var. *ophioscorodon* and interspersal with *A. longicuspis* accessions. These findings were in contrast to the genetic homogeneity of the *ophioscorodon* group (80 accessions were investigated by isozymes, seven of this by RAPDs), being genetically clearly distinct from *longicuspis*-type accessions as reported by Maaβ and Klaas (1995). The different results may be explained by the different morphological classification of the material prior to the molecular study, rather than by a misapplication of the RAPD markers in either case, since a comparable number of primers and markers per taxon was used in both laboratories. In the well-characterized German collection in Gatersleben, *ophioscorodon* was morphologically clearly distinguishable from *A. longicuspis* (Helm 1956; Maaβ and Klaas 1995) while Al-Zahim *et al*. (1997) reported difficulties in distinguishing *ophioscorodon* from *A. longicuspis* based solely on exserted anthers. An interspersal of var. *ophioscorodon* accessions either plants from the *longicuspis* group would explain these data.

Bradley *et al.* (1996) investigated a collection of 20 Australian garlic accessions with five RAPDs primers, resulting in 65 marker bands. The approach was well suited to grouping the major Australian cultivars according to bolting behaviour, early and late types, and places of origin.

Mota *et al.* (2004) studied the genetic diversity among 12 Brazilian cultivars considered as "noble" and "half-noble". They amplified 279 fragments using 80 primers. The cultivars "noble" and "half-noble" presented 57.1 and 54.2% of similarity, respectively.

More recently the diversity of Brazilian germplasm was analyzed (Buso *et al.* 2008) using 206 random amplified polymorphic DNA markers for analysis of the 17 most widely-grown garlic cultivars. Bootstrap analysis showed that the number of markers was efficient and sufficient to obtain a coefficient of variation of 10%. Similarity varied between 16 and 98% and cluster analysis showed that, in general, genetic similarities correlate with morphological characters of the cultivars and production cycle variation. This study emphasizes the suitability of RAPD analysis for garlic diversity analysis, which revealed that considerable genetic variation exists among the garlic cultivars tested. Additionally, RAPD markers used, seemed to be adequate for quality control after *in vitro* multiplication, showing that there was neither varietal mixing nor any detectable variability as a result of the manipulation process of the analyzed

material.

Also in 2008, the low genetic diversity among Chilean garlic accessions was pointed out (Paredes *et al.* 2008) RAPDs. The analysis of 68 accessions with 40 RAPD primers generated a total of 398 bands. They concluded that there is no association between the patterns generated by the primers employed and either the geographic origin of the clones or with morphological characters.

Volk and colleagues (2004) performed AFLP analysis on a 211 *Allium sativum* and *Allium longicuspis* accessions from USDA National Plant Germplasm System (NPGS) and commercial sources. They used several statistical approaches to evaluate how these clonal lineages are genetically differentiated and how these patterns of differentiation correspond to recognize phenotypic classifications. The analysis distinguished between many of the hardneck and softneck garlic types and show that softneck garlic types represent less genetic diversity than the hardneck types. The phylogenetic analysis of the genetic relationship of USA garlic varieties suggests that phenotypic categories correlate with genetic distance data. In addition the phylogeny suggests that *A. longicuspis* is indistinguishable from *A. sativum* as was found by Pooler and Simon (1993). In addition, it was impossible to correlate the data obtained with the geographical origin of the samples.

This study concluded that AFLP is useful and allows detection of duplicates for prioritizing the genotyped portion of the NPGS garlic collection for cryoconservation by assuming that a simple accession as well as genetically unique accessions should have priority over duplicate samples.

Rosales-Longo and Molina-Monterroso (2007) considered that genetic variability of garlic cultivated populations, in Guatemala was low based upon AFLP. Nine well differentiated genetic groups were confirmed and genetic variability was found to be a function of the site where garlic is grown. Higher genetic diversity was found among the "Criollo" samples in comparison with the diversity observed within the "Chilean" samples. Garlic plant genetic materials are now preserved at *in vitro* Germplasm Collection Bank at the ICTA's Biotechnology Unit.

One interesting point is presented by Ipek *et al.* (2003) who established a comparison among AFLP, RAPD and isozyme. They evaluated 45 garlic clones and three *A. longicuspis* clones and they compared AFLPs results with RAPD markers and isozymes. Three AFLP primer combinations generated a total of 183 polymorphic fragments. Although similarities between the clusters were low (>0.30), some clones within the clusters were very similar (>0.95) with AFLP analysis. Sixteen clones represented only six different banding patterns, within which they shared 100% polymorphic AFLPs, and RAPD markers, and likely are duplicates. In agreement with the results of others investigators, *A. longicuspis* and *A. sativum* clones were clustered together with no clear separation, suggesting these species are not genetically or specifically distinct. The topology of AFLP, RAPD, and isozyme dendrograms were similar, but RAPD and isozyme dendrograms reflect less and much less polymorphism, respectively demonstrating in this way that AFLP is an additional tool for fingerprinting and detailed assessment of genetic relationships in garlic.

Argentina

We have analysed more than 13 clones of the active collection of the EEA La Consulta (García-Lampasona and Burba 1999). Garlic plants were cultivated and DNA was extracted using Dellaporta (Dellaporta *et al*. 1983) and CTAB (cetyltrimethylammonium bromide) (Murray and Thompson 1980) methods. DNA extractions were carried out taking different tissues from the clove*:* leaf of growth, reservation leaf, disk and leaf. When evaluating the quality of the DNA, we concluded that the cleaning of the DNA dramatically decreased the quantity and the quality did not improve significantly.

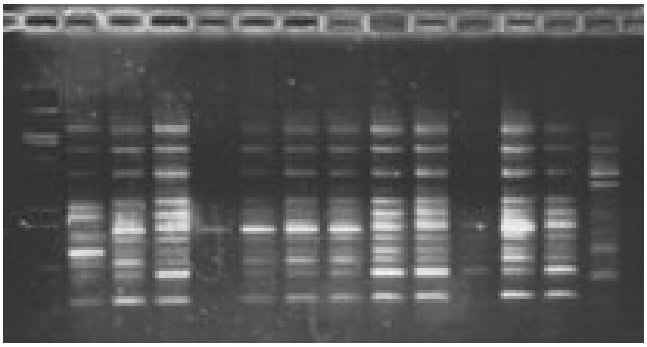

Fig. 1 Pattern of electrophoresis of genomic DNA amplification of garlic with the primer OPD09. Lane 1: l (*Hind*III-*Eco*RI), lane 2: *A. sativum* var. *ophioscorodon*, lanes 3 at 13: clones of *A. sativum* var. *sativum*, lane 14: *A. ampeloprasum* and lane 15: control without DNA. Reprinted from **García Lampasona S, Burba JL** (1999) Marcadores moleculares aplicados a la caracterización de germoplasma de ajo (*Allium sativum*) y ajo elefante (*A. ampeloprasum*). In: *Curso/Taller sobre Producción, Comercialización e Industrialización de Ajo*, Mendoza, INTA EEA La Consulta **6**, 61-62, 1999, with kind permission from INTA EEA La Consulta.

Amplification reactions were performed with the objective of characterizing garlic clones by means of the use of molecular markers RAPDs (Williams *et al*. 1990) to verify the identity and uniformity of the material subject to genetic improvement with the last end of guaranteeing the protection of the derived genetic creations of the classic or traditional genetic improvement. Forty decanucleotides were proven (series OPD and OPL) from Operon Technologies (Boulevard, Calif.) which were used as primers in the direct amplification for PCR.

RAPD molecular markers differentiated *A. sativum* var. *ophioscorodon* of *A. sativum* var. *sativum* and of *A. ampeloprasum* respectively. With the primers used it was not possible to find polymorphic bands that differentiate nine monoclones of *A. sativum* var. *sativum* (**Fig. 1**) (García Lampasona and Burba 1999). The results obtained approach the limits of the RAPD technique so we decided to use the technique of AFLP (Vos *et al*. 1995).

In a recent Argentine study, six primer combinations gave the best results in terms of polymorphic scorable bands per gel, identifying a total of 405 bands, of which, 398 showed a clear polymorphism. This represents 98.27% of the total bands. The amplified fragments ranged in size from approximately 0.1 to 0.5 kb. The results obtained were in agreement with those reported by Ipek *et al*. (2003) who found that the genetic variation in garlic clones ranged between 0.30 and 0.95. A UPGMA (unweighted pair-group method with arithmetic mean) dendrogram constructed by using 398 polymorphic AFLPs, revealed the existence of six arbitrary groups (A, B, C, D, E and F) and illustrated that six AFLP reactions differentiated all the clones commercially grown in Argentina (**Fig. 2**).

Argentinean classification is based, among other variables, upon dormancy period, since this feature is best associated with other characteristics like flowering, cold requirement and color (Burba 2008). In general, garlic clones were clustered according to the ecophysiological groups and bulb color. Taking into account this classification, the clones Colorado T and Fuego INTA (**Fig. 3A**) of the A group, belong to the ecophysiological group IVa (Burba 2008), which is characterized by its high cold requirement for dormancy break, long photoperiod requirement for bulb formation, long storage period, high pungency, presence of floral stalk and red cloves.

On the other hand, clusters B, C and D corresponded to ecophysiological group number III that show medium cold requirement, medium photoperiod requirement for bulb formation, medium storage period, medium pungency and white cloves. This group included Perla INTA (**Fig. 3B**), INTA (**Fig. 3C**), Nieve INTA (**Fig. 3D**), INCO 283, INCO

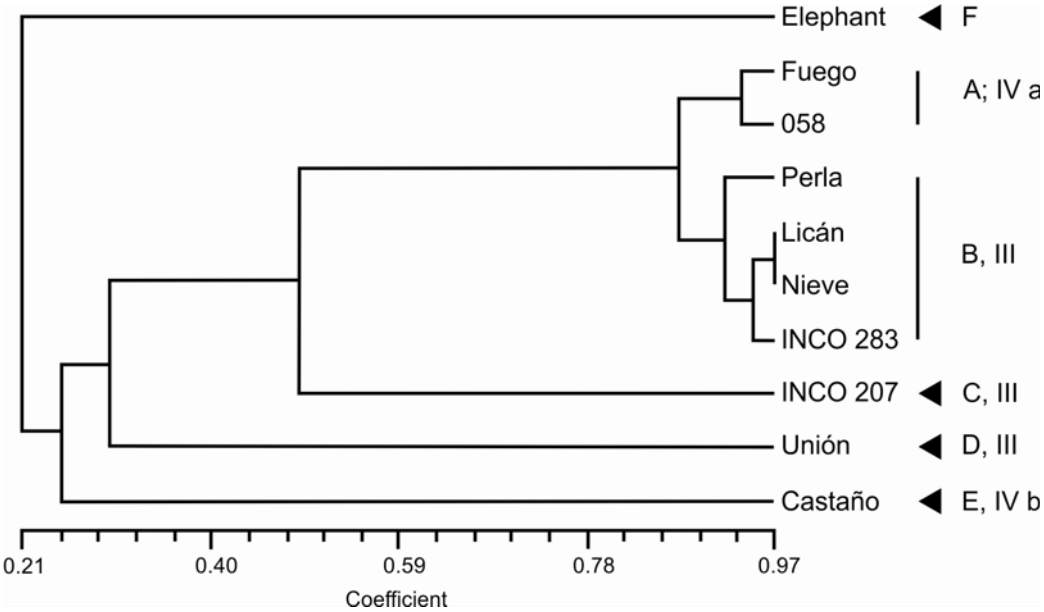

Fig. 2 Dendrogram of UPGMA cluster analysis representing the genetic similarity among garlic clones revealed by AFLP. Letters and roman numerals indicate six arbitrary and three physiological groups, respectively. Reprinted from **García Lampasona S, Martínez L, Burba JL** (2003) Genetic diversity in Argentinean selected garlic (*Allium sativum* L.) as assessed by AFLP (amplified fragment length polymorphism). *Euphytica* **132 (1)**, 115-119, with kind permission of Springer Science + BM, Berlin, Germany, ©2003.

207 and Unión (**Fig. 3E**), known as the "white" commercial type.

Our data did not separate Licán INTA (**Fig. 3C**) and Nieve INTA (**Fig. 3D**) at a coefficient of 0.97. These clones belong to the same ecophysiological group III. This suggests that these clones are less genetically distinct. Licán INTA (**Fig. 3C**) has white cloves with violet stripes and Nieve INTA (**Fig. 3D**) has white cloves. The occurrence of different colors in grapes with the same SSR pattern was already reported and could be due to very small genetic changes (Galet 1990). In garlic, this could also be attributable to small genetic changes which would be very difficult, however not impossible, to find with an appropriate AFLP primer combination. INCO 207 INTA and Unión (**Fig. 3E**), for example, were clustered separately from the rest of the clones at 0.495 and 0.30 similarity coefficient. This could result from great diversity in the original population.

An association between AFLP and geographical origin of Argentine clones is in agreement with Lallemand *et al* (1997), who found that the geographical origins of some cultivars were associated with their isoenzyme patterns. Al-Zahim *et al*. (1997) showed an association between morphological characters and polymorphic RAPD markers. These results, however, do not agree with Pooler and Simon (1993), who did not detect an association between isoenzyme pattern and geographical origin, while they detected an association with flower-related morphological characters. The same results were found by Ipek and Simon (1998) using AFLPs markers.

The original habitat of garlic coincides with the Tien Shan Mountains located in Central Asia, from which four subtypes of garlic were spread to the rest of the world. These include Asiatic, Continental, Mediterranean-European and Mediterranean-African subtypes (Engeland 1991). The Asiatic subtype was dispersed towards China and it was not evaluated in the Argentine study while the Continental subtype, represented by Castaño INTA (**Fig. 3F**), spread towards the north of Europe, it became known as "Russian". The Mediterranean-European or rocambole or "hardneck" subtype is represented by Fuego INTA (**Fig. 3A**) and Colorado T (058) in this study. They are clustered in the group A (**Fig. 2**) and are commercially denominated as "red". The Mediterranean-African or "softneck" subtype is represented by several clones: Nieve INTA (**Fig. 3D**), INCO 207, INCO 283, Perla INTA (**Fig. 3B**) and Unión (**Fig. 3E**). Those clones were introduced to South America, and clustered together in the groups B, C, D and E (**Fig. 2**). Perla INTA (**Fig. 3B**) is a typical member of physiological group number III and corresponds to the "American-white" subgroup. While Licán INTA (**Fig. 3C**), Nieve INTA (**Fig. 3D**) and INCO 283 belong to the same physiological group, they were separated in a different branch which corresponds to "Native-white" subgroup (Burba 2008).

The use of AFLP should allow differentiation not only between garlic subspecies (at 25% similarity) (**Fig. 2**), but also botanical varieties (at 29% similarity) and well-defined ecotype groups, such as the Argentinean physiological groups III, IV a, and b (at 91% similarity). These groups correspond with the French groups number III, I and IV and with the Japanese groups number 4 and 1, respectively (Burba 2008).

The accessions showed a range in levels of similarity from 0.24 to 0.97, using the coefficient of Jaccard, and this result in a dendrogram with six arbitrary groups. Accessions typically considered as different clones show similarities between 0.97 and 0.495. The garlic clones were clustered according to the physiological group and bulb color. We could detect an association between AFLP and the geographical origin of the clones.

This was the first report of AFLP used to characterize Argentinean garlic clones. The potential use of AFLP could allow not only the differentiation among species, but also between botanical varieties and well-defined ecotype groups. The utility of AFLPs in garlic identification was demonstrated.

In conclusion, AFLP is a useful marker for revealing genetic relationships and detecting morphologically similar garlic genotypes. Clones which were genetically close will need to be analyzed with more AFLP primer combinations, and preferably with other markers such as microsatellites.

MARKERS ASSOCIATED WITH BOLTING

Production of a visible flower stalks or bolting has been used as a major trait to categorize garlic clones. Analysis of mithocondrial genome variation with PCR revealed differences between bolting and non-bolting clones of garlic. Screening 333 garlic accessions from diverse geographic origins revealed a 1,403 bp mitochondrial DNA marker associated with bolting that they call BltM did not amplify in any of the 131 non-bolting clones, while amplification of this marker was observed in 127 of 130 (97.7%) garlic

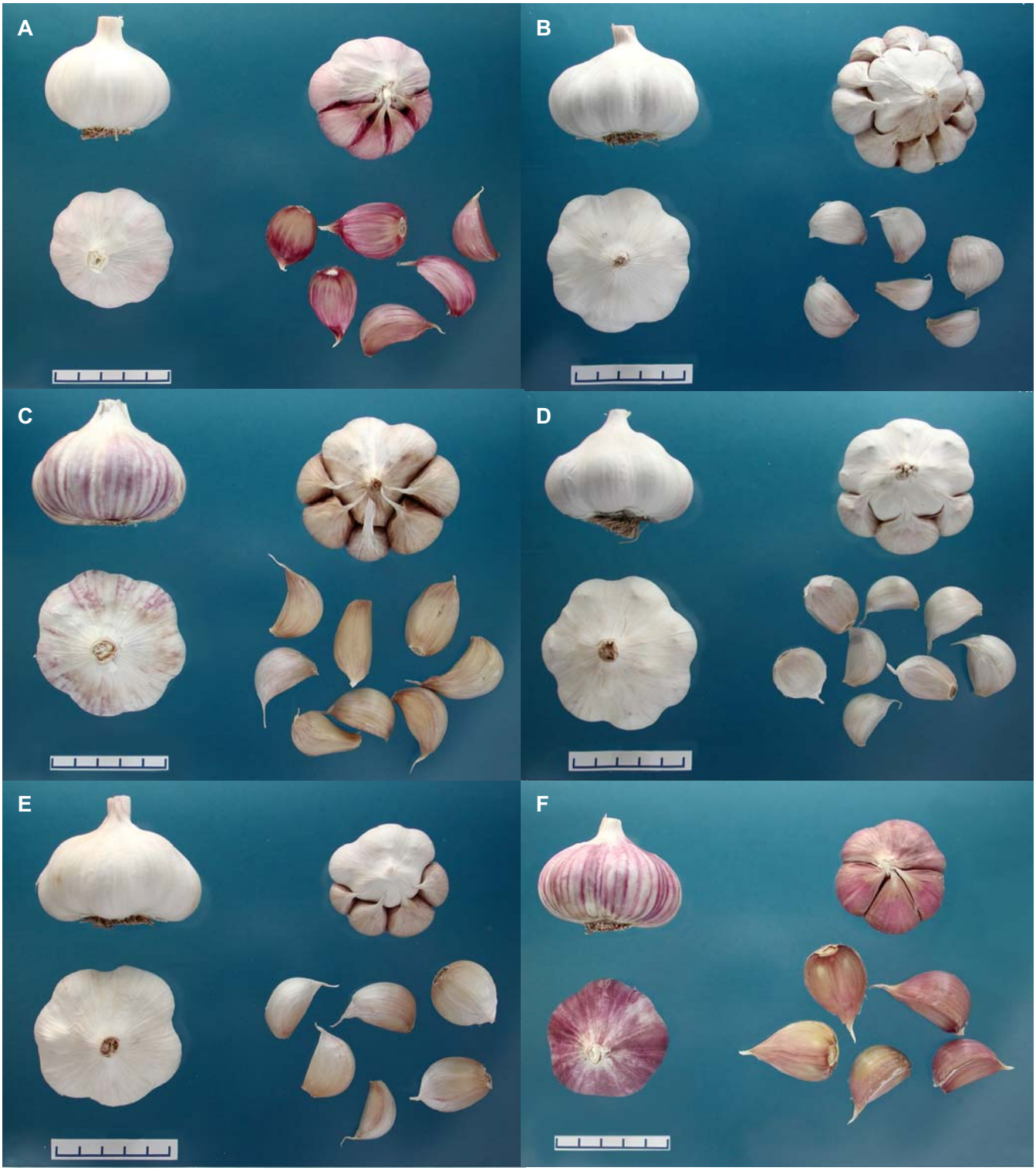

Fig. 3 (**A**) Fuego INTA. (**B**) Perla INTA. (**C**) Licán INTA. (**D**) Nieve INTA. (**E**) Unión. (**F**) Castaño INTA.

clones that bolted completely in Wisconsin. Seventy-two garlic clones bolted incompletely and this marker was not amplified in 69 (95.8%) of these clones. Due to the significant association of BltM with bolting. This PCR marker can be used to reliably discriminate completely bolting garlic clones from non-bolting and incomplete bolting ones. Sequence characterization of this marker revealed that BltM is a chimera involving both mitochondrial and chloroplast DNA. The DNA sequences including and flanking both the 5′ and 3′ ends of this marker are consistent with an approximately 4.8 kbp chloroplast DNA fragment having been inserted into the mitochondrial genome downstream from the mitochondrial *cox3* gene. Sequence alignment of the chloroplast gene in this chimeric region with the homologous sequence in GenBank indicate the presence of deletions, insertions, and single nucleotide polymorphisms in the coding sequences, resulting in putative incomplete open reading frames or frame shift mutations. Hence, they speculate that this insertion may have occurred long ago in the evolution of garlic (Ipek *et al*. 2007).

RECENT ADVANCES: fAFLP (FLUORESCENT AMPLIFIED FRAGMENT LENGTH POLYMORPHISM)

The fluorescence amplified fragment length polymorphism (fAFLP) assay is based on the amplification of restriction fragments from genomic DNA and the analysis of the amplified products using a genetic analyser and selective primers labelled with a fluorochrome instead of polyacrylamide

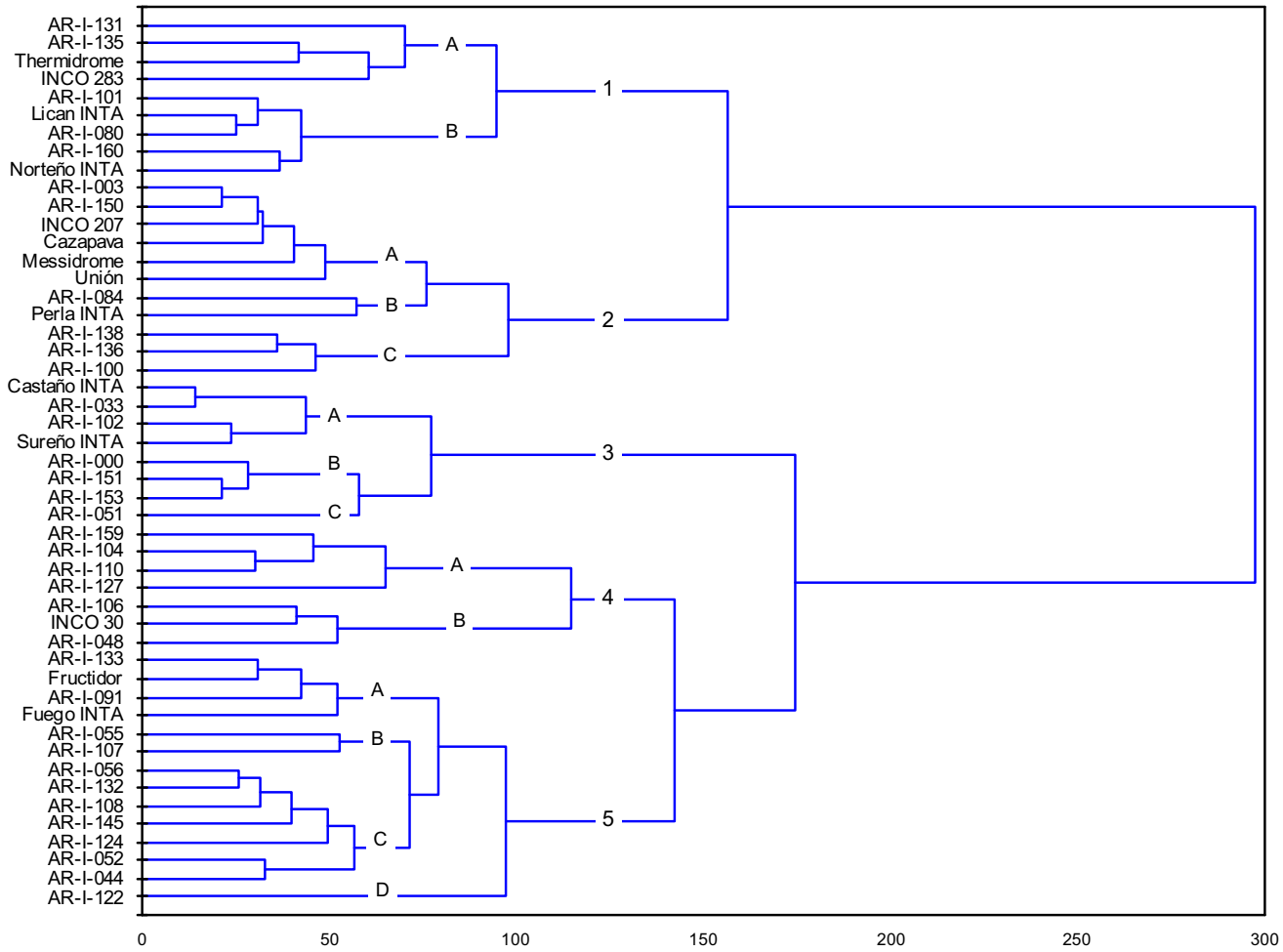

Fig. 4 Dendrogram that represents genetic similarity among accessions revealed by fAFLP. Numbers indicates five arbitrary groups.

gel electrophoresis and different staining methodologies. The principal advantage of fAFLP is the fully automatization of the process.

Forty nine (49) accessions of *Allium sativum* var. *sativum* from the Germplasm Bank of INTA La Consulta, Mendoza, Argentina were used in this study. The AFLP procedure (Vos *et al*. 1995) was performed as described by Berres (2001) with minor modifications. The modifications consisted on the use of fluorescent dyes linked to primers and the use of ABI Prism 3130, Applied Biosystems used for AFLP DNA analysis.

Fluorescent dyes attached to the DNA fragments were excited by laser and detected using a genetic analyzer Abi Prism 3130 (Applied Biosystems). The data (displayed as peaks in electropherogram files) were analyzed by using the Gene Mapper v 3.7 software (Applied Biosystems). The fragment sizes were determined by comparison with internal size standards (GeneScan-500), limiting analysis to fragments between 50 and 500 bp in size and allowing a resolution of ±1 bp. Reproducibility of peaks in electropherograms was checked by repeating fAFLP reactions on the plants examined in this study. The original gene frequencies and the population of origin of each allele copy of each individual are assumed to be unknown, so that they must be estimated from data. The obtained genotypes were input into *Structure 2.2* (Pritchard *et al*. 2000).

Our AFLP data identified five distinct groups of garlic with a high accuracy (**Fig. 4**). *Structure* assumes that there are a number of unknown populations, characterized by a set of allele frequencies at each locus. The software applies Markov chain Monte Carlo (MCMC) and Gibbs sampling methods, and the Metropolis-Hastings algorithm that are extremely useful for obtaining samples from a probability stationary distribution (Chib and Greenberg 1995, Gilks *et al*. 1996). Individuals in the sample are assigned to populations, or jointly to two or more populations if their genotypes indicate that they are admixed.

There were several individuals with apparently mixed ancestry. The number of populations supported by the data may depend on how different one would expect allele frequencies in the different populations, which is often difficult to specify *a priori*. Admixed individuals presumably contain chromosomal regions that derive from more than one population (Falush *et al*. 2003). When individuals have mixed ancestry, each genotyped allele comes from one or other of the populations and each individual is assumed to have inherited some proportion of its ancestry from each population.

When there is recent admixture or, with cultivated garlic that has had the sexual reproduction suppressed thousands years ago, we might expect to see strong correlations among linked loci. This occurs because an individual who is admixed will inherit large chromosomal segments from one population or another (Pritchard *et al*. 2000). Other situations that might cause additional populations to be inferred by *Structure* include a significant frequency of inbreeding, cryptic relatedness within the sample, or the presence of null alleles (Falush *et al*. 2003).

In most cases, accessions that fall within a physiological category are phenotypically similar, and belonged to the same genotypic group. In addition to answering questions about population histories, the characterization of genetically distinctive populations can assist in conservation of within-species diversity (Moritz 1994; Paetkau 1999; Rosenberg *et al*. 2001).

CONCLUDING REMARKS

Large germplasm collections involve field trials that usually are expensive and evaluation of some traits, such as quality

and yield stability can be expensive to assess. Molecular markers have proved to be a powerful tool in complementing bioassays and there are now many examples available to show the efficacy of such markers.

The use of molecular markers to track loci and genome regions in crop plants is now routinely applied in many breeding programs that are applicable to germplasm collections. The location of major loci is now known for many disease resistance genes, tolerances to abiotic stresses and quality traits. Improvements in marker screening techniques have also been important in facilitating the tracking of genes. For markers to be effective, they must be closely linked to the target locus and be able to detect polymorphisms in material likely to be used in a breeding program. For example, AFLP markers appear to frequently target repetitive regions of the genome. The stability of the sequence difference may also be an issue in some cases. SSRs are seen as being too unstable for some applications since the mutation rate may in some cases be high. The identification of the most appropriate marker system to use will vary greatly depending on the species, the objective of the marker work and resources available (Langridge and Chalmers 2005).

Referring to garlic, isozymes are limited by low numbers of polymorphic markers and RAPD variation may be due to nongenetic variables, but AFLPs have proven to be polymorphic and reliable. fAFLP opens new approaches to evaluating garlic molecular fingerprints.

Argentinean ecophysiological classification of garlic is based on dormancy period, since this feature is the best for its association with other characteristics like flowering, cold requirement and color (Burba 2008). In general, garlic clones were clustered according to the ecophysiological groups and bulb color.

We establish the first report of AFLP used to characterize Argentinean garlic clones. AFLP is a useful marker for revealing genetic relationships and detecting morphologically similar garlic genotypes.

Classification types, like ecophysiological may not be robust and may not reflect their true origin. Volk *et al.* (2004) found that of the 118 NPGS accessions, 24 had unique genotypes and of the overall 64% of the accessions were duplicates. We have identified some duplicated, and Argentine clones that do not genetically align with the described physiological and morphological categories. Plant and bulb characteristics are determined by growing season, winter conditions, location, as well as nutrient and water availability. Perhaps a ecophysiological phenotype extends to a number of genetically distinct accessions and may be, these accessions were phenotypically misclassified.

In short, despite the many complexities in the distribution of species, their genetic structure can often be approximated by a division into discrete populations, allowing assignment of individuals to populations with modest computational requirements. While our analysis of the genetic relationships of garlic varieties suggests that phenotypic categories correlate with genetic data, some categories require additional examination.

We can prioritize the genotyped portion of the garlic collection for cryoconservation assuming that a single accession representing each group of accessions as well as the genetically unique accessions should have priority over duplicate samples. Conservation of genetic diversity is of great importance toward ensuring that future breeding programs will have a large base to perform artificial selection. In our opinion it will be necessary to develop more versatile molecular markers for garlic characterization which will provide many markers to evaluate a great number of samples such SNPs and SSRs. However, garlic researchers have not developed these systems yet.

REFERENCES

Al-Zahim MH, Newbury HJ, Ford-Lloyd BV (1997) Classification of genetic variation in garlic (*Alllium sativum* L.) revealed by RAPD. *HortScience* **32**, 1102-1104

Berres M (2001) General fluorescent AFLP (fAFLP) protocols. University of Wisconsin Department of Zoology, Madison, Wisconsin. Available online: http://ravel.zoology.wisc.edu/sgaap/AFLP_html/fAFLP_Introduction.htm

Bradley KF, Rieger MA, Collins GG (1996) Classification of Australian garlic cultivars by DNA fingerprinting. *Australian Journal of Experimental Agriculture* **36**, 613-618

Burba JL (1997) Panorama mundial y nacional de poblaciones y cultivares de ajo. Posibilidades de adaptación. In: Burba JL (Ed) *50 Temas Sobre la Producción de ajo*, (1st Edn, Vol II), INTA EEA La Consulta, Mendoza, Argentina, pp 11-31

Burba JL (2008) Los grupos varietales del ajo (*Allium sativum* L.). Contribución para su entendimiento. *Horticultura Argentina* **27 (62)**, 20-27

Buso GSC, Paiva MR, Torres AC, Resende FV, Ferreira MA, Buso JA, Dusi AN (2008) Genetic diversity studies of Brazilian garlic cultivars and quality control of garlic-clover production. *Genetic and Molecular Research* **7 (2)**, 534-541

Chib S, Greenberg E (1995) Understanding the Metropoli-Hastings algorithm. *American Statistical* **49**, 327-335

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation. Version II. *Plant Molecular Biology Reporter* **1 (4)**, 19-21

Engeland RL (1991) *Growing Great Garlic: The Definitive Guide For Organic Gardeners And Small Farmers* (1st Edn), Filaree Productions, Okanogan, WA, 226 pp

Etoh T, Simon PW (2002) Diversity, fertility and seed production of garlic. In: Rabinowitch HD, Carrah I (Eds) *Allium Crop Science Recent Advances*, CABI, New York, pp 101-107

Evans IJ, James AM, Barnes SR (1983) Organization and evolution of repeated DNA sequences in closely related plant genomes. *Journal of Molecular Biology* **170**, 803-826

Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. *Genetics* **164**, 1567-1587

Fischer D, Bachmann K (2000) Onion microsatellites for germplasm analysis and their use in assessing intra- and interspecific relatedness within the subgenus *Rhizirideum*. *Theoretical and Applied Genetics* **101**, 153-164

Galet P (1990) *Cépages et Vignobles de France. L'Ampélographies Française* (2nd Edn, Vol II), Charles Dehan, Montpellier, France, 400 pp

García Lampasona S, Burba JL (1999) Marcadores moleculares aplicados a la caracterización de germoplasma de ajo (*Allium sativum*) y ajo elefante (*A. ampeloprasum*). In: *6to Curso/Taller sobre Producción, Comercialización e Industrialización de Ajo,* 23-26 June, 1999, INTA EEA La Consulta, Mendoza, Argentina, p 61-62 (Abstract)

García Lampasona S, Martínez L, Burba JL (2003) Genetic diversity in Argentinean selected garlic (*Allium sativum* L.) as assessed by AFLP (amplified fragment length polymorphism). *Euphytica* **132 (1)**, 115-119

Gilks WR, Richardson S, Spiegelhalter DJ (1996) Introducing Markov chain Monte Carlo. In: *Markov Chain Monte Carlo in Practice*, Gilks WR, Richardson S, Spiegelhalter DJ (Eds), Chapman and Hall, London, 488 pp

Harlan JB (1992) *Crops and Man* (2nd Edn), American Society of Agronomy and Crop Science Society of America, Madison, Wisconsin, USA, 284 pp

Helm J (1956) mDie zuWurz- und Speisezwecken kultivierten Arten der Gattun *Allium* L. *Kalturpflanze* **4**, 130-180

Ipek M, Simon PW (1998) Genetic diversity in garlic (*Allium sativum* L.) as assessed by amplified fragment length polymorphism (AFLP). In: *Proceedings of the 1998 National Onion (and other Allium) Research Conference*, 10-12 December, 1998, Sacramento, California, USA, p 110-120

Ipek M, Ipek A, Simon PW (2003) Comparison of AFLPs, RAPD markers, and isozymes for diversity assessment of garlic and detection of putative duplicates in germplasm collections. *Journal of the American Society for Horticultural Science* **128**, 246-252

Ipek M, Ipek A, Almquist SG, Simon PW (2005) Demonstration of linkage and development of the first low-density genetic map of garlic, based on AFLP markers. *Theoretical and Applied Genetics* **110**, 228-236

Ipek M, Ipek A, Senalik D, Simon PW (2007) Characterization of an unusual cytoplasmic chimera detected in bolting garlic clones. *Journal of the American Society for Horticultural Science* **132 (5)**, 664-669

Lallemand J, Messiaen CM, Briad F, Etoh T (1997) Delimitation of varietal groups in garlic (*Allium sativum* L.) by morphological, physiological and biochemical characters. *Acta Horticulturae* **433**, 123-132

Langridge P, Chalmers K (2005) Identification and application of molecular markers. In: Lörz H, Wenzel G (Eds) *Molecular Marker Systems in Plant Breeding and Crop Improvement*, *Series: Biotechnology in Agriculture and Forestry* (1st Edn, Vol 55), Springer, Heidelberg, Germany, pp 3-22

Maaβ HI, Klaas M (1995) Infraspecific differentiation of garlic (*Allium sativum* L.) by isozyme and RAPD markers. *Theoretical and Applied Genetics* **91**, 89-97

Matus I, González MI, del Pozo A (1999) Evaluation of phenotypic variation in a Chilean collection of garlic (*Allium sativum* L.) clones using multivariate analysis. *Plant Genetic Resources Newsletter* **117**, 31-36

Maughan PJ, Saghai Maroof MA, Buss GR, Huestis GM (1996) Amplified fragment length polymorphism (AFLP) in soybean: Species diversity, inheritance, and near-isogenic line analysis. *Theoretical and Applied Genetics* **93**,

392-401

Messiaen CM, Cohat J, Leroux JPL, Pichon M, Beyres A (1993) Les *Allium* alimentaries reproduits par voie végétative. INRA Éditions, Paris, France, 230 pp

Moritz C (1994) Defining 'evolutionarily significant units' for conservation. *Trends in Ecology and Evolution* **9**, 373-375

Mota JH, de Souza RJ, Yuri JE, de Resende GM, Paiva LV (2004) Diversidade genética de cultivares de alho (*Allium sativum* L.) por meio de marcador molecular RAPD. *Ciências Agrotececnicas, Lavras* **29 (4)**, 764-770

Murray JM, Thompson W (1980) Rapid isolation of high-molecular weight plant DNA. *Nucleic Acids Research* **8**, 4321-4325

Paetkau D (1999) Using genetics to identify intraspecific conservation units: A critique of current methods. *Conservation Biology* **13**, 1507-1509

Paredes M, Becerra V, González MI (2008) Low genetic diversity among garlic (*Allium sativum* L.) accessions detected using random amplified polymorphic DNA (RAPD). *Chilean Journal of Agricultural Research* **68 (1)**, 3-12

Paul S, Wachira FN, Powell W, Waugh R (1997) Diversity and genetic differentiation among populations of Indian and Kenyan tea (*Camellia sinensis* (L.) O. Kuntze) revealed by AFLP markers. *Theoretical and Applied Genetics* **94**, 255-263

P**ooler MR, Simon PW** (1993) Characterization and classification of isozyme and morphological variation in a diverse collection of garlic clones. *Euphytica* **68**, 121-130

Powell W, Machray G, Provan J (1996) Polymorphism revealed by simple sequence repeats. *Trends in Plant Science* **1**, 215-222

Pritchard JK, Stephens M, Donnelly PJ (2000) Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-959

Rosales-Longo FU, Molina-Monterroso LG (2007) Diversidad genética de poblaciones de ajo (*Allium sativum* L.) cultivadas en Guatemala, definida por marcadores de ADN. *Agronomía Mesoamericana* **18 (1)**, 85-92

Rosenberg NA, Woolf E, Pritchard JK, Schaap T, Gefel D (2001) Distinctive genetic signatures in the Libyan Jews. *Proceedings of the National Academy of Sciences USA* **98**, 858-863

Sánchez G, Restrepo S, Duque MC, Fregene M, Bonierbale M, Verdier V (1999) AFLP assessment of genetic variability in cassava accessions (*Manihot esculenta*) resistant and susceptible to the cassava bacterial blight (CBB). *Genome* **42**, 163-172

Sharma SK, Knox MR, Ellis THN (1996) AFLP analysis of the diversity and phylogeny of *Lens* and its comparison with RAPD analysis. *Theoretical and Applied Genetics* **93**, 751-758

Shim SI, Jorgensen RB (2000) Genetic structure in cultivated and wild carrots (*Daucus carota* L.) revealed by AFLP analysis. *Theoretical and Applied Genetics* **101**, 227-233

Simon PW, Jenderek MM (2003) Flowering, seed production, and the genesis of garlic breeding. In: Janick (Ed) *Plant Breeding Reviews* (Vol II), Wiley, New York, USA, pp 211-244

Tohme JD, Gonzalez O, Beebe S, Duque MC (1996) AFLP analysis of gene pools of a wild bean core collection. *Crop Science* **36**, 1375-1384

Ved Brat S (1965) Genetic systems in *Allium*. I. Chromosome variation. *Chromosoma (Berlin)* **16**, 486-499

Volk GM, Henk AD, Richards CM (2004) Genetic diversity among U.S. garlic clones as detected using AFLP methods. *Journal of the American Society for Horticultural Science* **129**, 559-569

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Research* **23**, 4407-4414

Williams JKG, Kubelik AR, Livak KJ, Rafalski JA, Trogey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531-6535

Yee E, Kidwell KK, Sills GR, Lumpkin TA (1999) Diversity among selected *Vigna angularis* (azuki) accessions on the basis of RAPD and AFLP markers. *Crop Science* **39**, 268-275

Zabeau M, Vos O (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Application number 92402629.7, Publication number EP 0534858 A1, Bulletin 93/13

Zewdie Y, Havey MJ, Prince JP, Jenderek MM (2005) The first genetic linkages among expressed regions of the garlic genome. *Journal of the American Society for Horticultural Sciences* **130 (4)**, 569-574