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Short communication

Fingerprinting of three typical *macrosperma* Italian lentil (*Lens culinaris* Medik.) landraces using fluorescence-based AFLP markers

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ABSTRACT

Italian lentil landraces are principally cultivated for self or local consumption. Most of them are disappearing, particularly *macrosperma* types by being less required by the market. A pre-requisite for the conservation and the efficient use of genetic resources is the better understanding of the extent and the distribution of the existing genetic variation, useful for future breeding programmes. Our study was undertaken to analyse and quantify the genetic diversity within and among three *macrosperma* Italian lentil landraces (Onano, Altamura and Villalba), using fluorescent AFLP markers. AFLP markers generated information to differentiate among closely related genotypes and group within the same cluster individuals belonging to the same landrace. The total genetic diversity (H_T), the genetic diversity within population (H_S) and the extent of differentiation between populations (D_{ST}) were 0.198, 0.155 and 0.043, respectively. The fixation index (G_{ST} = 0.219) showed that about 78% of the observed total genetic variation can be attributed to within population differences and around 22% is due to differences among populations. The gene flow estimate (N_m = 1.774) and the mean genetic distance value (0.077) suggested narrow genetic base among the analysed populations, confirming the tendency of Italian lentil landraces to group together. The present study showed that fluorescence-based AFLP technique is a biotechnological tool that can provide significant insights for research in genetic diversity of lentil landraces and their subsequent conservation and utilization in breeding programs.

1. Introduction

Lentil (*Lens culinaris* Medik.) is considered to be one of the most nutritious grain legume crops, providing a cheap source of dietary proteins and a good source of minerals. Due to its ancient domestication (Piergiovanni, 2000), a wide diversity of this crop was over millennia expressed in a myriad of landraces (Erskine, 1997). In the past, lentil was widely cultivated in Italy allowing the rising of a huge number of local populations, but socio-economic changes, occurring in the last 60 years, reduced drastically the lentil growing area by 92%, making Italy an importing country (FAOSTAT, 2006). Nowadays, Italian lentil landraces are principally cultivated for self or local consumption; as a result, most of them have disappeared and others are at the edge of the extinction, particularly *macrosperma* types (over 6 mm in diameter), less required by Italian market than *microsperma* ones (less than 6 mm). This loss of agro-biodiversity limits future options, through the loss of genetic information and genetic material that could be introduced by breeding into new improved varieties. Of great interest was the discovery that lentils of subspecies *macrosperma* have significantly higher saponin content than seeds of the subspecies *microsperma* (Ruiz et al., 1997). Saponins are a class of bioactive compounds with diverse good properties such as the inhibition of growth and sporulation of a wide range of fungi (Gestetner et al., 1971), the reduction of plasma cholesterol levels in humans (Sidhu and Oakenfull, 1986) and the exhibition of anticancer activity (Konoshima et al., 1992). The high content of saponins is an important added value for *macrosperma* landraces that could be used to develop breeding programs with the aim of improving the nutritional quality and fungal resistance of lentils.

In our opinion, special attention should be paid to the safeguard of the three *macrosperma* landraces Altamura (Bari, Apulia), Villalba (Caltanissetta, Sicily), and Onano (Viterbo, Latium) on which Italian intensive lentil production was relied (Piergiovanni, 2000). Altamura lentils were greatly appreciated for the large and flat seeds with light green coat and superior taste (Bozzini et al., 1988; Piergiovanni, 2000). The high regard for Villalba lentils was related to the excellent seed quality (Sarno et al., 1988) and to a very high adaptation to the semi-arid environment of Central Sicily (Gallo et al., 1997). Onano lentils were largely exported abroad (Piergiovanni, 2000) and the high appreciation for their large and light green seeds were confirmed

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Fig. 1. Geographic location of the three Italian lentil landraces analysed.

by rewards at international expositions (Pro Loco Onanese, 1990). A pre-requisite for the conservation and the efficient use of genetic resources is a detailed study both on the extent and the distribution of the existing genetic variation. Genetic molecular markers can provide a relatively unbiased method of quantifying such genetic diversity. Even if morphological variation within each Italian landrace is quite appreciable, only one extensive study has recently been done, using ISSRs, to investigate whether variation exists also at the DNA level (Sonnante and Pignone, 2007). Nevertheless, polymorphism generated by ISSR markers is limited in comparison to that produced by fluorescent AFLPs (Hodkinson et al., 2002). The current research aims at analysing and quantifying the genetic diversity within and among Onano, Altamura, and Villalba lentil landraces, using fluorescent AFLP markers.

2. Materials and methods

2.1. Plant materials and DNA extraction

Italian *macrosperma* lentil landraces Onano, Altamura, and Villalba (Fig. 1), were evaluated by AFLPs on a single plant DNA basis (total of 84 samples).

Total genomic DNA was isolated from leaves of 10-day-old seedlings following NucleoSpin Plant kit (MACHEREY-NAGEL, Germany) procedure. The concentration of DNA samples was determined both by optical density reading at 260 nm (Sambrook et al., 1989) and electrophoresis on 1% (w/v) agarose gel.

2.2. AFLP analysis

The AFLP analysis was carried out according to the procedure developed by Vos et al. (1995), with minor modifications using AFLP™ Plant Mapping kit for Regular Plant Genome (Applied Biosystems, California, USA) and following the manufacturer's instructions. Six primer combinations were used for the selective amplifications: *Eco*RI-ACA/*Mse*I-CAC, *Eco*RI-ACT/*Mse*I-CTA, *Eco*RI-AGC/*Mse*I-CTA *Eco*RI-AAC/*Mse*I-CAG, *Eco*RI-ACC/*Mse*I-CAT and *Eco*RI-ACT/*Mse*I-CAA (Table 1). The *Eco*RI-NNN primers were labelled with fluorescent dyes (Applied Biosystems, AFLP™ Selective Amplification Start-Up Module).

Table 1
Total number of bands (TNB), number of polymorphic bands (NPB), and percentage of polymorphic bands (%*P*) obtained per AFLP primer.

Primer sequence (5′ → 3′)	TNB	NPB	%*P*
*Eco*RI-ACA/*Mse*I-CAC	95	85	89.5
*Eco*RI-ACT/*Mse*I-CTA	85	78	91.8
*Eco*RI-AGC/*Mse*I-CTA	127	115	90.6
*Eco*RI-AAC/*Mse*I-CAG	81	66	81.5
*Eco*RI-ACC/*Mse*I-CAT	129	118	91.5
*Eco*RI-ACT/*Mse*I-CAA	115	103	89.6
Total	632	565	89.4

The amplified products from selective amplifications were loaded and run on the automatic sequencer ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems). Data were analysed using GeneMapper 3.7 software.

2.3. Data analysis

In the AFLP analysis the fragment size along with the peak height and area of each amplified fragment were assigned by using the Local Southern Method provided by the GeneMapper Software on the basis of internal standard (GeneScan™-500 LIZ® Size Standard, Applied Biosystems) contained in each sample from the analysed genotypes. Only DNA fragments ranging from 100 to 500 bp in size and showing a peak height greater than 100 were scored as present (1) or absent (0) and entered into a binary qualitative data matrix. The average AFLP marker allele frequency (p_i) and its relative standard error, were calculated for each single population separately and over all landrace populations. The presence/absence data matrix of the AFLP phenotypes was analysed using POPGENE version 1.32 (Yeh et al., 1997) to estimate genetic diversity parameters. The observed number of alleles (n_o) and the effective number of alleles (n_e) according to Kimura and Crow (1964) and number (N_{pl}) and % of polymorphic loci were calculated. The polymorphism degree was calculated over all populations using Shannon's information index (I) of phenotypic diversity (Lewontin, 1974).

Genetic diversity (H) and population differentiation (D_{ST}) statistics of Nei (1973) were used to summarise AFLP marker data among the tested genotypes. Let H_T be the total genetic diversity over all loci and all populations considered together and let H_S be the average over all populations of H for each population, the proportion of diversity expressed between populations (G_{ST}) was estimated as D_{ST}/H_T, where D_{ST} is the among populations differentiation computed as $H_T - H_S$ and thus $G_{ST} = 1 - H_S/H_T$. The level of gene flow (N_m) was derived and interpreted according to McDermott and McDonald (1993).

Genetic distance (GD) estimates between lentil populations were calculated for all AFLP marker loci, by using Nei's (1978) unbiased genetic distance coefficient.

Moreover, genetic similarity (GS) of Dice (1945) was estimated for all pairs of the individuals. The between populations mean genetic similarity estimate was obtained by averaging individual GS estimates using the whole set of plants belonging to the compared populations. Also the within population mean genetic similarity estimate was calculated taking into account all plants representing that population. Dendrogram was constructed on the basis of the similarity matrix data applying the un-weighted pair-group arithmetic average method (UPGMA) cluster analysis. The data were also subjected to Bootstrap Analysis (WinBoot software; Yap and Nelson, 1996), using 1000 iterations, to estimate the reliability of the clustering pattern. All the calculations for genetic

Table 2
Single-and multi-population genetic variation statistics for all AFLP markers including sample size (s), number (N_{pl}) and % of polymorphic loci, observed (n_o) and effective (n_e) number of alleles per locus, mean marker allele frequency (p_i) with standard error, Nei's genetic diversity (H), Shannon's information index (I), and Dice genetic similarity (GS).

	s	N_{pl}	$\%_{pl}$	n_o	n_e	$p_i \pm$ S.E.	H	I	GS
Altamura	29	405	63.0	1.62	1.28	0.410 ± 0.041	0.167	0.259	0.819
Onano	30	374	58.2	1.58	1.28	0.433 ± 0.035	0.149	0.232	0.855
Villalba	25	321	49.9	1.50	1.30	0.460 ± 0.037	0.147	0.224	0.860
Overall	84	565	89.4	1.88	1.32	0.433 ± 0.030	0.197	0.311	0.792

similarity analyses were conducted by using the appropriate routines of NTSYS-PC software, version 2.11a (Exeter Publishing, Setauket, NY; Rohlf, 2002).

3. Results and discussion

The current study represents an investigation of the genetic diversity displayed within and among the three *macrosperma* Italian landraces (Altamura, Onano, and Villalba) on which the intensive production was based when Italy was one of the major lentil producers in the Mediterranean basin. The high discriminative fluorescence-based AFLP markers were used for the analysis. Among the several marker systems, AFLPs (Vos et al., 1995) offer the advantages of reproducibility and the generation of large number of polymorphic markers spanning the whole target genome without prior knowledge of it. Fluorescent AFLPs have been successfully used for characterisation and evaluation of genetic relationships in many crops (Hartl and Seefelder, 1998; Massawe et al., 2002; Tara Satyavathi et al., 2006).

The six primer combinations selected to screen the lentil genotypes resulted in a total of 632 amplification products, 89.4% of which being polymorphic (Table 1), showing a good level of polymorphism among the genotypes analysed. The number of amplification products generated by individual primer pairs ranged from 81 (*Eco*RI-AAC/*Mse*I-CAG) to 129 (*Eco*RI-ACC/*Mse*I-CAT) with an average of 105 fragments per primer pair. Sharma et al. (1996), studying the diversity of 54 lentil accessions, using radioactive AFLP, found fewer informative bands per primer (37). This disagreement could be due to the fluorescent labelling and to the automated fragment detection technology that offers significant improvements over radioactive labelling methods by increasing the scoring accuracy (Mitchell et al., 1997; Sharma et al., 2000). In fact, Tara Satyavathi et al. (2006) using fluorescent AFLPs to assess genetic diversity in 72 soybean varieties, under cultivation in India, obtained 1319 products of which 1257 were polymorphic (95%) from 12 AFLP primer pairs. The average number of fragments per primer pair was even 109.92.

Descriptive statistics and genetic variation estimates over all AFLP markers for each lentil landrace and over the three landraces are reported in Table 2. The total number of polymorphic loci was 405 (63.0%) for Altamura, 374 (58.2%) for Onano, 321 (49.9%) for Villalba and 565 (89.4%) for all genotypes. Within each population, the observed number of marker alleles per locus (n_o) ranged from 1.50 (Villalba) to 1.62 (Altamura) with 1.88 for all types, whereas the effective number of marker alleles per locus (n_e) ranged from 1.28 (Altamura and Onano) to 1.30 (Villalba) within each landrace and 1.32 for all populations (Table 2). The Nei's unbiased genetic diversity (H) estimates were calculated for all AFLP loci and each of the three lentil population. The average intra-population genetic diversity was 0.154 with 0.167 for Altamura, 0.149 for Onano, and 0.147 for Villalba. The Shannon's information index (I) over all landraces and AFLP marker loci was 0.311, with a range of 0.224 for Villalba to 0.259 for Altamura (Table 2). Comparison of genetic diversity estimates shows that Villalba could be considered as the most genetically uniform population.

Genetic diversity statistics and gene flow estimates based on AFLP loci are reported in Table 3. The proportion of the among-landraces genetic diversity was as low as G_{ST} = 0.219 meaning that about 78% of the total genetic diversity was due to DNA polymorphism within a population whereas only 22% of the total variation existed among populations. The low extent of differentiation between the three lentil landraces (D_{ST} = 0.043) and the relatively high gene flow estimate (N_m = 1.774) suggested a narrow genetic base among Onano, Altamura, and Villalba populations. In contrast, Sonnante and Pignone (2007) using ISSRs revealed that a higher percentage of the genetic differentiation takes place among landraces while a lesser amount resides within them. We could ascribe these dissimilar results to the different molecular marker technique (ISSRs) used, and to the different sample size analysed in their study. The higher number of polymorphic loci within each landrace probably pointed out to higher genetic diversity estimated within the landrace when using fluorescent AFLPs.

Nei's unbiased genetic distances and Dice's genetic similarities were estimated for all pairwise comparison among the three landraces and are reported in Table 4. The value of genetic similarity coefficients (0.71–0.75) as well as the low estimates of genetic distance (0.052–0.097) among the three landraces confirmed the sharing of a common gene pool. This phenomenon could be attributed to the influence of human activity such as seed exchange between farmers or unanticipated admixtures. Our results are consistent with the findings of Sharma et al. (1995) who observed a narrow genetic base among the cultivated lentils tested using RAPDs. Similarly, Sonnante and Pignone (2001), analysing a collection of lentil using molecular tools, found an indication that Italian accessions have a tendency to cluster all together.

The relationships among genotypes and populations have been visualised in the UPGMA dendrogram generated from Dice (1945) genetic similarity estimates (Fig. 2), and a bootstrap analysis was conducted to validate dendrogram structure (1000 cycles). The similarity tree showed three main clusters: the first one included individuals belonging to the landrace Altamura, the second one involved only genotypes belonging to the landrace Onano, whereas the third one included genotypes belonging to the landrace

Table 3
Nei's genetic diversity statistics and gene flow for all AFLP loci including sample size (s).

	s	H_T	H_S	D_{ST}	G_{ST}	N_m
Overall	84	0.198	0.155	0.043	0.219	1.779
S.D.		0.031	0.020			

Table 4
Matrices of Dice genetic similarity (above diagonal) and Nei genetic distance (below diagonal).

	Altamura	Onano	Villalba
Altamura	–	0.75	0.71
Onano	0.052	–	0.74
Villalba	0.097	0.082	–

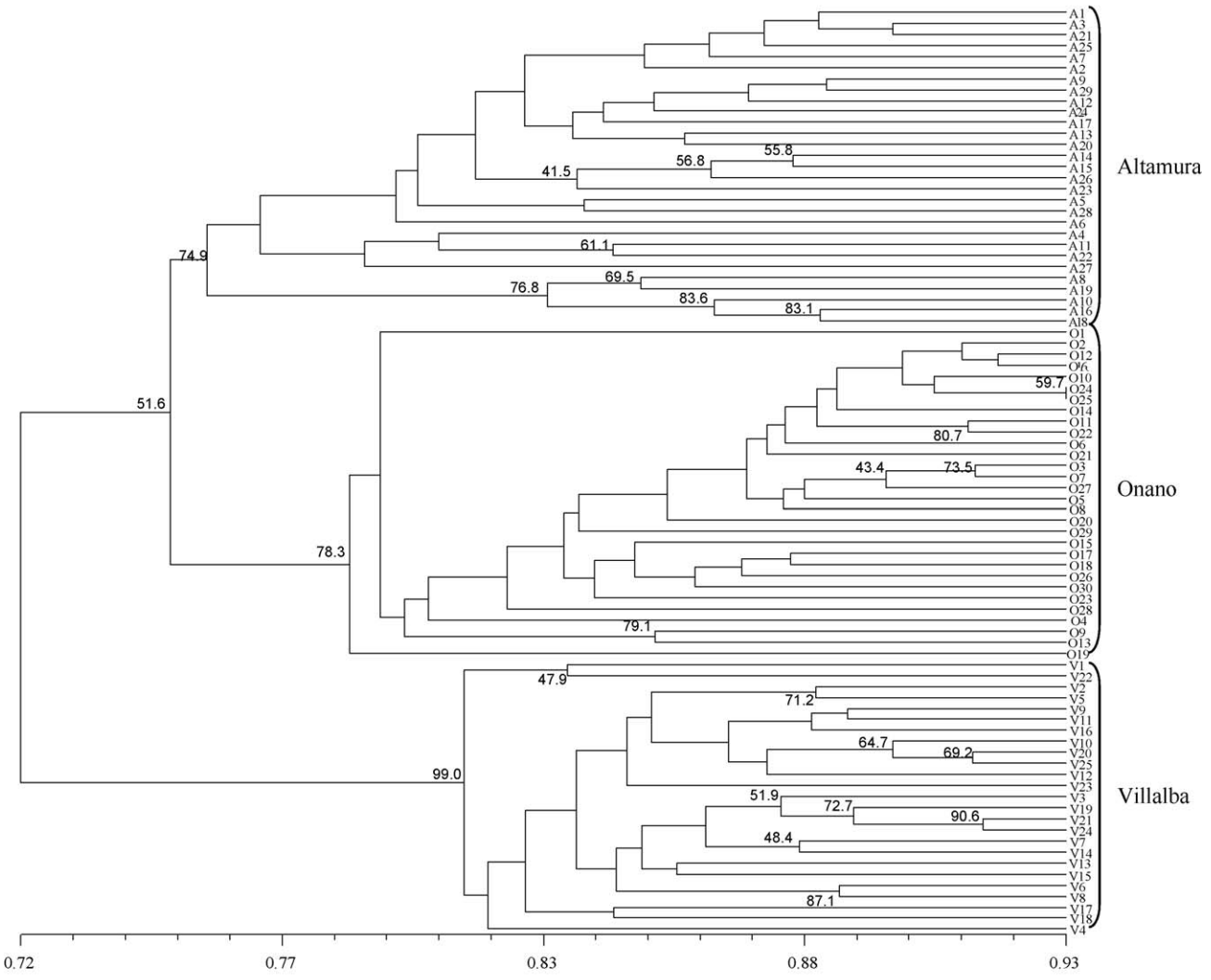

Fig. 2. The UPGMA dendrogram of the single plant DNA samples obtained from the genetic similarity matrix based on AFLP markers. Numbers in percentages at the nodes indicate the bootstrap values of the consensus tree tested (branches lacking the value received < 40% bootstrap support).

Villalba. The Sicilian landrace separated from the other two populations at a low genetic similarity value leading to an underling relative genetic divergence from Altamura and Onano landraces. In the AFLP analysis each individual was characterised by a unique profile, showing that AFLP are suitable markers for discriminating genotypes belonging to the same population. Moreover, AFLP data were informative enough to group together all individuals coming from the same landrace and discriminating among Onano, Altamura and Villalba landraces. The high level of polymorphism revealed within each local lentil variety was in agreement with a recent study (Piergiovanni and Taranto, 2005) showing that Italian lentil germplasm was characterised by a relevant genetic variation when seed storage proteins were analysed by electrophoresis (SDS-PAGE).

4. Conclusions

We reported the first use of a high-throughput; semi-automated fluorescence-based AFLP marker system to fingerprint Italian lentil landraces. This study provides clear evidence about the versatility and precision of this biotechnological tool to understand the distribution of genetic variation within and between landraces. This is a key aspect in the efficient conservation, characterisation and utilization of landraces like Onano, Altamura and Villalba, which are under extinction risk despite their high nutritional value and adaptability to intensive agricultural systems. The low level of genetic distance exhibited by Altamura, Onano, and Villalba populations suggests a common origin, confirming the tendency of Italian lentil landraces to group together when compared to foreign germplasm.

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References

Bozzini, A., Iannelli, P., Rossi, L., 1988. Valutazione di germoplasma di lenticchia di recente introduzione. L'Informatore Agrario 44, 39–45.

Dice, L.R., 1945. Measures of the amount of ecological association between species. Ecology 26, 297–302.
Erskine, W., 1997. Lessons for breeders from landraces of lentil. Euphytica 93, 107–112.
FAOSTAT, 2006. Food and Agricultural Organization, Statistical Databases, www.fao.org.
Gallo, G., Barbera, A.C., Venora, G., 1997. Valutazione agronomica di popolazioni di lenticchia (*Lens culinaris* Medik.) in ambienti semi-aridi.In: Proc. III Convegno Nazionale sulla Biodiversità, 16–17 Giugno, Reggio Calabria, pp. 279–288.
Gestetner, B., Assa, Y., Henis, Y., Birk, Y., Bondi, A., 1971. Lucerne saponins IV. Relationship between their chemical constitution, and haemolitic and anti-fungal activities. J. Sci. Food Agric. 22, 168–172.
Hartl, L., Seefelder, S., 1998. Diversity of selected hop cultivars detected by fluorescent AFLPs. Theor. Appl. Genet. 96, 112–116.
Hodkinson, T.R., Chase, M.W., Renvoize, S.A., 2002. Characterization of a genetic resource collection for *Miscanthus* (Saccharinae, Andropogoneae, Poaceae) using AFLP and ISSR PCR. Ann. Bot. 89, 627–636.
Kimura, M., Crow, J.F., 1964. The number of alleles that can be maintained in a finite population. Genetics 49, 725–738.
Konoshima, T., Kokumai, M., Kozuka, M., 1992. Antitumor promoting activities of afromosin and soyasaponin I isolated from Wistaria *brachybotrys*. J. Nat. Prod. 55, 1776–1778.
Lewontin, R.C., 1974. The Genetic Basis of Evolutionary Change. Columbia University Press, New York, USA.
Massawe, F.J., Dickinson, M., Roberts, J.A., Azam-Ali, S.N., 2002. Genetic diversity in bambara groundnut (*Vigna subterranea* (L.) Verdc) landraces revealed by AFLP markers. Genome 45, 1175–1180.
McDermott, J.M., McDonald, B.A., 1993. Gene flow in plant pathosystems. Annu. Rev. Phytopathol. 31, 353–373.
Mitchell, S.E., Kresovich, S., Jester, C.A., Hernandez, C.J., Szewc McFadden, A.K., 1997. Application of multiplex PCR and fluorescence-based, semi-automated allele-sizing technology for genotyping plant genetic resources. Crop Sci. 37, 617–624.
Nei, M., 1973. Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. U.S.A. 70, 3321–3323.
Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89, 583–590.
Piergiovanni, A.R., 2000. The evolution of lentil (*Lens culinaris* Medik.) cultivation in Italy and its effects on the survival of autochthonous populations. Genet. Resour. Crop Evol. 47, 305–314.
Piergiovanni, A.R., Taranto, G., 2005. Assessment of the genetic variation in Italian lentil populations by electrophoresis (SDS-PAGE) of seed storage proteins. PGR Newslett. 141, 33–38.
Pro Loco Onanese, 1990. Sagra della lenticchia onanese. Gigli, Viterbo, Italy.
Rohlf, F.J., 2002. NTSYSpc: Numerical Taxonomy System Ver. 2.1. Exeter Publishing, Ltd., Setauket, NY.
Ruiz, R.G., Price, K.R., Rose, M.E., Fenwick, G.R., 1997. Effect of seed size and testa colour on saponin content of Spanish lentil seed. Food Chem. 58, 223–226.
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sarno, R., Stringi, L., Amato, G., Pristina, L., 1988. L'epoca di semina ed il genotipo nella coltivazione della lenticchia in ambiente semi-arido. L'Informatore Agrario 44, 51–54.
Sharma, S.K., Dawson, I.K., Waugh, R., 1995. Relationships among cultivated and wild lentils revealed by RAPD analysis. Theor. Appl. Genet. 91, 647–654.
Sharma, S.K., Knox, M.R., Ellis, T.H.N., 1996. AFLP analysis of the diversity and phylogeny of *Lens* and its comparison with RAPD analysis. Theor. Appl. Genet. 93, 751–758.
Sharma, A., Sharma, R., Machii, H., 2000. Assessment of genetic diversity in a *Morus* germplasm collection using fluorescence-based AFLP markers. Theor. Appl. Genet. 101, 1049–1055.
Sidhu, G.S., Oakenfull, D.G., 1986. A mechanism for the hypocholesterolaemic activity of saponins. Br. J. Nutr. 55, 643–649.
Sonnante, G., Pignone, D., 2001. Assessment of genetic variation in a collection of lentil using molecular tools. Euphytica 120, 301–307.
Sonnante, G., Pignone, D., 2007. The major Italian landraces of lentil (*Lens culinaris* Medik.): their molecular diversity and possible origin. Genet. Resour. Crop Evol. 54, 1023–1031.
Tara Satyavathi, C., Bhat, K.V., Bharadway, C., Tiwari, S.P., Chaudhury, V.K., 2006. AFLP analysis of genetic diversity in Indian soybean [*Glycine max* (L.) Merr.] varieties. Genet. Resour. Crop Evol. 53, 1069–1079.
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. Nucl. Acids Res. 23, 4407–4414.
Yap, V., Nelson, R.J., 1996. WinBoot: A Program for Performing Boostrap Analysis of Binary Data to Determine the Confidence Limits of UPGMA-based Dendrograms. International Rice Research Institute, Manila, Philippines.
Yeh, F.C., Yang, R.C., Boyle, T., 1997. POPGENE Version 1.21. CIFOR and University of Alberta, Edmonton, Alberta, Canada.