NPC Natural Product Communications

2006
Vol. 1
No. 3
223 - 228

AFLP Analysis for Genetic Diversity in *Capsicum annuum* and Related Species

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Received: December 22nd, 2005; Accepted: January 24th, 2006

Intra- and inter-specific genetic variation analysis was conducted using amplified fragment length polymorphism (AFLP) profiling in *Capsicum* accessions in the germplasms collected from different geographical locations in India. A total of 24 accessions were investigated belonging to six species, namely *C. annuum*, *C. baccatum*, *C. chinence*, *C. eximium*, *C. frutescens* and *C. luteum*. Average similarity within the 15 accessions of *C. annuum* was highest (100 %) between accessions CIMAP/CA45 and CIMAP/CA49 obtained from IISR, Kerala and 43 % among the species CIMAP/CC1 and CIMAP/CB2. In this analysis, accessions were clustered more pronouncedly according to their geographical locations than to their taxonomic labels. A great degree of intermixing of present day domesticated chillies is evident from the present study.

Keywords: *Capsicum* germplasm, genetic diversity, AFLP, principal component analysis.

The genus *Capsicum* is distributed throughout the tropics and is represented by 30 species [1] among which *C. annuum*, *C. frutescens*, *C. chinence*, *C. baccatum* and *C. pubescens* are widely cultivated. *Capsicum* species exhibit a high degree of morphological variation in flower and fruit (colour, shape and size). The species by nature is facultatively cross-pollinated. The Indian germplasm is mainly represented by two species, *C. annuum* and *C. frutescens*, with a number of varieties cultivated throughout the country [2], but mainly in the southern states, nearer to the tropics, where the climate is friendly to *C. annuum*. The Portuguese introduced *Capsicum* species into India in the 16th century [3-4], but since then, the genus might have undergone different evolutionary changes through intended and unintended breeding and selection. Although considerable efforts have gone into breeding of new morphotypes and chemotypes, and into agronomical evaluation in India, a systematic evaluation of Indian germplasms in terms of genetic analysis has yet to be carried out. In the present investigation, we utilized AFLP markers to classify differentially mostly the Indian germplasms in comparison with a few accessions collected from outside India.

Principal component analysis: The AFLP analysis of the 24 accessions with selected 27-primer combinations revealed a total of 401 polymorphic DNA fragments with an average of 14.8 polymorphic loci per primer combination. Accessions CIMAP/CA45 and CIMAP/CA49 collected from IISR, Karanataka were morphologically dissimilar (drooping solitary fruits and upright clustered fruits respectively), but shared almost all the amplified fragments showing highest similarity (100 %) between themselves. In the component plot, all 24 accessions grouped in seven clusters, according to their geographical distributions, with few exceptions where they clustered together with morphologically related accessions. Out of these, accessions CIMAP/CA4, CIMAP/CA13, CIMAP/CA14, CIMAP/CA17 and CIMAP/CA34 formed one group. All these were collected from north India, except CIMAP/CA34, which was obtained from south India (Andhra Pradesh). Similarly, accessions CIMAP/CA44, CIMAP/ CA45, CIMAP/CA49 and CIMAP/CA55 formed one group and were collected from the south (IISR, Kerala), except CIMAP/CA55, which was obtained from north India (Uttaranchal). Accessions CIMAP/CA16, CIMAP/CA27, CIMAP /CA33, CIMAP/CA36 and CIMAP/CA41 formed a

separate group and were collected from Assam, Lucknow (U. P.), Anantpur and Guntur (A. P.), respectively, which are widely different geographical areas of India. Besides *C. annuum*, accessions of other species (CIMAP/CB1, CIMAP/CB2 and CIMAP/CC1) were collected from South-India (IIHR, Karanataka) and formed one cluster along with *C. annuum* accession CIMAP/CA60. Accessions CIMAP/CF1 and CIMAP/CL, obtained from IIHR, Karanataka, formed another cluster. Likewise, accessions CIMAP/CC2 and CIMAP/CE01, obtained from the botanical garden in Nijmegen, the Netherlands, formed a separate cluster. A separate cluster was also formed by accessions CIMAP/CF2 and CIMAP/CF3, collected from Assam.

In AFLP analysis, 3 major clusters of *C. annuum* were observed, as shown in figure 1, which lie apart by a large distance. Only one accession of *C. annuum* (CIMAP/CA60) clustered with the two accessions of *C. baccatum* and one of *C. chinense*. The other *C. chinense* accession was grouped with that of *C. eximium*. In this analysis, the two *C. baccatum* accessions were found to be closer to *C. chinense* accession CIMAP/CC1. The two accessions are distinctly different and present in two different clusters, indicating two lines of evolution.

In the first group (A) of *C. annuum*, except CIMAP/CA55, all were growing in similar environmental conditions. The exception may be due to the migration of seeds through human interventions to different geographical regions. Also, CIMAP/CB1, CIMAP/CB2, CIMAP/CA60 and CIMAP/CC1 were collected from the same geographical location and were growing in similar present analysis. Cluster D of the other extreme represents accessions from similar environmental conditions, except CIMAP/CA34, and this exception may be interpreted as a recent migration. The cluster C, in the middle, having accessions CIMAP/CA16, CIMAP/CA41, CIMAP/CA36, CIMAP/CA33 and CIMAP/CA27, were from different geographical locations, which were neither from northern nor southern India. The other accessions (taxa) grouped according to their geographical location and not according to their taxonomic name, as indicated by AFLP clustering. In this investigation, considering AFLP would reveal the polymorphism present in repetitive DNA elements in the genus *Capsicum*. Though it might be possible that some of the AFLP fragments have come from genes that are unique , their share was still predicted to be less compared with repetitive elements. Alternatively, the

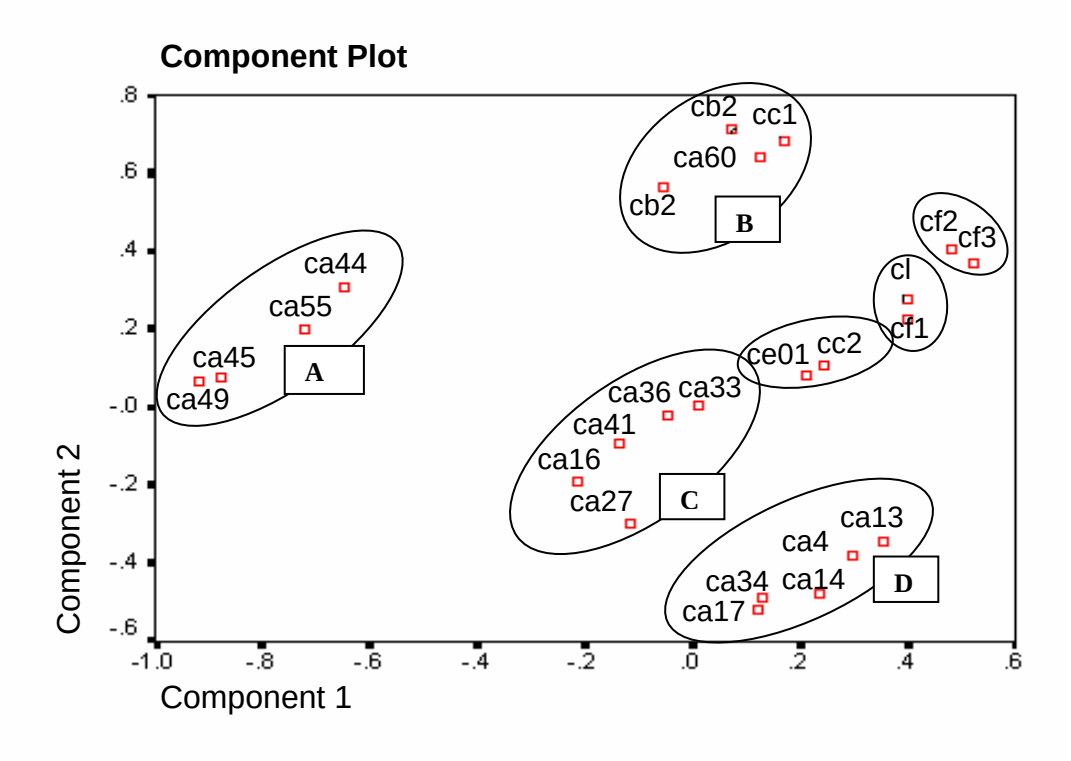

Figure 1. AFLP principal component analysis plot.

intermixing of genomes may be a common feature when different genotypes grow within the same geographical location for some duration, as the genus is facultatively cross-pollinated.

In *C. annuum* germplasms, as analyzed by Paran et al. [5] through AFLP and RAPD, divergent groups for small fruit cultivars and large fruit cultivars were detected. In this analysis, a limited variation was observed among blocky type cultivars. In the present analysis, we could not detect such a type of correlation, although we have used a higher number of primers for analysis of germplasms. Lefebvre et al. [6] have indicated a triangular relationship between molecular and morphological characters linking polygenic inheritance of traits, which was used to compute phenotypic distances. The correlation coefficient between phenotypic and molecular distances depends on the association between marker loci and quantitative trait loci. Accessions of 21 cultivated and wild *Capsicum* were analyzed [7] using RFLP markers and it was found that an accession of *C. chinence* grouped with a *C. baccatum* cluster from the same geographical location (Brazil). It was proposed that the two genotypes had undergone cross-fertilization [7]. In our investigation, we also detected clustering of *C. chinence* and *C. baccatum* in the same group. *C. annuum*, *C. chinence* and *C. frutescens* are expected to be more closely related, on the basis of flower morphology [8-9]. The presence of both accessions of *C. baccatum* in the same group as *C. annuum* and *C. chinence* is clearly an indication of cross-fertilization at the geographical location where they were grown. The species were maintaining their morphological characteristics and retaining their specific status, but there were only small differences in the genome. The differential clustering may also indicate that the essential characters are not affected by these cross-fertilizations. It has been reported earlier that the present day chilies are evolved through cross-fertilization and intermixing facilitated by similar chromosome numbers [6].

Variation among and within *Capsicum* species was studied by analyzing 134 accessions maintained at the Asian Vegetable Research and Development Centre (AVRDC) in Taiwan [10]. Diagnostic RAPD markers were identified to discriminate between the *Capsicum* species adding tools for taxonomic classification. This analysis of Indian germplasms has helped to reveal that, although the species characters are maintained through self-fertilization, lots of differences exist in the genome for other economical characters, which can be exploited in the future for breeding.

Experimental

Plant accessions: A total of 24 accessions of *Capsicum* germplasm, representing 6 species viz., *Capsicum annuum* L, *C. baccatum* var. *baccatum* L., *C. baccatum* var. *pendulum* L., *C. frutescens* L. and *C. luteum* were collected in the form of fruits and seeds from different localities and institutes (Indian Institute of Spices Research-IISR, Indian Institute of Horticulture Research-IIHR). Two species, *C. chinense* Jacq., and *C. eximium* Hunz., were the gift of Nijmegen Botanical Garden, the Netherlands (Table 1). These accessions, now grown and maintained at CIMAP field gene bank and in Green House, were used for AFLP analysis.

Amplified fragment length polymorphism analysis: DNA was isolated from leaf tissue essentially according to the protocol described by Khanuja et al. [11] and quantified fluorimetrically using a DyNa Quant 200 Fluorometer (Hoefer, USA). Genomic DNA was restricted using two restriction endonucleases EcoRI and Tru9I (an isoschizomer of MseI) and double stranded adaptors were ligated to the ends of DNA fragments, generating template DNA for subsequent PCR amplifications (preselective followed by selective). Restriction and ligation reactions were carried out simultaneously in a single reaction tube [12]. For the reaction, an enzyme master mix for 24 reactions was prepared containing 2.4 µl 10X T_4 DNA ligase buffer, 2.4 µl 0.5 M NaCl, 1.2 µl 1mg/ml BSA, 2.4 µl Tru9I (10U/µl), 10.2 µl EcoRI (12U/µl), 1.2 µl T_4 DNA ligase (20U/µl high concentration) and 4.2 µl H_2O. The restriction-ligation reaction consisted of 500 ng of DNA (5.5µl), 1µl 10X T_4 DNA ligase buffer, 1µl 0.5 M NaCl, 0.5µl 1mg/ml BSA, 1µl MseI adaptors (PE Biosystems), 1µl EcoRI adaptors (PE Biosystems) and 1µl enzyme master mix, as described above. The reaction mix was incubated overnight at room temperature and subsequently diluted 20 fold with $T_{10}E_{0.1}$ buffer.

The ligated adaptors served as primer binding sites for a low level selection in preselective amplification of the restriction fragments. The MseI complementary primer had a 3'-C and the EcoRI complementary primer a 3'-A. Only the genomic

Table 1: Collection details of *Capsicum* accessions used in AFLP analysis.

S. No.	Accession No.	Place of collection	Taxonomic labels
1	CIMAP/CA4	CIMAP Genebank, U. P.	*C. annuum*
2	CIMAP/CA13	Haldwani, Uttaranchal	*C. annuum*
3	CIMAP/CA14	CIMAP Genebank	*C. annuum*
4	CIMAP/CA16	Assam	*C. annuum*
5	CIMAP/CA 17	Ajmgarh, U. P.	*C. annuum*
6	CIMAP/CA27	Lucknow, U. P.	*C. annuum*
7	CIMAP/CA33	Lucknow, U. P.	*C. annuum*
8	CIMAP/CA34	Anantapur, A. P.	*C. annuum*
9	CIMAP/CA36	Anantapur, A. P.	*C. annuum*
10	CIMAP/CA41	Guntur, A. P.	*C. annuum*
11	CIMAP/CA44	IISR, Kerala	*C. annuum*
12	CIMAP/CA45	IISR, Kerala	*C. annuum*
13	CIMAP/CA49	IISR, Kerala	*C. annuum*
14	CIMAP/CA55	Purara, Uttaranchal	*C. annuum*
15	CIMAP/CA60	IIHR, Karanataka	*C. annuum*
16	CIMAP/CB1	IIHR, Karanataka	*C. baccatum* var. *baccatum*
17	CIMAP/CB2	IIHR, Karanataka	*C. baccatum* var. *pendulum*
18	CIMAP/CC1	IIHR, Karanataka	*C. chinence*
19	CIMAP/CC2	Nijmegen, The Netherland	*C. chinence*
20	CIMAP/CE01	Nijmegen, The Netherland	*C. eximium*
21	CIMAP/CF1	IIHR, Karanataka	*C. frutescens*
22	CIMAP/CF2	Assam	*C. frutescens*
23	CIMAP/CF3	Assam	*C. frutescens*
24	CIMAP/CL	IIHR, Karanataka	*C. luteum*

fragments having an adaptor on each end amplified exponentially during PCR. The preselective amplification mixture was prepared by adding 4 µl of 20 fold diluted DNA from the restriction–ligation reaction, 0.5 µl AFLP preselective primer (EcoRI, PE Biosystems), 0.5 µl AFLP preselective primer (MseI, PE Biosystems) and 15 µl AFLP core mix. The preselective amplification was carried out in a thermal cycler programmed at: 72°C for 2 min; 20 cycles of 94°C for 20 sec, 56°C for 30 sec and 72°C for 2 min; 60°C for 30 min; and 4°C for infinity.

The preamplified DNA was diluted 20 fold with $T_{10}E_{0.1}$ buffer and selective amplifications were carried out using different Mse I and EcoRI primer combinations (PE Biosystems). Primers chosen for the amplifications were from sixteen available AFLP selective primers (eight fluorescently labeled EcoRI primers and eight unlabeled MseI primers). The EcoRI primers contained three selective nucleotides with the sequence 5'-[Dye-Primer-Axx]-3', while the MseI primers had the selective nucleotides starting with C *i.e.* 5'-[Primer-Cxx]-3'. The "Explorer" gel for all 64 reactions was run using an accession of *C. annuum* (CIMAP/CA27) to determine the most responsive primer pairs for the *Capsicum* genome. Multiplexing of PCR reactions was followed so as to set up all the 64 (8x8) reactions in 24 tubes.

Table 2: 27 AFLP primer combinations used for *Capsicum* germplasm evaluation.

S No.	Primer combination	
1	MseI-CAC	EcoRI-ACA EcoRI-AGG EcoRI-ACC
2	MseI-CAG	EcoRI-ACA EcoRI-AGG EcoRI-ACC
3	MseI-CAT	EcoRI-ACT EcoRI-AAG EcoRI-AAC
4	MseI-CAT	EcoRI-ACA EcoRI-AGG EcoRI-ACC
5	MseI-CTC	EcoRI-ACA EcoRI-AGG EcoRI-ACC
6	MseI-CTG	EcoRI-ACA EcoRI-AGG EcoRI-ACC
7	MseI-CTT	EcoRI-ACA EcoRI-AGG EcoRI-ACC
8	MseI-CAG	EcoRI-ACT EcoRI-ACG EcoRI-AGC
9	MseI-CTC	EcoRI-ACT EcoRI-ACG EcoRI-AAC

For selective amplification, the reactions were set up as follows: 3 µl of 20 fold diluted preselective amplification reaction product, 15 µl AFLP core mix, 1 µl MseI primer 5'-[Primer-Cxx]-3', 1.5µl EcoRI primers 5'-[Dye-Primer-Axx]-3' {0.5 µl of three EcoRI primers each were pooled here}. Selective amplification was carried out in a thermal cycler programmed for: 94°C for 2 min; 10 cycles of

Table 3: Genetic similarity indices of 24 *Capsicum* accessions.

CIMAP /CA4	CIMAP /CA13	CIMAP /CA14	CIMAP /CA16	CIMAP /CA17	CIMAP /CA27	CIMAP /CA33	CIMAP /CA34	CIMAP /CA36	CIMAP /CA41	CIMAP /CA44	CIMAP /CA45	CIMAP /CA49	CIMAP /CA55	CIMAP /CA60	CIMAP /CB1	CIMAP /CB2	CIMAP /CC1	CIMAP /CC2	CIMAP /CE01	CIMAP /CF1	CIMAP /CF2	CIMAP /CF3	CIMAP /CL
1																							
0.316	1																						
0.167	0.338	1																					
0.145	0.056	0.097	1																				
0.160	0.212	0.307	0.204	1																			
0.236	0.148	0.128	0.417	0.219	1																		
0.116	0.114	0.037	0.031	0.100	0.121	1																	
0.152	0.088	0.188	0.160	0.241	0.273	0.071	1																
0.100	0.098	0.087	0.048	0.155	0.120	0.095	0.113	1															
0.016	0.000	0.137	0.085	0.048	0.094	0.126	0.115	0.089	1														
0.076	0.087	0.078	0.132	0.052	0.106	0.062	0.008	0.217	0.051	1													
0.096	0.145	0.229	0.320	0.157	0.204	0.228	0.121	0.169	0.147	0.628	1												
0.162	0.109	0.113	0.228	0.146	0.334	0.131	0.123	0.227	0.150	0.479	1	1											
0.181	0.157	0.083	0.289	0.234	0.381	0.208	0.150	0.202	0.200	0.540	0.346	0.527	1										
0.036	0.035	0.026	0.061	0.109	0.068	0.055	0.040	0.094	0.068	0.095	0.182	0.215	0.335	1									
0.009	0.047	0.062	0.135	0.110	0.039	0.083	0.040	0.037	0.029	0.138	0.235	0.250	0.268	0.291	1								
0.034	0.033	0.022	0.052	0.008	0.071	0.032	0.025	0.063	0.104	0.103	0.183	0.210	0.232	0.194	0.164	1							
0.033	0.045	0.099	0.029	0.032	0.082	0.093	0.038	0.049	0.013	0.124	0.193	0.124	0.262	0.162	0.174	0.430	1						
0.255	0.289	0.158	0.275	0.127	0.298	0.185	0.117	0.150	0.127	0.205	0.152	0.096	0.092	0.338	0.251	0.189	0.269	1					
0.155	0.033	0.106	0.103	0.202	0.111	0.114	0.080	0.224	0.113	0.151	0.059	0.045	0.047	0.184	0.206	0.107	0.231	0.130	1				
0.285	0.210	0.195	0.299	0.219	0.243	0.196	0.165	0.340	0.141	0.267	0.103	0.114	0.201	0.264	0.221	0.247	0.304	0.202	0.156	1			
0.235	0.309	0.190	0.208	0.213	0.288	0.191	0.182	0.185	0.172	0.327	0.104	0.079	0.173	0.361	0.252	0.359	0.320	0.197	0.153	0.341	1		
0.254	0.281	0.188	0.252	0.182	0.316	0.237	0.211	0.223	0.124	0.240	0.172	0.073	0.129	0.423	0.308	0.286	0.274	0.213	0.183	0.359	0.377	1	
0.239	0.237	0.292	0.184	0.178	0.257	0.232	0.196	0.219	0.132	0.253	0.169	0.107	0.208	0.317	0.274	0.234	0.278	0.164	0.080	0.306	0.364	0.397	1

94°C for 20 sec, 66°C (-1°C / cycle) for 30 sec, 72°Cfor 2 min; 20 cycles 94°C for 20 sec, 56°C for 30 sec, 72°C for 2 min; 60°C for 30 min; and 4°C for infinity. The samples were loaded on a 5% Long Ranger polyacrylamide gel on the ABI Prism 377 DNA sequencer. The selective amplification reaction product (3 μl) was mixed with 4 μl of loading buffer {500ROX size standard (10%), blue dextran (10%), deionized formamide (80%)}, from which 1.5 μl was finally loaded onto the gel.

A total of 27 primer combinations, as given in table 2, was chosen according to the number of fragments amplified and suitability for multiplexing after analyzing the explorer gel. All the accessions were then subjected to selective amplification using these primer combinations. For AFLP reactions and the AFLP amplification modules the guidelines supplied by PE Biosystems, USA, were used.

Data analysis methods: The peak analysis was performed using Gene Scan Analysis *v 3.1* software (PE Biosystems, USA). A similarity matrix was obtained after multivariant analysis using Nei and Li's coefficient [13]. Principal Component Analysis (PCA) was carried out using similarity coefficients of different taxa by extracting two component matrices and plotting them against each other to find out the grouping of different taxa using SPSS *v 7.5* software (SPSS, Inc.).

Acknowledgement - We thank to Dr. Gerard, The Netherlands and Dr. Madhvi Reddy, Bangalore for their donation of germplasms. This work was supported by grants and a fellowship from the Council of Scientific and Industrial Research (CSIR), INDIA.

References

[1] Hunziker AT. (**1958**) A synopsis of the genus *Capsicum*. 8th Congres International de Botanique, Paris – 1954. *Comptes Rendus des Seances et Rapports et Communications Deposes lors du Congres*. p. 73-74.

[2] Singh J. (**1993**) Improvement of chillies. Advances in Horticulture. (5) *Vegetable crops*: Part-1, 70-84.

[3] Purseglove JW. (**1968**) Tropical crops. Dicotyledons 2. Longman, Green & Co. London. 524-530.

[4] Heiser CB. (**1976**) Pepper *Capsicum* (Solanaceae). In: Evolution of Crop Plants (Simmond NW. Ed.) Longman, London: 265-268.

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

[6] Lefebvre V, Goffinet B, Chauvet JC, Caromel B, Signoret P, Brand R, Palloix A. (**2001**) Evaluation of genetic distances between pepper inbred lines for cultivar protection purposes: comparison of AFLP, RAPD and phenotypic data. *Theoretical and Applied Genetics*, ***102***, 741–750.

[7] Prince JP, Lackney VK, Angeles C, Blauth JR, Kyle MM. (**1995**) A survey of DNA polymorphism within the genus *Capsicum* and the fingerprinting of pepper cultivars. *Genome*, ***38***, 224-231.

[8] Pickersgill B. (**1988**) The genus *Capsicum*: a multidisciplinary approach to the taxonomy of cultivated and wild plants. *Biologisches Zentralblatt Band*, ***107***, 381-389.

[9] Jensen RJ, McLeod MJ, Eshbaugh WH, Guttman SI. (**1979**) Numerical taxonomic analyses of allozymic variation in *Capsicum* (Solanaceae). *Taxon*, ***28***, 315-327.

[10] Rodriguez JM, Berke T, Engle L, Nienhuis J. (**1999**). Variation among and within *Capsicum* species revealed by RAPD markers. *Theoretical and Applied Genetics*, ***99***, 147-156.

[11] Khanuja SPS, Shasany AK, Darokar MP, Kumar S. (**1999**) Rapid isolation of PCR amplifiable DNA from the dry and fresh amples of plants producing large amounts of secondary metabolites and essential oils by modified CTAB procedure. *Plant Molecular Biology Reporter*, ***17***, 74.

[12] Vos P, Hogers R, Bleeker M, Reijans M, van de Lee I, 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.

[13] Nei M. and Li WH (**1979**) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences USA*, ***76***, 5269-5273.