7. AFLP ANALYSIS OF *S. obtusifolia* PROVENANCES

A number of molecular biological tools are in modern trend to detect polymorphism for determining differences in nucleotide sequences. These powerful tools provide efficient molecular markers for evaluating genetic diversity and phylogenetic study dealing only with DNA or RNA and without going into the details of pleiotropic effects, environmental conditions or pedigree records (Seefelder et al., 2000; Tao et al., 2009). For assessing within and between genetic variations of plant populations, molecular markers-based tools have been found to be very effective (Powell et al., 1995). Several DNA based molecular analyses that are in vogue, include SSR, ISSR, RAPD, AFLP, RFLP. SNP, SCoT, ITS and SCAR in medicinal plants have been discussed that are effective in discriminating the plant species at variety and at lower levels by detecting variations in DNA sequences (Williams et al., 1990; Morgante and Olivieri, 1993; Meudt and Clarke, 2007). Among all the aforesaid techniques, AFLP developed by Zabeau and Vos (1993) emerges as the most popular marker tool, analyzes a greater part of the genome and produces a greater percentage of independent polymorphic loci in one analysis (Papa et al., 2005; Sathyanarayana et al., 2011; Kim et al., 2013). As AFLP technique is generic in nature, no prior sequencing information is required for genomic DNA libraries or designing primers and can even identify 20-100 loci per assay. A very less amount of DNA (100-500 ng) is sufficient for the assay; thus, detecting genetic variations at very minor levels (Bhat et al., 2005). AFLP technique is much more efficient than RAPD and RFLP (Garcia-Mas et al., 2000) and now-a-days became marker of choice (Jones et al., 1997; Milbourne et al., 1997; Barker et al., 1999). For detecting infra-specific variations AFLP proved to be the one of the most robust and efficient technique with maximum reproducibility (Patzak 2001; Hodkinson et al.,

2002; Archak et al., 2003). Intraspecific variations by AFLP techniques have been studied in the species of *Populus maximowiczii* (Rajora and Dancik, 1995a).

Senna obtusifolia (L.) Irwin and Barneby of the family Leguminosae is considered as a medicinal plant with nutraceutical properties for the presence of a host of secondary metaboloits contributing to an array of medicinal properties for decades. *Senna obtusifolia* is widely accepted by pharmaceutical industries throughout the world for its immense medicinal properties. Several workers studied genetic relationships by using different molecular techniques like, AFLP, ISSR and RAPD among several species under the genera *Cassia* and *Senna*, revealing their phylogenetic relationship (Shanat et al., 2017; Mao et al., 2017; Acharya et al., 2011). Mao et al. (2017) carried out ISSR and ScoT primers analyses in *S. obtusifolia* from China. Growing of a species on a landmass with any number of individuals, however, not having a continuous distribution and occurring as an isolated community, cause to develop subtle genetic deviation with respect to the individuals of the same species of a distantly placed community. Such a situation raises enough points to designate the community as a 'provenance' with genetic identity showing intraspecific diversity. Cosidered *S. obtusifolia* samples are supposed to be the prospective provenances with some extent of genetic difference. Till date there is no report on the intraspecific genetic diversity or even congruity among the individuals of *Senna obtusifolia*, growing in different parts India, in the light of the results revealed through molecular technique. Genetic difference might have an implication in causing difference at the level of different biochemical traits of the individuals and so having a chance to get difference in the amount of production of bioactive molecules, too, amongst the plants under consideration. With the availability of such difference selection may be practiced among the provenances to sort out the best one in regard of productivity. In

consideration of such notion, AFLP analysis of considered accessions has been carried out to reveal the genetic diversity among them, if any.

7.1 MATERIALS AND METHODS

7.1.1 Materials

Seed samples of all the considered 20 accessions were collected from different provenances and were used as germplasms for the AFLP experiments. Thirty random seeds from each provenance were selected and each line was germinated separately in Hyco trays with sterile coir peat in normal germinating conditions. Ten random samples were chosen on appearance of true leaves excluding the cotyledonary leaves from them and collected for performing AFLP to assess genetic variations. The objective here was to find out the genetic variations and elucidate distances between the plants of different provenances.

7.1.2 Methods

7.1.2.1 DNA Extraction

The leaf samples were collected from the plants chosen from the Hyco trays. DNA extraction was done using a CTAB based method (Weising et al., 1995) with the plant DNA kit procured from MDI Membrane Technologies, Ambala Cantonment, India. Quantification of all the DNA samples was done on agarose gel by comparing with uncut lambda DNA of known concentration. The final concentrations of DNA were adjusted around 100 ng/µl. The DNA quantifications on agarose gel of all the 20 accessions DNA are shown in the Figure 7.1.

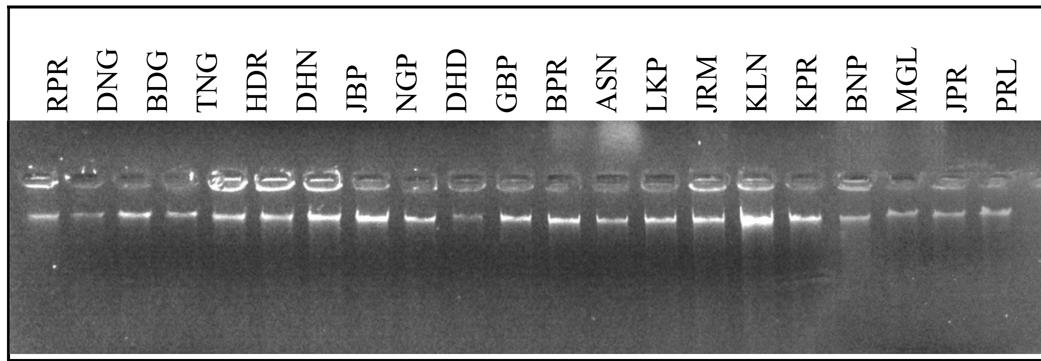


Figure 7.1 *S. obtusifolia* Genomic DNA quantification on agarose gel

7.1.2.2 AFLP Assay

In this study, the protocol developed by Zabeau and Vos (1993) was used to perform the AFLP analysis. The steps of the protocol are as follows:

7.1.2.3 Restriction Digestion of Genomic DNA

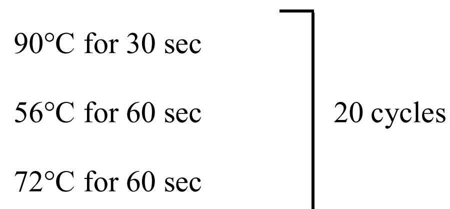
Approximately 300 ng Genomic DNA was absorbed with MseI and EcoRI (1.25units/µl each) in 25µl reaction volume.

7.1.2.4 Adapter Ligation

The DNA template for PCR amplification was generated by legating the restricted digested fragments with MseI and EcoRI adapters.

7.1.2.5 Pre-amplification

The products were diluted first in 1:10 ratio. Then primers complementary to restriction sites and adapter sequences were used to pre-amplify the DNA template, following the parameter setting:



On the gel and smears, the presence of an aliquot of the pre-amplified product ensured the good quality of the AFLP libraries. The Pre-amplification of adaptor ligated libraries are shown in Figure 7.2.

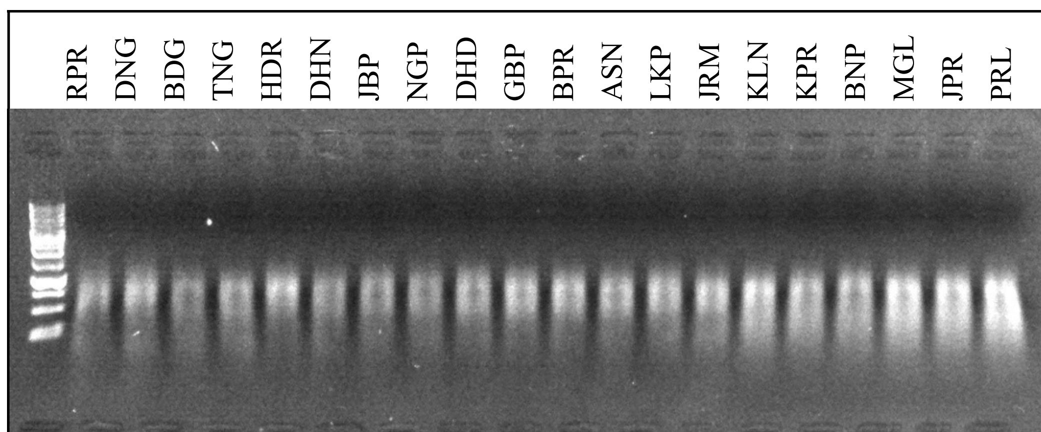


Figure 7.2 Pre-amplification of adaptor ligated libraries

7.1.2.6 Primer Sequences

Table 7.1 and Table 7.2 present the nucleotide AFLP primers sequence used for this study. Only one selective nucleotide was present in the pre-amplification primers. However, there were three to four selective nucleotides in the amplification primers.

Table 7.1 AFLP analysis pre-amplification primers sequence

Preamp-Primers	Code	Sequence*
Eco RI+1	E-A	5'-X-A 3'
Mse I+0	M-0	5'-Y A-3'

* X: GAC TGC GTA CCA ATT C and Y: GAT GAG TCC TGA GTA A

Table 7.2 Selective Primers

Preamp-Primers	Code*	Sequence*
Eco RI+2 – AG (IR-700 Labeled)	E-AG	5'-X AG-3'
Eco RI+2 – AT (IR-800 Labeled)	E-AT	5'-X AT-3'
Eco RI+3 – ACA (IR-700 Labeled)	E-ACA	5'-X ACA-3'
Eco RI+3 – AAC (IR-800 Labeled)	E-AAC	5'-X AAC-3'
Eco RI+3 – ACT (IR-800 Labeled)	E-ACT	5'-X ACT-3'
Mse I+3 – CAG	M-CAG	5'-Y CAG-3'
Mse I+3 – CTA	M-CTA	5'-Y CTA-3'

* X: GAC TGC GTA CCA ATT C and Y: GAT GAG TCC TGA GTA A

7.1.2.7 Primer Labeling

For selective amplification of the pre-amplified products, the EcoRI primers were fluorescently labeled with commercially available IR700 and IR800 dye.

7.1.2.8 Selective Amplification

The products were further diluted. The selective amplification then used the pre-amplified AFLP library as a template. EcoRI +2 and EcoRI + 3 were used in combination with MseI + 3 primers. The cycle parameters for the selective amplification are given below.

94°C for 30 sec
65°C for 30 sec
72°C for 60 sec
] 01 cycle

The annealing cycle started after this and it is repeated 12 times. The annealing temperature was decreased by 0.7°C in each cycle. Thereafter, the process is repeated for 23 cycles with the parameter setting given below.

94°C for 30 sec
56°C for 30 sec
72°C for 60 sec
] 23 cycles

Equal volume of 98% formamide dye was added at the end of the polymerase chain reaction. The sample is then added to 6% polyacrylamide gel electrophoresis and the AFLP fragments were analyzed on Licor 4300 DNA analyzer.

7.1.2.9 Jaccard's Coefficient of Genetic Similarity

Jaccard's coefficients provides a measure of genetic similarity of binary variables obtained from the AFLP scores. For any two individuals *a* and *b*, the Jaccard's coefficient (Jaccard, 1908), denoted by GS_J, is calculated as follows:

$$GS_J = \frac{x}{(x + y + z)}$$

where 'x' denotes the number of bands common to a and b, 'y' denotes the number of bands absent in b and present in a, and 'z' denotes the number of bands present in b and absent in a. On the other hand, Jaccard's distance provides a measure of dissimilarity among binary variables.

7.1.2.10 Hierarchical Clustering Using UPGMA

UPGMA is a clustering method particularly useful to identify the species/samples closely related to their geographical locations. The method was proposed by Sokal and Michener (1957). It works based on the agglomerative strategy. This strategy groups the most similar genotypes first. The initial groups formed are then merged according to their similarity.

7.2 RESULTS AND DISCUSSIONS

7.2.1 Results

The DNA fingerprints (Figure 7.3 - Figure 7.12) of *Senna obtusifolia* randomly collected from twenty different provenances, characterized at the DNA level, showed prominent genomic similarities and dissimilarities among the provenances of the species. Different marker combinations were employed in unique combinations to produce more informative data sets in this study. For the AFLP analysis, the DNA fingerprint profile conducted with 10 different EcoRI/MseI primer amalgamations were used. Scoring of bands were done manually for presence (marked as 1) or absence (marked as 0) throughout all the samples in the experiment. The summary is presented in Table 7.3. The fingerprints generated a scoring of total 453 bands, out of which 150 polymorphic bands showed an average 33.11% polymorphism among all the 20

accessions. Primer combination of E-AAC/M-CAG showed the maximum number of polymorphic bands (50%), while the combination E-ACT/M-CTA produced minimum polymorphic bands (20.8%). The other primer combinations E-AAC/M-CTA, E-ACA/M-CAG, E-ACA/M-CTA, E-ACT/M-CAG, E-AG/M-CAG, E-AG/M-CTA, E-AT/M-CAG, E-AT/M-CTA produced 47.5%, 24%, 25.9%, 25.4%, 28.9%, 38.9%, 29%, 31.1% respectively. The data matrix obtained from the results used to calculate genetic similarity by using different coefficients were as proposed by Nei and Li (1979), Sokal and Michener (1958), and Jaccard (1908), with the data pooled from the experiments.

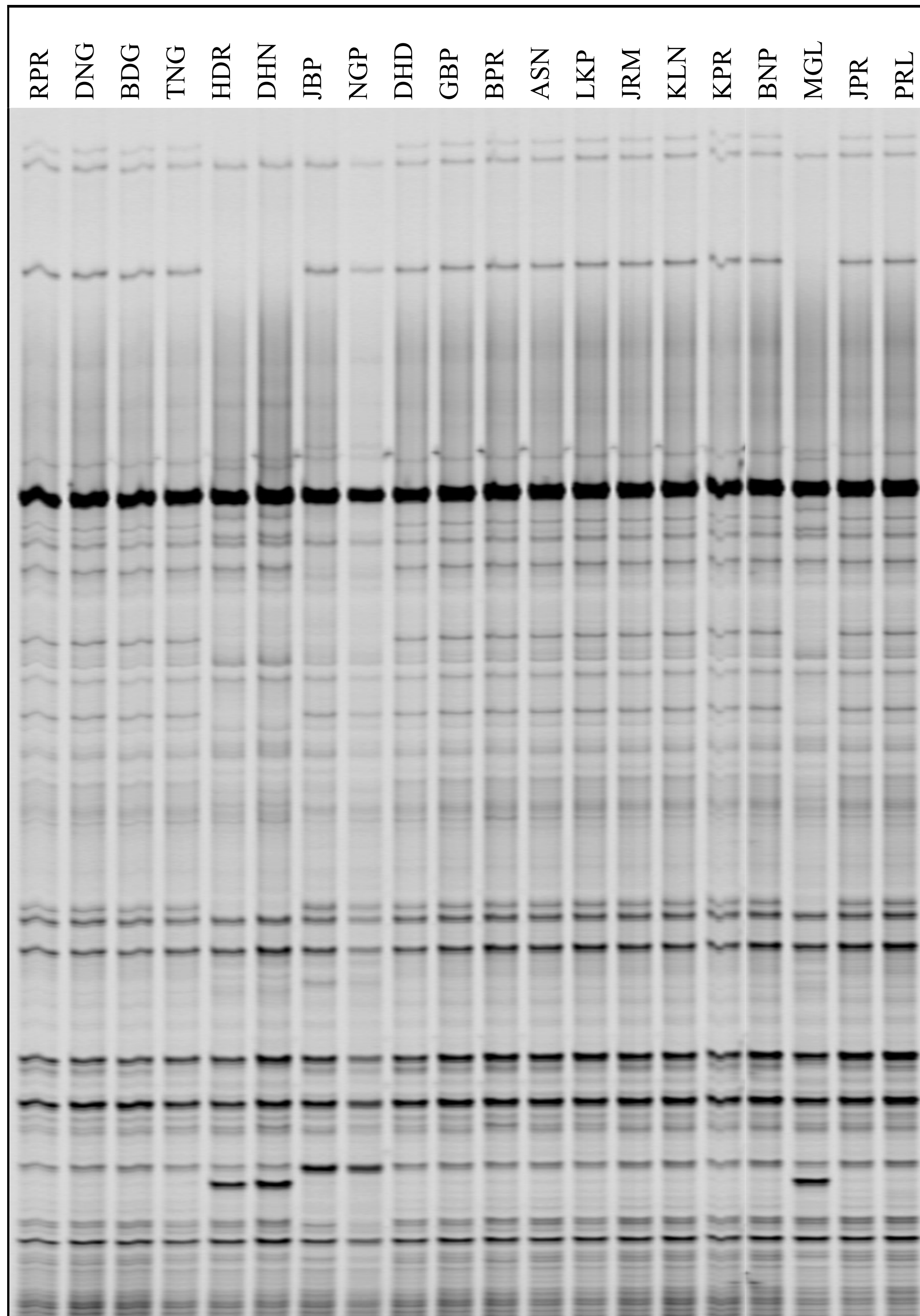


Figure 7.3 DNA Fingerprint (AFLP Primer Pair E-AAC/M-CAG)

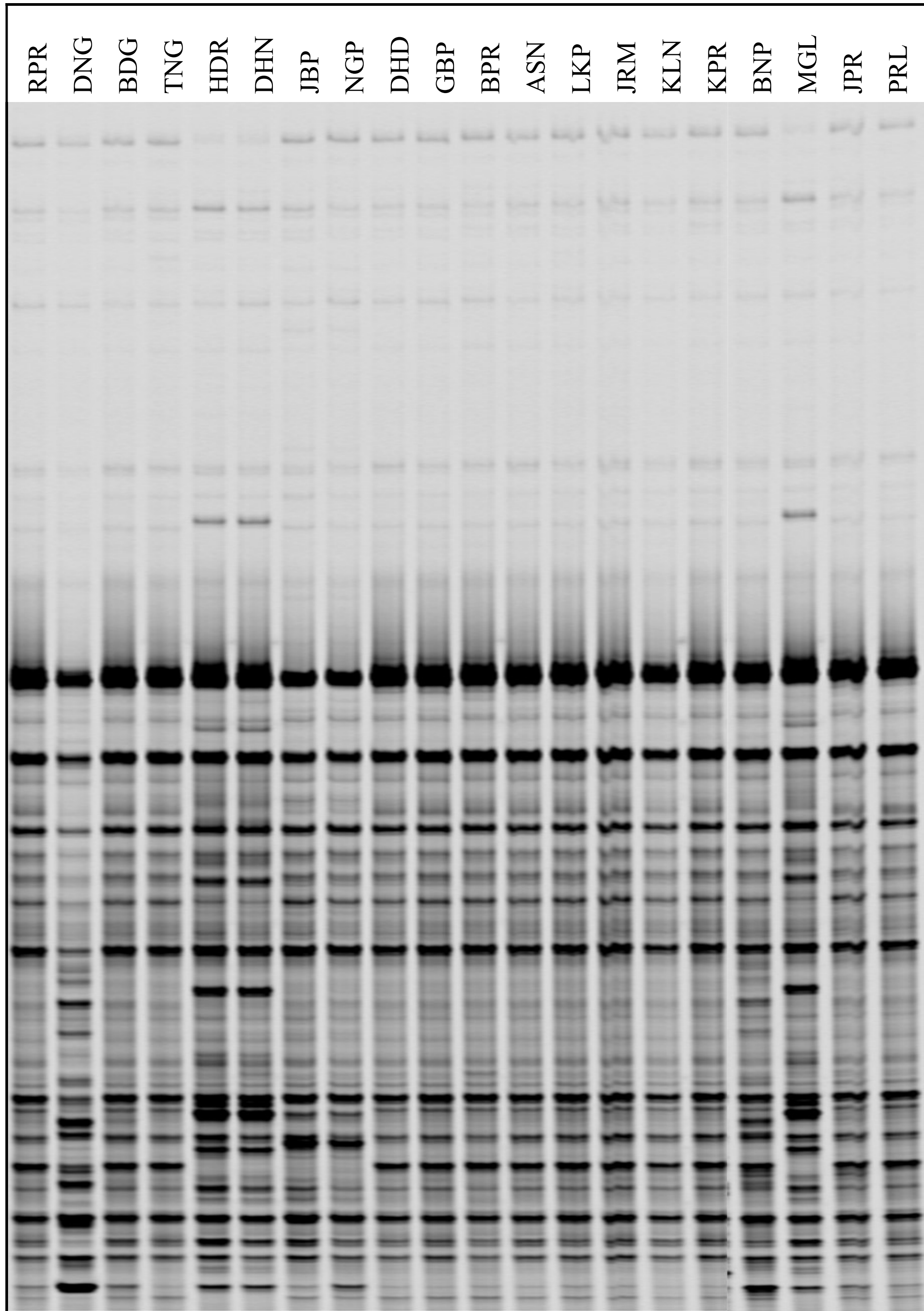


Figure 7.4 DNA Fingerprint (AFLP Primer Pair E-AAC/M-CTA)

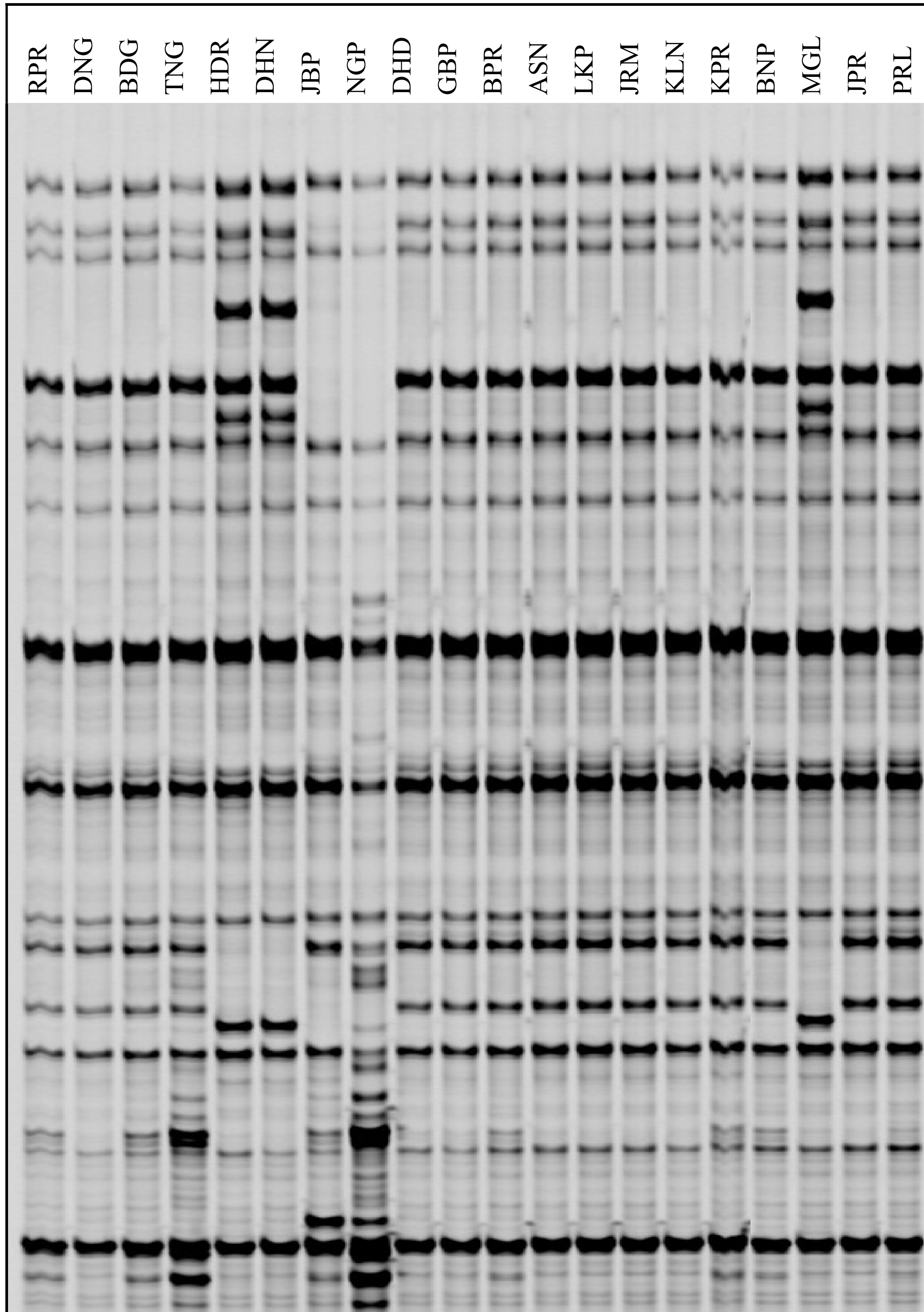


Figure 7.5 DNA Fingerprint (AFLP Primer Pair E-ACA/M-CAG)

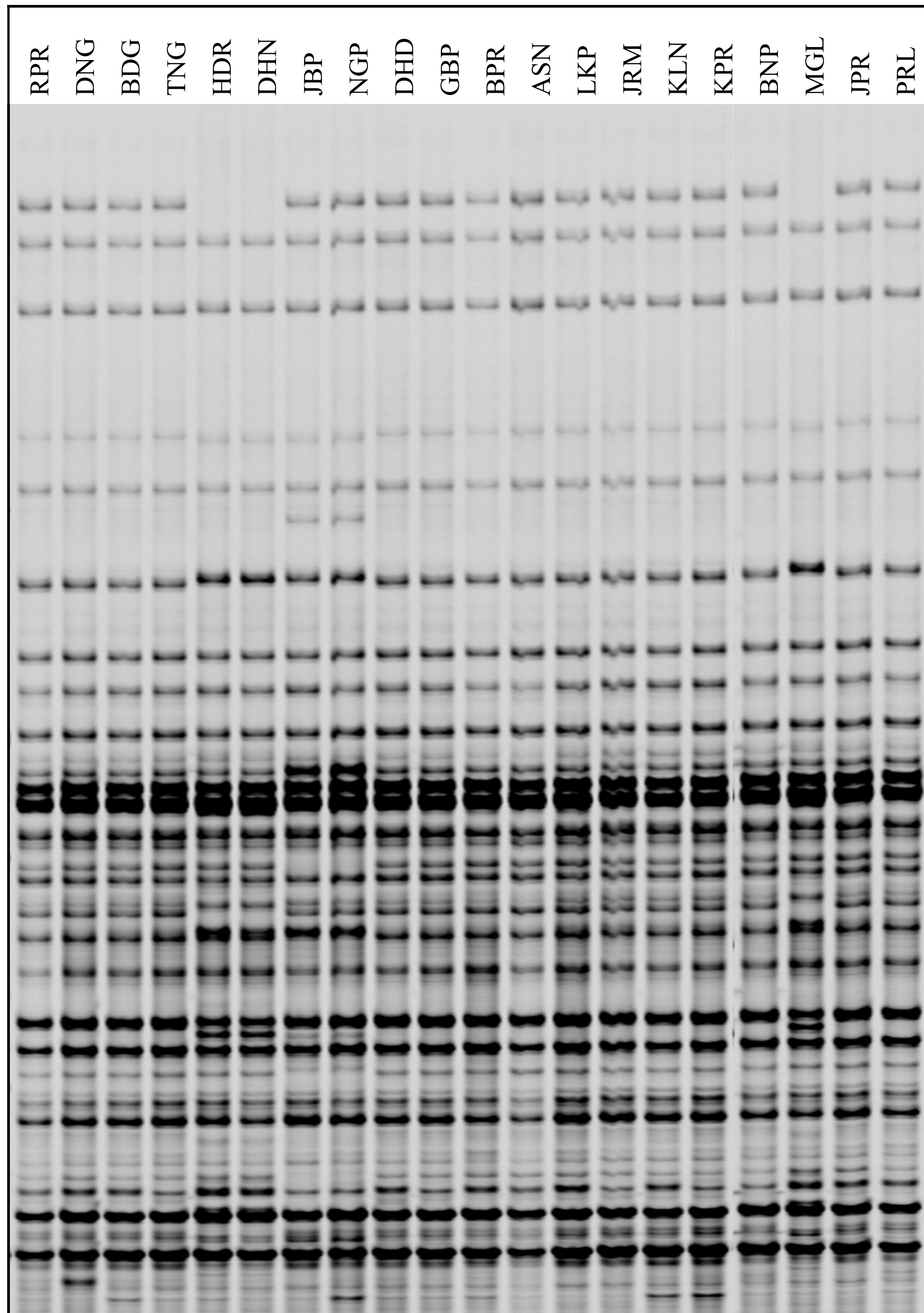


Figure 7.6 DNA Fingerprint (AFLP Primer Pair E-ACA/M-CTA)

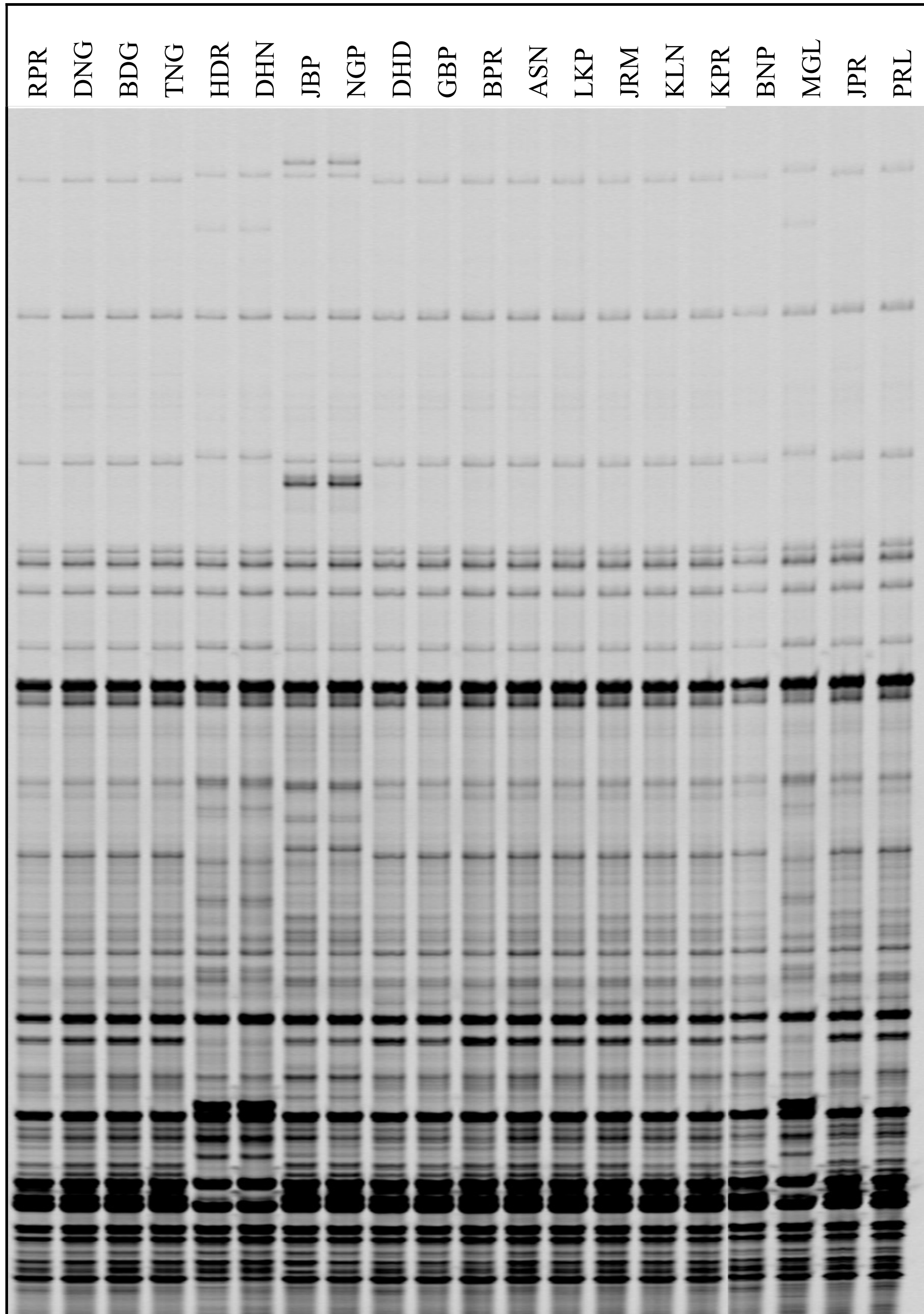


Figure 7.7 DNA Fingerprint (AFLP Primer Pair E-ACT/M-CAG)

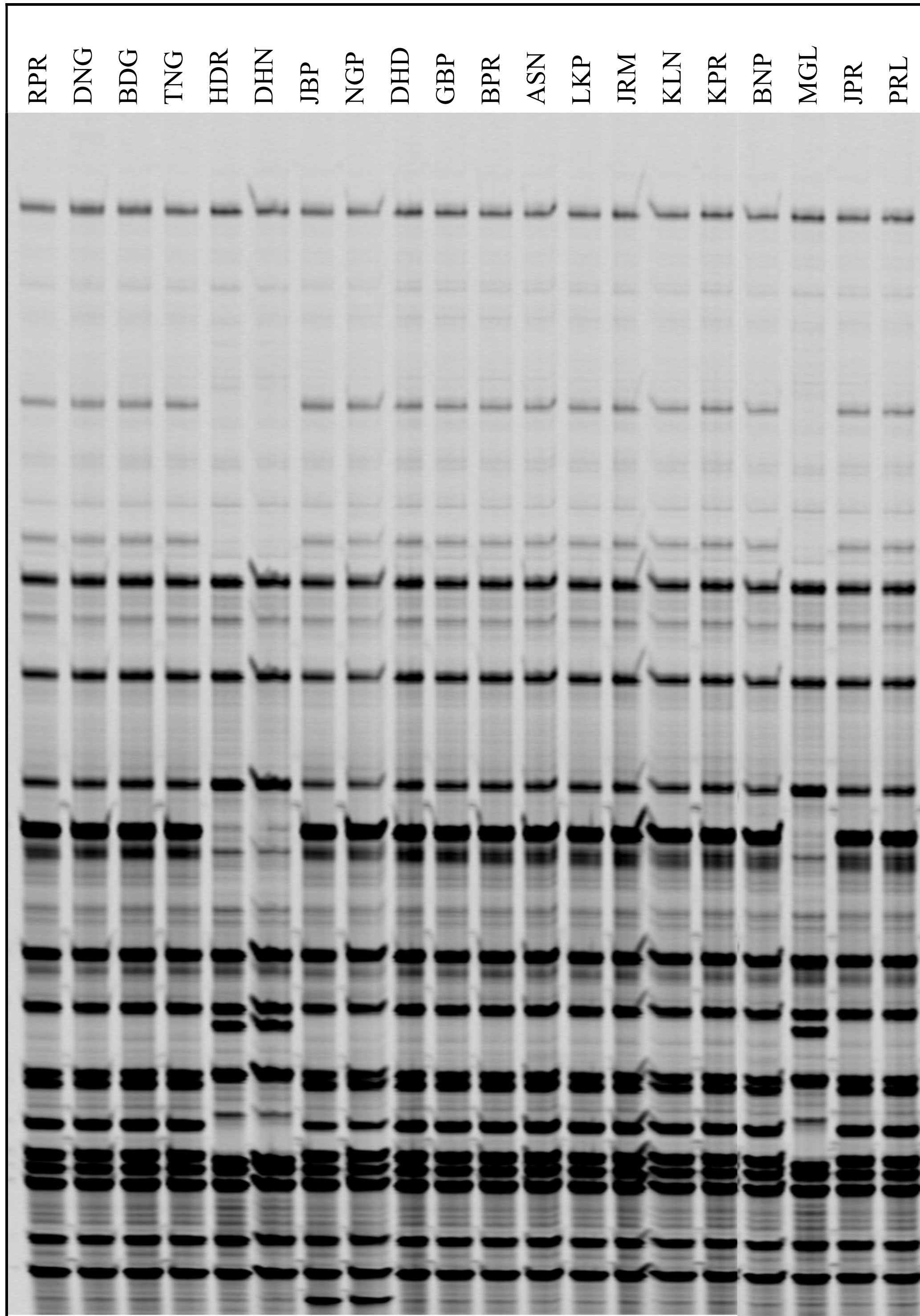


Figure 7.8 DNA Fingerprint (AFLP Primer Pair E-ACT/M-CTA)

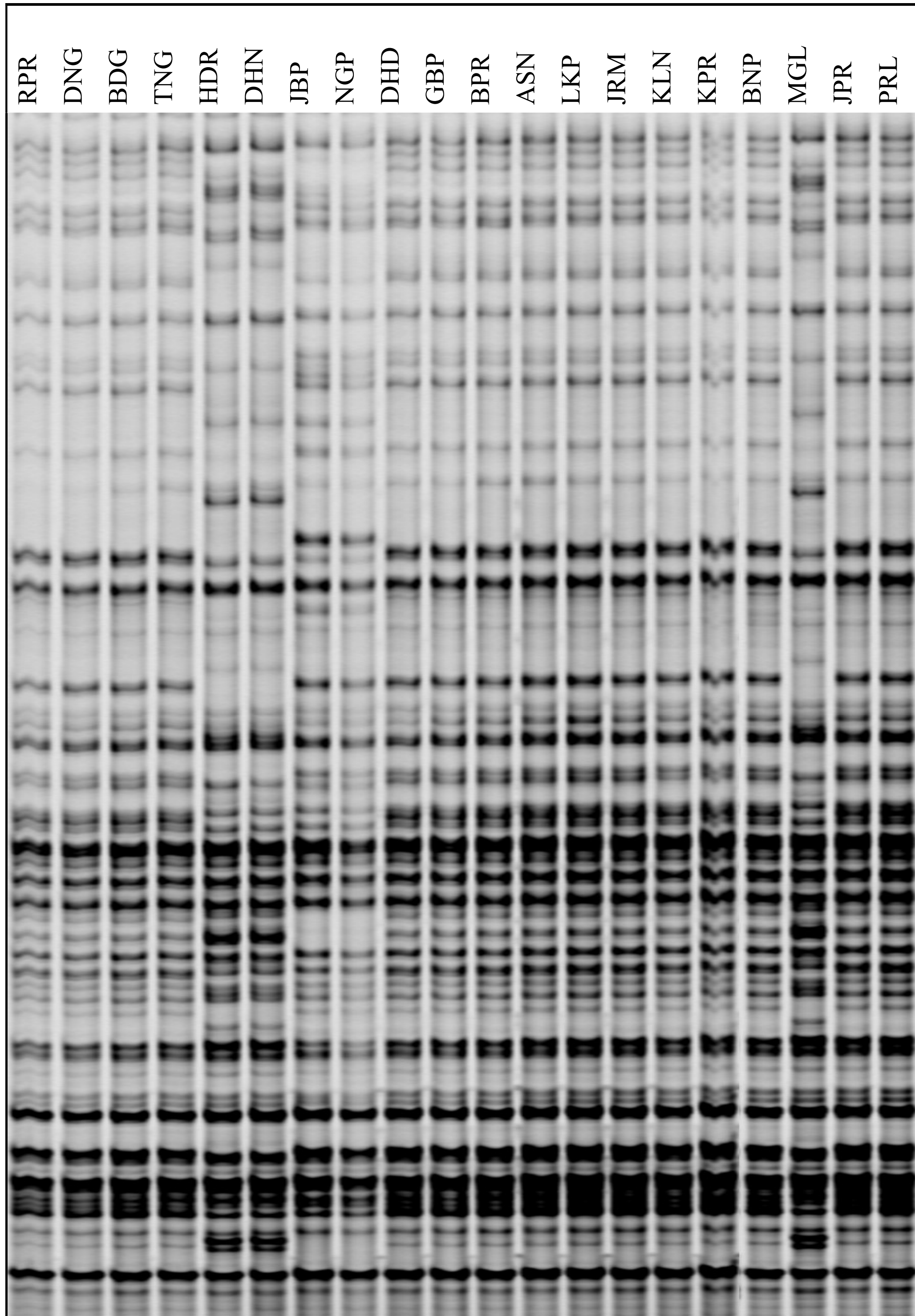


Figure 7.9 DNA Fingerprint (AFLP Primer Pair E-AG/M-CAG)

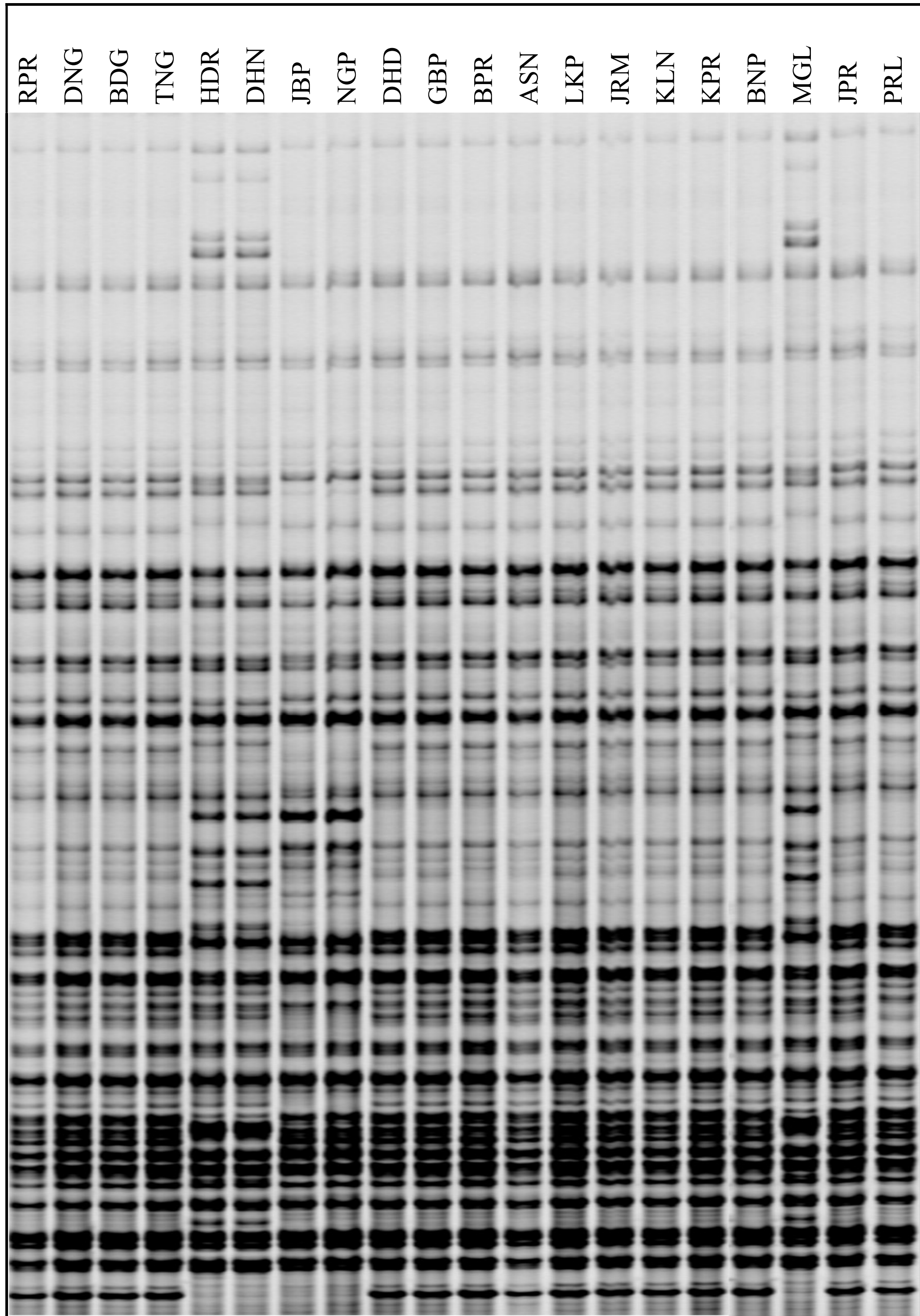


Figure 7.10 DNA Fingerprint (AFLP Primer Pair E-AG/M-CTA)

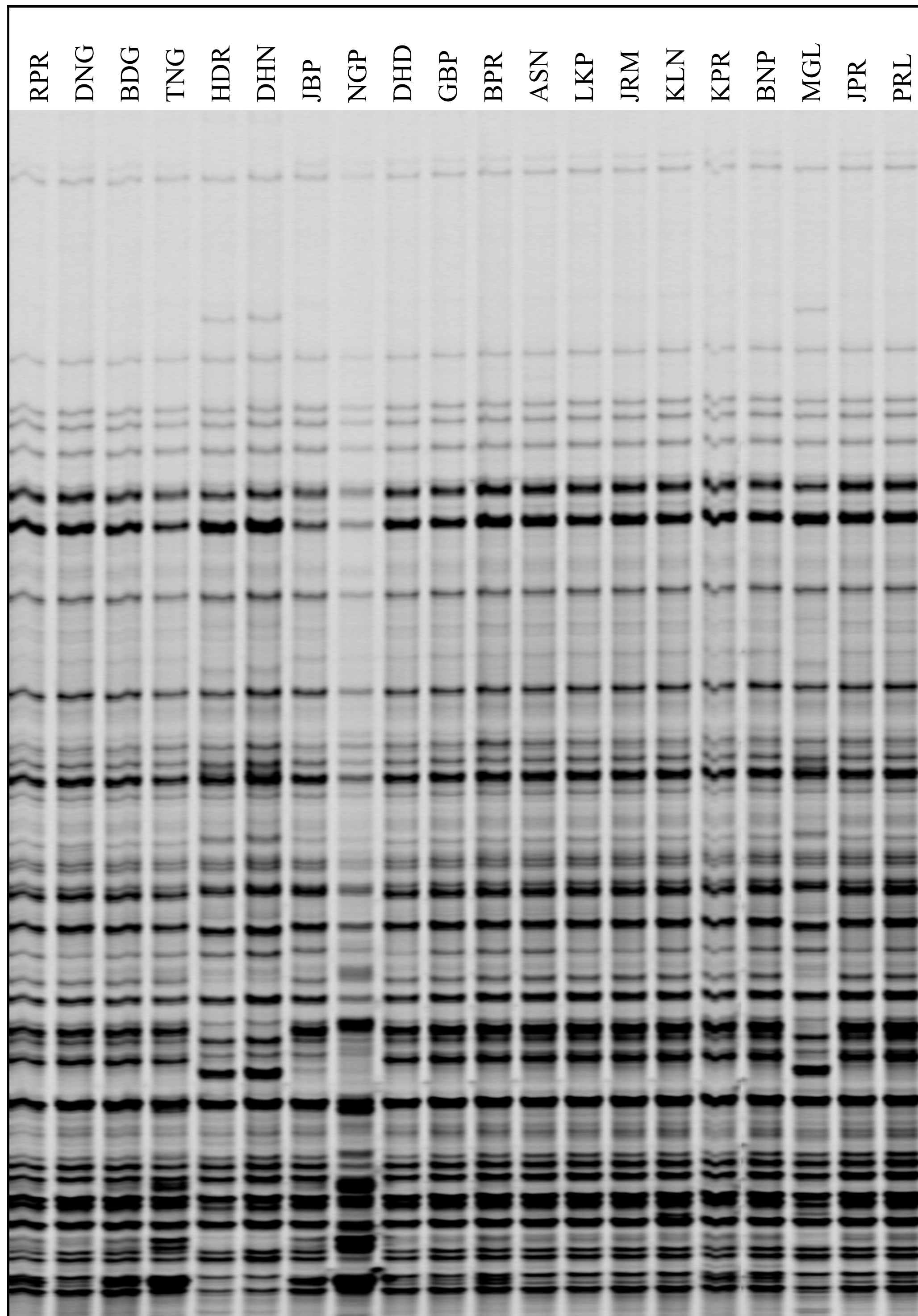


Figure 7.11 DNA Fingerprint (AFLP Primer Pair E-AT/M-CAG)

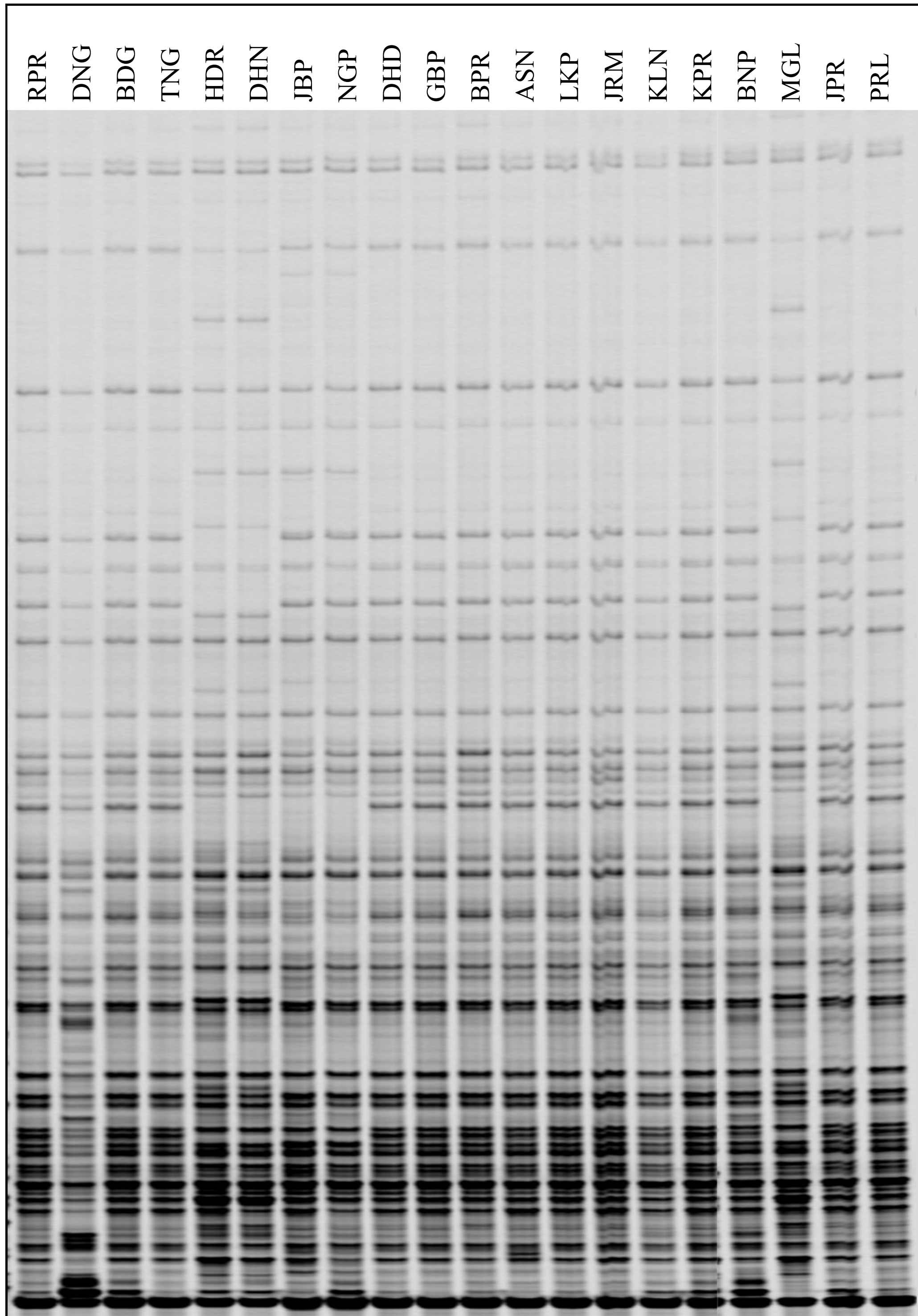


Figure 7.12 DNA Fingerprint (AFLP Primer Pair E-AT/M-CTA)

Table 7.3 AFLP polymorphic bands scored for the primer combinations of pre and selective-amplification

Primer Pairs	Total Band Scored	Polymorphic Bands	Percentage Polymorphism
E-AAC/M-CAG	58	29	50.0
E-AAC/M-CTA	40	19	47.5
E-ACA/M-CAG	50	17	24.0
E-ACA/M-CTA	43	11	25.9
E-ACT/M-CAG	59	15	25.4
E-ACT/M-CTA	53	11	20.8
E-AG/M-CAG	38	11	28.9
E-AG/M-CTA	36	14	38.9
E-AT/M-CAG	31	9	29.0
E-AT/M-CTA	45	14	31.1
Total	453	150	-
Average	45.3	15	33.11
Minimum	31	9	20.8
Maximum	59	29	50.0

The Jaccard's distance matrix obtained for the AFLP scores for the twenty provenances of *S. obtusifolia* are presented in Table 7.4. The resulting genetic distance matrix revealed that the distances vary from 0.00255 to 0.25287. The average variation for all the samples is 0.08651. The minimum genetic divergence occured for Raipur and Bandhavgarh sample pairs with a value of 0.00255. The other two pairs of samples having the next minimum divergence were Lakshmikantapur and Jhargram with a value of 0.00257, and Asansol and Jhargram with a value of 0.00258. The highest genetic divergence occurred for Haridwar and Nagpur sample pairs. Grossly, the distance matrix indicated that relatively very minimum genetic diversity exists among the

provenances of *S. obtusifolia*, though they represent the communities quite distantly placed throughout India.

Table 7.4 Jaccard's distance matrix of AFLP scores

	RPR	DNG	BDG	TNG	HDR	DHN	JBP	NGP	DHD	GBP	BPR	ASN	LKP	JRM	KLN	KPR	BNP	MGL	JPR
DNG	0.04040																		
BDG	0.00255	0.03788																	
TNG	0.01772	0.05263	0.01519																
HDR	0.23341	0.23908	0.23516	0.23516															
DHN	0.22299	0.22864	0.22477	0.23690	0.01302														
JBP	0.09429	0.12315	0.09653	0.11057	0.23310	0.22248													
NGP	0.12195	0.14563	0.11951	0.11951	0.25287	0.24654	0.03125												
DHD	0.02030	0.05038	0.01777	0.03275	0.23394	0.22350	0.09926	0.11302											
GBP	0.01527	0.04545	0.01272	0.02778	0.23799	0.22759	0.10396	0.12225	0.01023										
BPR	0.01018	0.04534	0.00763	0.02273	0.23744	0.22706	0.09901	0.11736	0.01020	0.00512									
ASN	0.02036	0.04557	0.01781	0.03283	0.24256	0.23218	0.10891	0.12714	0.02041	0.01535	0.01531								
LKP	0.01527	0.04051	0.01272	0.02778	0.23799	0.22759	0.10396	0.12225	0.01531	0.01023	0.01020	0.00514							
JRM	0.01781	0.04304	0.01527	0.03030	0.24027	0.22989	0.10644	0.12469	0.01786	0.01279	0.01276	0.00258	0.00257						
KLN	0.02030	0.05038	0.01777	0.03275	0.24201	0.23165	0.10864	0.12683	0.02036	0.01531	0.01527	0.01535	0.01023	0.01279					
KPR	0.01272	0.04293	0.01018	0.02525	0.23165	0.22120	0.10149	0.11980	0.01276	0.00767	0.00765	0.01279	0.00767	0.01023	0.01276				
BNP	0.02015	0.03030	0.01763	0.03250	0.23235	0.22197	0.10319	0.12136	0.02519	0.02020	0.01515	0.02525	0.02020	0.02273	0.02519	0.01768			
MGL	0.22449	0.23409	0.22222	0.22222	0.05290	0.06500	0.22811	0.23973	0.22096	0.22096	0.22449	0.22551	0.22096	0.22323	0.22096	0.22273	0.21946		
JPR	0.01018	0.04040	0.00763	0.02273	0.23744	0.22706	0.10370	0.12195	0.01527	0.01020	0.01018	0.01531	0.01020	0.01276	0.01527	0.00765	0.02015	0.22045	
PRL	0.01018	0.04040	0.00763	0.02273	0.23744	0.22706	0.09901	0.11736	0.01527	0.01020	0.01018	0.01531	0.01020	0.01276	0.01527	0.00765	0.02015	0.22045	0.00510

Further study of the genetic relationship of twenty provenances of *S. obtusifolia* for the hierarchical clustering by using UPGMA is presented in Figure 7.13.

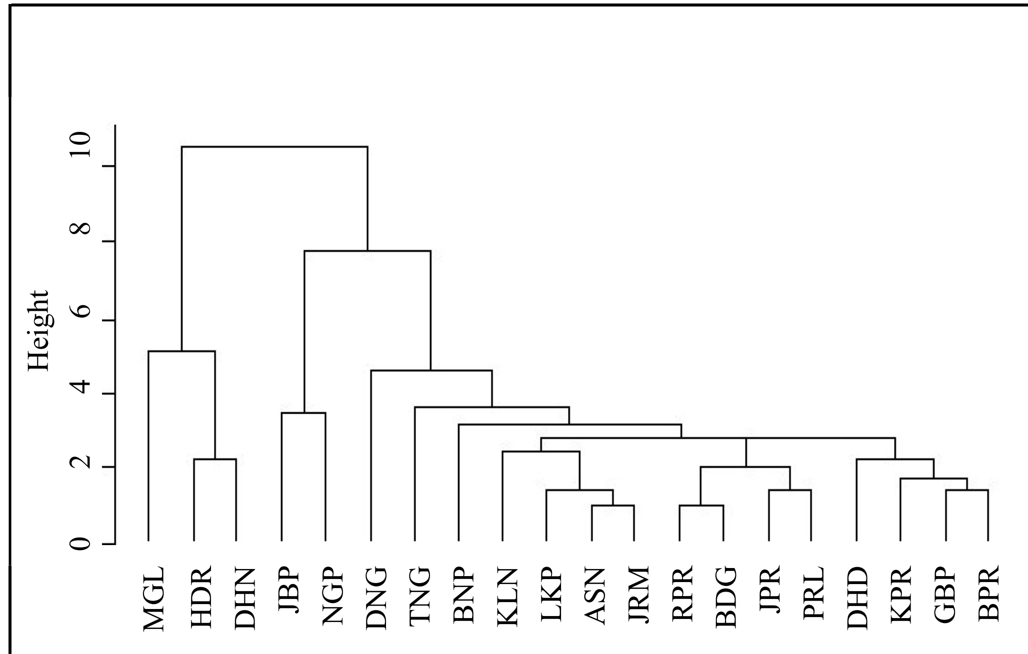


Figure 7.13 UPGMA dendrogram showing the genetic diversities of AFLP fingerprints of 20 accessions

The UPGMA clustering arranged the twenty samples of *S. obtusifolia* primarily into three groups. The first group contains three samples collected from Mangalore (MGL), Haridwar (HDR) and Dehradun (DHN). Out of these three provenances, Haridwar (HDR) and Dehradun (DHN) were noted to be genetically closer than that of Mangalore (MGL). The sample from Mangalore (MGL) marginally differed from the other two. The second group contains the samples from Jabalpur (JBP) and Nagpur (NGP). These two samples had shown no variation. The third group contains the rest fifteen provenances. Interestingly, the third group can further be classified into few more subgroups. The first subgroup contains the sample from Devendranagar (DNG) and the remaining fourteen others as two distinct classes. This implies that the Devendranagar

(DNG) provenance showed marginal variation from the remaining fourteen samples. The next subgroup identifies the sample from Tatanagar (TNG) as slightly different from the remaining thirteen. Similarly, the next subgroup identified the sample from Bishnupur (BNP) as slightly genetically different from the remaining twelve. Finally, these twelve samples are clustered into three more subgroups. The first subgroup contains the provenances of Kalyani (KLN), Lakshmikantapur (LKP), Asansol (ASN) and Jhargram (JRM). Out of these four, the samples from Asansol (ASN) and Jhargram (JRM) are genetically closer than the others. The second subgroup contains the samples collected from Raipur (RPR), Bandhavgarh (BDG), Jaipur (JPR) and Purulia (PRL). Here, the samples from Raipur (RPR) and Bandhavgarh (BDG) lie in close proximity. Also, the samples from Jaipur (JPR) and Purulia (PRL) are genetically similar. However, there is a nominal difference between these two pairs. The last subgroup contains the samples collected from Dahod (DHD), Kharagpur (KPR), Gobindapur (GBP) and Bolpur (BPR). In this subgroup, the Gobindapur (GBP) and Bolpur (BPR) provenances were found to be quite congruent.

7.2.2 Discussions

In the present study 10 different marker combinations with EcoRI/MseI showed a moderate nature of diversity among the different provenances, under study. Though the genetic distance among twenty provenances of *S. obtusifolia*, ranging between 0.00255 and 0.25287 of Jaccard's coefficient show subtle variation amongst them, the detail account of the relationship in the light of UPGMA reveals the relative positions of the provenances based on a genetic attribute. Dendrogram constructed from the data pooled through the analysis of the provenances, grown in the same field having same physical and chemical environment, showed whatever variations, was of permanent nature due to the changes in the genome, accumulated through years.

The use of molecular characterization of genetic elements has been most effectively used in plenty of plant materials with a purpose of understanding the most plausible and stable relationship between them (Mao et al., 2017; Marakli, 2018; Sihanat et al., 2017). Mohanty et al. (2010) rightly delineated 28 wild species under the genus *Cassia*, a very closely related taxon to *Senna*, with the aid of RAPD, ISSR and SSR. Likewise, Mao et al (2017) most effectively identified *S. obtusifolia* and *Senna occidentalis* seeds based on the results obtained with the use of 100 ISSR and 85 SCoT primers. Such information fortifies the utility of the molecular marker AFLP, too, for deriving the genetic relationship between the conspecific as well, as congeneric taxa. Mao et al (2017) could find absolute genetic homogeneity amongst 20 different collections of *S. obtusifolia* and 16 collections of *S. occidentalis* and also quite a fair extent of nearness between the two congeneric species. In the light of such evidences the relationship revealed among twenty provenances of *S. obtusifolia* proves to be the most valued information for understanding diversity in them and also the extent of proximity between provenances. Relationship derived through AFLP study among 20 provenances may be corroborated with other traits of them and the information obtained therefrom may act as a cue to trace difference in morphology and the ability in producing bioactive molecules.