Assessment of genetic diversity within and among populations of *Tylophora rotundifolia* using RAPD markers

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Abstract

Medicinal plants are increasingly endangered. The survival index of a species is greatly determined by the percentage of polymorphism and the gene flow between the populations. Assessing genetic diversity is considered vital for formulating conservation statergies of endangered species such as *Tylophora rotundifolia.* RAPD was employed to assess genetic diversity. Nei's gene diversity (H_E) ranged from 0.1778 to 0.5 and the average expected heterozygosity (H_E) was 0.2643. The mean Shannon indices (*Ho*) was 0.3985. Principal coordinate analysis shows the first two components account for 61.17% of the total variation. The average percentage of polymorphic bands is 46% and Polymorphism Information Content was 0.57. The data indicate a restricted gene flow among populations and calls for immediae conservation measures.

Key words: conservation, genetic diversity, RAPD markers, *Tylophora rotundifolia*

Introduction

Tylophora rotundifolia Ham.ex Wight is an important indigenous medicinal plant found in restricted localities (Shoolpaneshwer sanctuary) in South Gujarat, India (Fig.1). It is a twining perennial, mostly unbranched, hairy herb with coriaceous, broadly ovate or subrotund, and petiolate leaves. It produces numerous flowers from January to July and long, ovate seeds (Cooke, 1908; Shah, 1978; Almeida, 2001).The tribal medicinal men of Gujarat have been using *Tylophora rotundifolia* root extracts for chest pain, indigestion and as an emetic for insect bite.

The said species do not have a scattered population and are listed as endangered. It is over exploited for its medicinal value to an extent that now it can be found in restricted regions in the inner recesses of Shoolpaneshwer sanctuary in South Gujarat (D'Cruz, 2003). The loss of one plant species means its depletion from the biological Gene Bank (Haas, 2008).

The main goal of biodiversity studies is the preservation of existing genetic diversity. There could possibly be a genetic basis for the endangered nature if a species has very low allele frequency. Survival of a species depends on the maintenance of genetic variability within and among populations that accommodate new selection pressures brought about by environmental changes. It is very important to understand the complex processes involved in the long term evolutionary history of species such as genetic drift, mutation, gene flow within the populations (Jayanti and Mandal, 2001; Faisal *et al*.,

2007). Restricted populations have lower genetic diversity than widespread species. The species under study is threatened possibly by the increase of agriculture, cattle grazing and ethnomedicinal usage. This may suggest a species-specific reason for the reduced diversity in small populations of *Tylophora rotundifolia*. Information on how genetic variation is distributed among the existing population of an endangered species can be used in designing recovery programmes as it is an irreplaceable resource. Moreover, opting for conservation measures depends on their economic and medicinal importance to individuals, a particular community or society at large. Hence protecting an endangered species such as *Tylophora rotundifolia* becomes an individual conservation priority as well.

At present, no study has been carried out to assess the genetic diversity and to correlate it with the existing endangered nature of *Tylophora rotundifolia*. Keeping this in mind, the present study was undertaken with the objectives of locating, collecting and generating species-specific fingerprints for *Tylophora rotundifolia* and to assess genetic diversity expressed as percentage polymorphism and evaluate significant variations within and among different subpopulations using RAPD. Moreover, the data could be used to examine genetic relationship (identity and distance) within and among populations and correlate this data with the threatened status of the species under study. Further this could be used to suggest management choices for efficient conservation that could help in maintaining genetic variation within the species of *Tylophora rotundifolia* using the baseline information generated.

Materials and Methods

Collection of plant materials

The study area was visited regularly during the monsoon season and plant materials have been identified and verified with the help of taxonomists. Tender leaves of *Tylophora rotundifolia* were collected exclusively from 12 locations from the Shoolpaneshwar sanctuary of the Dediapada forests in South Gujarat (Table 1). If more than one sample was collected from a site, it was treated as a single accession for analysis. Care has been taken to maintain a geographical distance of about 5-8 kilometres between each collection site in order to reduce genetic similarity in accessions and to minimize sampling error while interpreting the data.

DNA isolation and primer screening

Extraction of total genomic DNA was carried out from 100 mgs of tender, fresh leaves using the Nucleon PhytoPure DNA extraction kit (GE Healthcare), following the manufacturer's instructions. The extracted DNA was quantified spectrophotometrically and the DNA pellets were stored at 4°C until further analysis.

PCR and agarose gel electrophoresis

RAPD-PCR reactions were carried out using the Ready-To-Go RAPD analysis beads (GE Healthcare) which are room temperature stable beads optimized for RAPD

reactions. Each bead contains thermostable polymerases (AmpliTaq™ DNA polymerase and Stoffel fragment), dNTPs (0.4 mM each dNTP), BSA (2.5 µg) and buffer (3mM Magnesium Chloride, 30mM Potassium Chloride, and 10 mM Tris (pH 8.3) in a 25 µl reaction volume. To this 25 pmol of a single RAPD primer and 50 ng of template DNA were added. The 20 primers were selected from the OPI RAPD primer kit (Qiagen Operon, Germany). Amplification was carried out on a Techne Master thermocycler, Ver B. 1.7. The machine was programmed to 1 cycle of 5 minutes of initial denaturation at 94°C. This was followed by 40 cycles of 1 minute at 94°C for denaturation, 1 min at 36°C for annealing, and 2 min at 72°C for extension. This was then followed by a final extension at 72°C for 7 min. The amplification products were separated by electrophoresis in 1.6% (w/v) agarose gels with 0.5X TBE buffer, stained by 0.5 µg/ml of ethidium bromide and photographed under exposure to UV light using a digital camera. A 100 bp DNA ladder (GE Healthcare, 27-4007-01) was also loaded on one side of the gel as a band size standard. PCR-RAPD analysis was repeated at least three times and only the primers producing strong and reproducible bands were used in the analysis.

Data analysis

The RAPD data were analyzed using NTSYS-PC (version 2.02) (Numerical Taxonomy and Multivariate Analysis System) computer package (Rolf, 1998). Each RAPD fragments was treated as a unit character and was scored presence or absence of the band (1 or 0). The 1/0 matrix was prepared for all fragments scored and the data were used to generate Jaccard's similarity coefficients for RAPD bands (Jaccard, 1908). The

Jaccard's coefficients were subjected to unweighted pair-group method using arithmetical averages (UPGMA) to generate a dendrogram using linkage procedure. The Jaccard's similarity matrix was then used as the basis for ordination by Principal Coordinates Analysis (PCoA), which was performed to show the distribution of the genotypes in a scatter plot using the software MVSP version 3.13n (Multivariate Statistical Package; http://www.kovcomp.com/mvsp). Polymorphism Information Content (PIC) for each RAPD locus was calculated based on the number of bands/primer, as described by Weir, using the formula PIC = $1\text{-}Pi^2$, where *Pi* is the frequency of the *i*th band in the genotype examined (Weir, 1990). PIC compares the polymorphism levels across markers and is used to determine the usefulness of markers for specific studies.

Two other softwares such as Popgene (Yea, 1999) and TFPGA (Tools for Population Genetic Analysis) V.1.7 (Miller, 1997) designed for dominant markers like RAPD were also used to analyse the data. Inorder to estimate the genetic variation within and among populations of *T. rotundifolia* the Shannon index was calculated using the formula $Ho = -\Sigma pi \log_2 pi$ (Lewontin, 1972), where *H*o is diversity and *pi* is the frequency of a particular RAPD band. It must be noted that heterozygous individuals cannot be detected directly by RAPD as RAPD data is dominant data. However, the Shannon index is suitable for RAPD data as it is insensitive to this bias (Meekins, 2001; Tsuda *et al*., 2004). Various measures of heterozygosity were also calculated. Total heterozygosity, $H_T = 2qi\ (1\text{-}qi) + \text{Var}\ (qi)$ where *qi* is the frequency of the null allele at *i*th locus in a population. H_T was calculated with Lynch and Milligan's correction (Lynch and Milligan, 1994). Mean heterozygosity within population (*Hs*), diversity among

populations ($D_{ST} = H_T - H_S$) and the coefficient of population differentiation ($G_{ST} = D_{ST}/H_T$) were also calculted.

Result

All ambiguous RAPD bands were excluded from scoring in order to avoid fragments that could be artefact. Several polymorphic bands were observed for each primer in the range of 350-3300 bp. A total of 152 bands were scored for the 20 RAPD primers out of which 73 bands were polymorphic. The number of bands per primer ranged between 4 to13 with a mean of 7.6. Though the percentage of polymorphic bands for each primer ranged from 11 to 86%, the average polymorphism was 46%. The average polymorphic information content (PIC) was 0.7283 (Table 2). Representative RAPD patterns generated by primers OPI 1,2,11,6,7 and OPI 16 are shown in Figure 2(a-e). The pairwise Jaccard's coefficients genetic similarity matrix was prepared on the basis of RAPD data. The genetic similarity coefficients among all 12 populations varied from 0.61 (between zones 1 and 12) to 0.91 (between zones 4 and 5; Table 3).

Nei's gene diversity (H_E) was calculated using Popgene. It ranged from 0.1778 to 0.5 and the average proportion of loci expected to be heterozygous was 0.2643. Shannon indices (H_o) were also calculated to find genetic variation within population. It ranged from 0.1408 to 0.6862 and the mean was 0.3985 with S. D. of 0.2622.

To understand overall genetic relationships among *Tylophora rotundifolia* populations, a cluster analysis was carried out based on similarity coefficients generated from 152 RAPD bands using UPGMA. This was further used for developing a dendrogram (Fig. 3). Though all accessions could be grouped into one cluster at 65%, two major clusters were detected at 83% similarity level. The first cluster contained accessions 2, 3, 4, 5, 8 (Zhadoli, Gadad Ghat, Panya Ghat, Chopadi and Gichad) and the second cluster has accessions 6, 7 (Duthor, Dumkhal). The UPGMA based cluster analysis is a good indication of the genetic relationship existing among the populations of *Tylophora rotundifolia.* Moreover, decline in the effective allele frequency expose the threatened nature of the species.

In order to determine the genetic relationships among sample collection zones, a PCoA analysis based on the Euclidean matrix was carried out. The first (Eigen value = 1.021, percentage of variance = 33.26) and second (Eigen value = 0.4, percentage of variance = 13.04) axis with a cumulative variance of 46.3% was observed. Plots of the first two coordinates are used for generating a PCoA graph (Fig. 4). Ewens-Watterson test for Neutrality showed that except four, all loci are neutral ($p > 0.01$). The PCoA analysis is a good indication of the genetic relationships among the species and the neutrality test depicts the vulnerability of the species in the existing environmental stress conditions.

Discussion

Genetic diversity of endangered species has always enthused evolutionary and conservation biologists. The ability of a species to adapt to environmental changes depends greatly on the genetic diversity in the species (Neel and Ellstrand, 2003; Anand *et al.,* 2004). Narrowing of gene pool and reduced genetic diversity pose challenges in the selection pressure brought in by environmental changes (Caro and Laurenson, 1994). The use of RAPD technique to detect genetic variation at the level of DNA was found to be sensitive and powerful in *Tylophora rotundifolia*. The low genetic diversity observed in *Tylophora rotundifolia* may be reflecting the survival pressure the species is facing currently. The average polymorphism information content (PIC) value of 0.7283 corroborates the endangered nature of this species (Cuc *et al.*, 2008).

Analysis shows that *Tylophora rotundifolia* had a low level of heterozygosity. The average Nei's gene diversity (H_E) of 0.2643 and mean Shannon indices (H_o) of 0.3985 indicate a low genetic variation within the population. The observed isolation of population to patches in the Shoolpaneshwar sanctuary is a significant prediction of heterozygosity. The observed [1.7895 (S. D. = 0.4189)] and the effective allele frequency [1.4512 (S. D. = 0.3812)] was low, which further support a low genetic variation within the species. Such a narrow variation can be detrimental to the sustainability of the species. Pairwise similarities estimates based on Jaccard's coefficient (ranging from 0.61 to 0.91) is highly significant with regard to genetic diversity among various populations of *Tylophora rotundifolia*.

The RAPD data showed close linkage between the populations of 2, 3, 4, 5, 8 (Zhadoli, Gadad Ghat, Panya Ghat, Chopadi and Gichad) and populations of 6 and 7 (Duthor, Dumkhal). The high pairwise similarity and low gene diversity suggests a limited genetic variation (Archak *et al*., 2002) in the *Tylophora rotundifolia* population.

Existence of low genetic diversity within species has been mostly attributed to self pollination, unless other environmental pressures are influencing genetic diversity (Archak *et al.,* 2002). The basis of RAPD based genetic diversity is high mutation, inversion, deletion and near neutrality of alleles. RAPD polymorphism should be governed by mutation drift balance. Small populations with low gene diversity will be affected much by lack of gene flow, limited gene drift and reduced mutation rate. Therefore, a high genetic diversity further supports greater differentiation (Nagylaki, 1998; Anand *et at.*, 2004). The low levels of differentiation in *Tylophora rotundifolia* may be attributed to a common genetic pool, lack of differential selection pressure, diminished mutation rate and short life span of the species. More genetic diversity could result from higher cross pollination and higher effective population size. Increased effective population size may result from higher densities of *Tylophora rotundifolia* in the Shoolpaneshwar region. However, this is not possible since the species survives for about 8 months and is completely wiped out by the end of August.

Conclusion

Human activities and digging of the plant out for making medicines, agriculture and cattle grazing, lack of pollinating agents can damage the habitats and reduce it to

island-like distributions (Belletti *et al.,* 2008). If the area of population is large, the probability of crossing among the individual plants increases which results in the retention of genetic variation. Additionally, current gene flow through pollen or seed movement among the clonal populations may prevent differentiation due to reduced population size. If RAPD diversity is a reliable indicator of population variability, then anthropogenic fragmentation may lead to an increased extinction risk for *Tylophora rotundifolia*. Therefore awareness programmes should be conducted on a war footing and adequate species propagation should be carried out to save this species from extinction. Though many individuals of the species have been recorded in the earlier studies, they have disappeared gradually along with environmental changes in their habitat and are presently restricted to isolated patches in the interiors of the Shoolpaneshwar sanctuary. Further, the study implies that more variation needs to be introduced in the existing population for species persistence. Therefore, the immediate approach and need of the hour may be to preserve the local genotypes by collecting seeds from representative ecotypes, introducing more of micropropagated plants with variation to increase the actual population and allow cross breeding.

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Table 1. Location of collection of plant material.

Code no	***Tylophora rotundifolia***
1	Hinalo
2	Zhadoli
3	Gadad ghat
4	Panya ghat
5	Chopadi
6	Duthor
7	Dumkhal
8	Gichad
9	Gangapur
10	Mathasar
11	Morjadi
12	Namgir

Table 2. Primers with their sequences used for RAPD analysis of *Tylophora rotundifolia*, and the total number of bands, polymorphic amplification products, percentage of polymorphism yielded by each primer and polymorphism information content (PIC).

Primer	Sequence 5' to 3'	Mol. wt range (bp)	Polymorphic bands	Total bands	% Polymorphism	PIC
OPI-01	ACCTGGACAC	3000 -900	1	5	20	0.9375
OPI-02	GGAGGAGAGG	2200 - 600	2	7	28.57	0.4375
OPI-03	CAGAAGCCCA	1600 - 400	1	9	11.11	0.75
OPI-04	CCGCCTAGTC	3000 - 800	3	8	37.50	0.6875
OPI-05	TGTTCCACGG	3300 - 700	6	8	75.00	0.825
OPI-06	AAGGCGGCAG	1400 - 600	2	8	25.00	0.5937
OPI-07	CAGCGACAAG	1200 - 400	2	7	28.57	0.6875
OPI-08	TTTGCCCGGT	1600 - 800	4	5	80.00	0.8125
OPI-09	TGGAGAGCAG	1400 - 400	3	9	33.33	0.7083
OPI-10	ACAACGCGAG	1800 - 400	8	10	80.00	0.6796
OPI-11	ACATGCCGTG	1400 - 550	3	7	42.86	0.6458
OPI-12	AGAGGGCACA	1400 - 550	2	6	33.33	0.8437
OPI-13	CTGGGGCTGA	2000 - 550	5	6	83.33	0.5
OPI-14	TGACGGCGGT	1800 - 550	1	4	25.00	0.75
OPI-15	TCATCCGAGG	3000 - 900	6	7	85.71	0.8125
OPI-16	TCTCCGCCCT	2000 -500	3	8	37.50	0.9375
OPI-17	GGTGGTGATG	1800 -350	2	10	20.00	0.5937
OPI-18	TGCCCAGCCT	2500 - 500	7	11	63.64	0.7321
OPI-19	AATGCGGGAG	3300 - 400	11	13	84.62	0.6953
OPI-20	AAAGTGCGGG	1200 - 600	1	4	25.00	0.9375
		Total	**73**	**152**	**920.08**	**14.5672**
		mean/primer		**7.6**		
		Average Polymorphism			**46.%**	**0.7283**

Table 3. Similarity matrix for Jaccard's coefficient for *Tylophora rotundifolia*: range of values from 0 to 1.0 indicating increasing similarity. Numbers (1-12) in the table represent location listed in Table 1.

	1	2	3	4	5	6	7	8	9	10	11	12
1	1.00											
2	0.69	1.00										
3	0.79	0.83	1.00									
4	0.78	0.86	0.85	1.00								
5	0.76	0.85	0.85	0.91	1.00							
6	0.69	0.80	0.76	0.79	0.79	1.00						
7	0.75	0.72	0.78	0.77	0.79	0.86	1.00					
8	0.84	0.78	0.85	0.83	0.83	0.74	0.78	1.00				
9	0.66	0.71	0.67	0.75	0.72	0.66	0.68	0.72	1.00			
10	0.63	0.64	0.66	0.68	0.70	0.67	0.64	0.63	0.67	1.00		
11	0.70	0.66	0.68	0.69	0.67	0.64	0.64	0.74	0.76	0.64	1.00	
12	0.61	0.75	0.65	0.70	0.71	0.68	0.64	0.68	0.76	0.62	0.78	1.00

Figure legends

Figure 1. Shoolpaneshwer sanctuary located in the south of Gujarat, India.

Figure 2.a. RAPD profile generated for four *T. rotundifolia* accessions using primer OPI-1 (lane 1, 2, 3, 4) and with OPI-2 (lane 5, 6, 7, 8).

Figure 2.b. RAPD profile generated for eight *T. rotundifolia* accessions using primer OPI-11. Arrows indicate some of the polymorphic bands scored.

Figure.2.c. RAPD profile generated for four *T. rotundifolia* accessions using primer OPI-6 (lane 1,2,3,4). Left most lane is 100bp ladder.

Figure.2.d. RAPD profile generated for four *T. rotundifolia* accessions using primer OPI-7 (lane 1,2,3,4). Left most lane is 100bp ladder.

Figure.2.e. RAPD profile generated for four *T. rotundifolia* accessions using primer OPI-16 (lane 1,2,3,4). Left most lane is 100bp ladder.

Figure. 3. Average linkage cluster analysis of 12 accessions of *T. rotundifolia* using RAPD data from a 12x12 matrix of similarity indices. The bar on the top represents similarity index based on Jaccard's coeficients. Numbers indicates the code of collection site.

Figure. 4. Principal coordinate analysis of 12 accessions of *T. rotundifolia* based on Jaccard's similarity matrix. Numbers in the plot represent locations listed in Table 1.

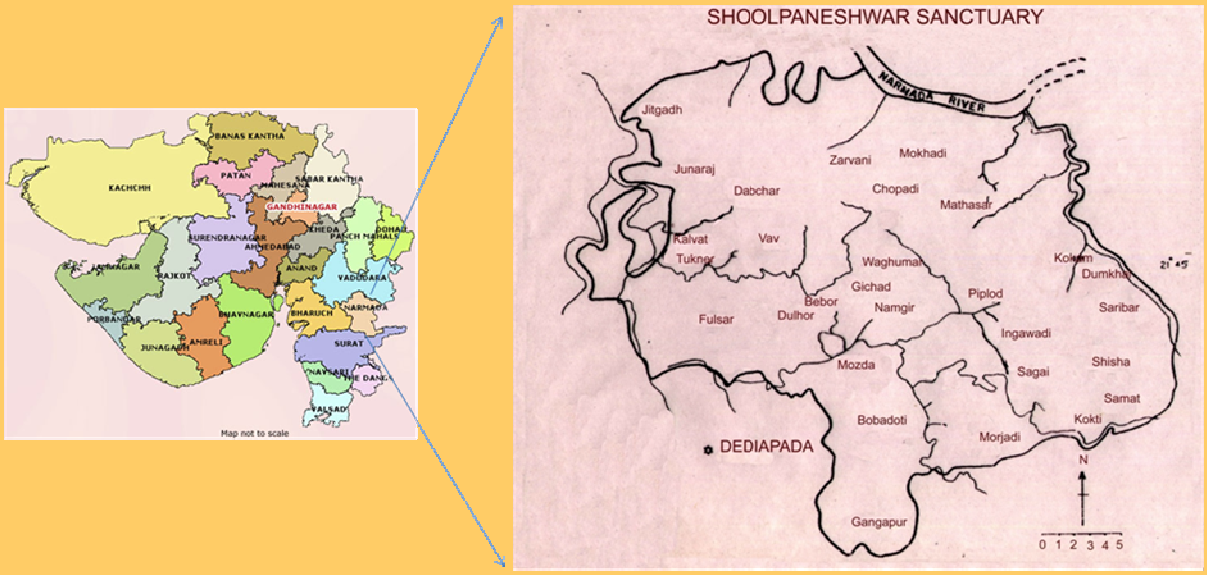


Figure 1. Sebastian *et al*. 2010

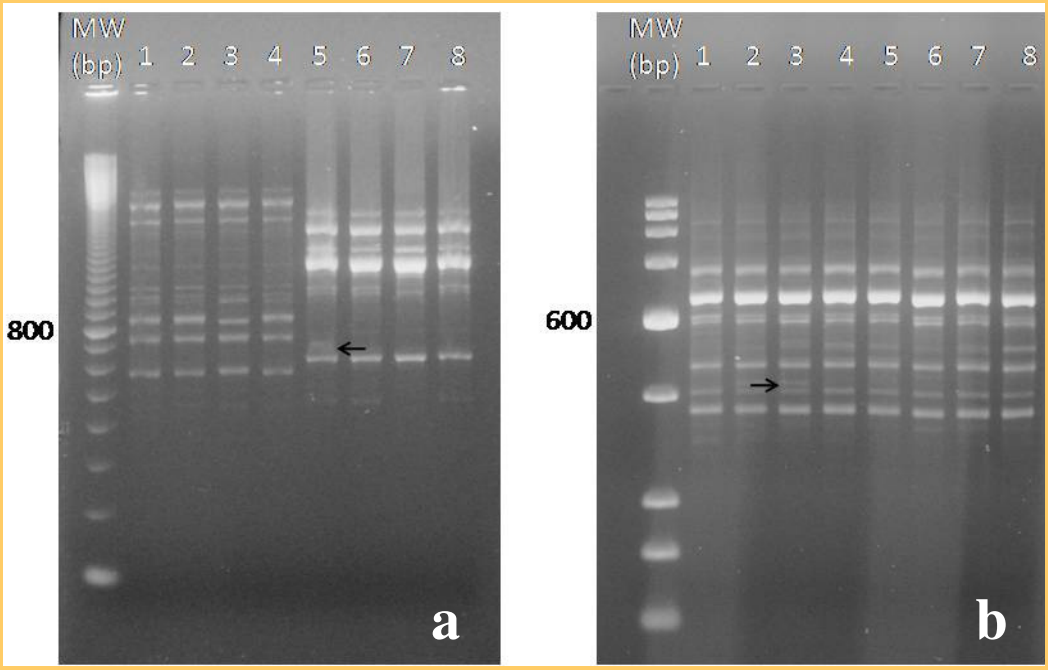
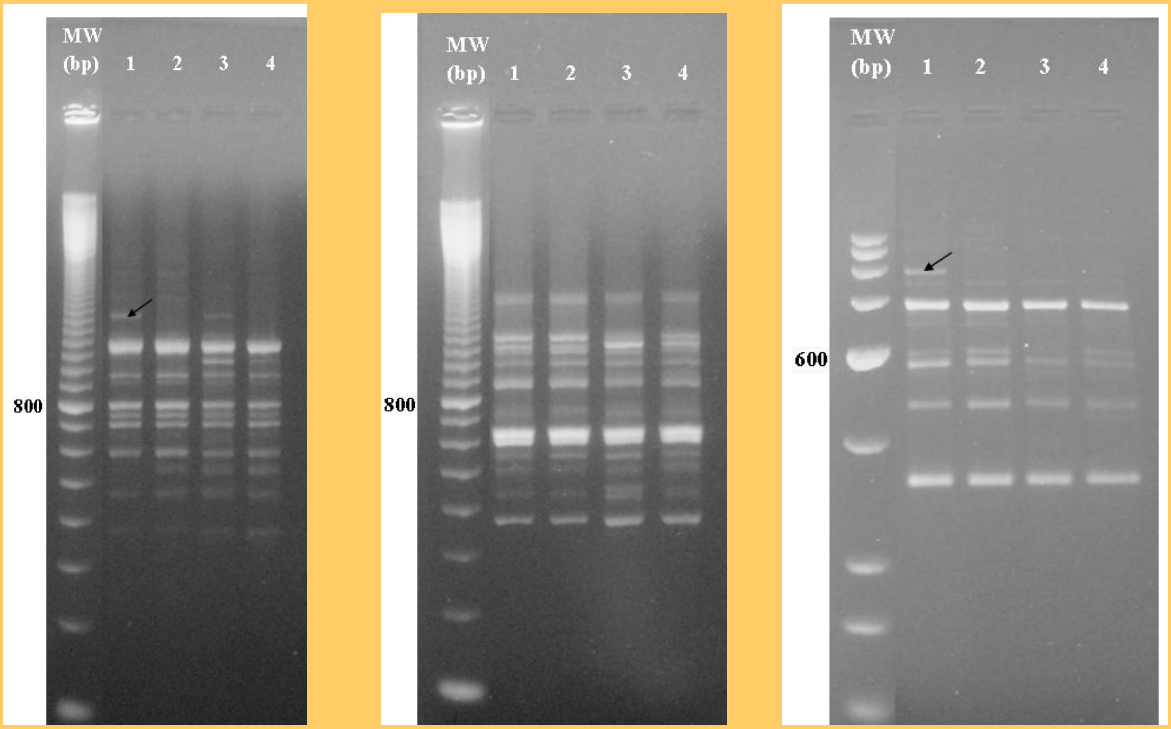


Figure 2.a,b. Sebastian *et al*. 2010



(c) **(d)** **(e)**

Figure 2.c,d,e. Sebastian *et al*. 2010

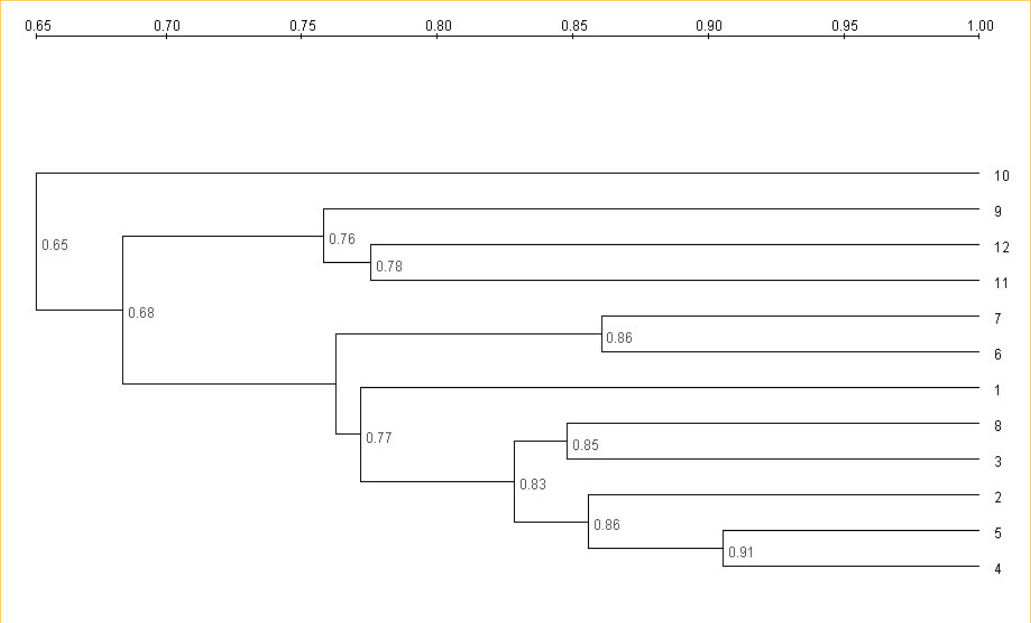


Figure 3. Sebastian *et al*. 2010

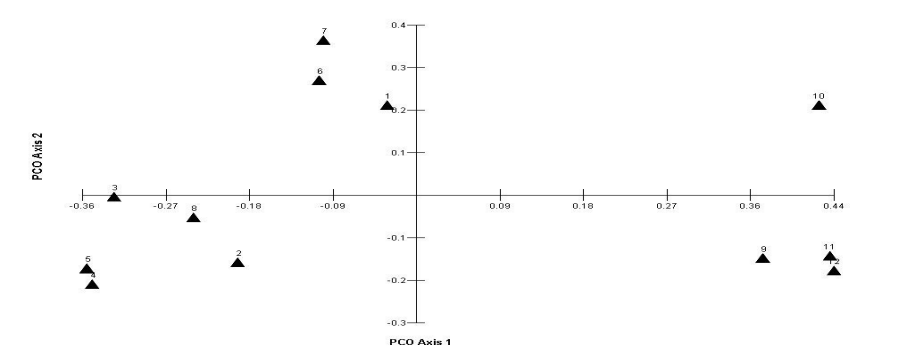


Figure 4. Sebastian *et al*. 2010