Current Trends in Biotechnology and Pharmacy
Vol. 10 (1) 5-12 January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

Genetic Polymorphism of *Tylophora* Species of Goa as Revealed by RAPD

Uma Masur[1*], P. Hima Kumari[2], Kailas Gokhale[1] and U.M.X. Sangodkar[3]
[1]Parvatibai Chowgule College of Arts and Science, Gogol, Margao-Goa-403602, India
[2]Department of Genetics, Osmania University, Hyderabad-500 007, India
[3]Department of Marine Biotechnology, Goa University, Bambolim, Panajim, Goa, India
*For Correspondence - umapatil20@yahoo.co.in

Abstract

Tylophora indica is an important medicinal plant and is used against dysentery, diarrhoea, humoral and bronchial asthma, allergic rhinitis, cold and cough. The plant is also known for anticancer properties. Other species of *Tylophora* predominantly found in Goa is *T. dalzelli*. It has been reported as a local herb for the treatment of asthma. Hence the main objective of this study is to know the genetic relationship in these two species. To achieve this, randomly amplified polymorphic DNA (RAPD) was performed. A total of six *Tylophora* variants were collected from various regions of Goa and analyzed using RAPD with OPF 1-10 primers. Genetic similarities were determined between and within the collected species through Jaccard's coefficient system. RAPD primers generated 39 amplification products, of which all 39 were polymorphic. Results from cluster analysis and linkage distance, indicate 38% genetic similarity at the interspecifc level between *T. indica* whereas 10% between two *T. dalzelli* variants. UPGMA cluster analysis revealed similar results.Our study indicated that *T. indica* collected from Valpoi was the most diverse one among all the samples.These results based on the molecular markers have shown high genetic diversity among thecollected species.

Keywords: *Tylophora indica*, *Tylophora dalzelli*, RAPD, medicinal plant, genetic polymorphism.

Introduction

Tylophora indica belongs to the family Asclepiadaceae. It consists of 130 genera and 2000 species. *T. indica* was studied extensively for its antiasthmatic and allergic rhinitis problems (1). *Tylophora indica* is one of the herbs, and mostly used against asthma. Pharmacological investigations have also confirmed the anti-asthmatic properties of its leaf extract (1). The major alkaloid tylophorine has been reported to have immunosuppressive, anti-inflammatory effect (2). The leaf and stem extract having tylophoridine are known to possess anti-leukemic properties also (3). Thus, the plant has been reported to be anti-asthmatic, anti-allergic, hepato protective and immuno modulator (4-5). Significant activity of the extracts of stem and leaf were observed against two standard transplantable tumors, lymphoid leukemia L1210 and lymhocytic leukemiaP388 (6). Hence, there is already a concern about over exploitation and decline in the wild population of *T. indica* species (7). A closely related species is *T. dalzelli*, which is commonly found as a roadside weed. *T. dalzelli* has been reported to be used as a local herb for the treatment of asthma in Goa (8,12). *T. dalzelli* with more or less has same pharmacological properties but the genetic relationship of these two species is not known.

DNA-based molecular markers have been proposed as an excellent approach for identifying geographical variation, genetic diversity, phylogenetic relationship, authentication of plant

species, pharmacognostic characterization, species characterization and genetic mapping in medicinal plants (9). Accordingly, the RAPD work carried out on three different species belonging to the family Asclepiadaceae for comparative analysis gave an important clue about the genetic diversity as well as close affinities (10). Similarly, RAPD and ISSRcarried out on *in vitro* generated microshoot through somatic embryogenesis of *Tylophora* showed genomic bands like that of the mother plant signifying uniformity and resemblance to the mother plant (11). Though pharmacognostic studies on *T. dalzelli* were carried out (13) there was no literature on the comparative analysis of secondary metabolites present and their use to treat asthma and other diseases.In the present study, random amplified polymorphic DNA (RAPD) was performed on *T. indica* and *T. dalzelli* species to know their genetic diversity and to determine whether *T. dalzelli* could be also used as a medicinal plant for treating asthma.

Materials and Methods

***Plant collection*:** Six species of *Tylophora* used in this study were collected from five different populations belonging to five different geographical areas of North Goa (NG) and South of Goa (SG), like Pernem (P), Rivona (R), Valpoi (V), Nuvem (C) and Margao (M) (Table 1) and were grown ex-situ. Geographical data of the collection points was identified (Table 1) (14). The plants were brought to the lab andidentified with the help ofThe Flora of the Presidency of Bombay and Flora of Goa, Diu, Daman, Dadra and Nagarhaveli (15- 16). They were also confirmed at the Botany Department, Goa University, Goa. Leaves were harvested after one month and transported to the Plant Genetics Laboratory, Department of Genetics, Osmania University, Hyderabad, for DNA isolation and RAPD analysis.

***Isolation of genomic DNA*:**The plant genomic DNA was isolated according to the protocol of Doyle and Doyle (1987) (17). About 1 g of young leaf tissue was homogenized in liquid nitrogen and the powder was mixed in 5 ml of pre-heated (65^0C) CTAB buffer containing 1% polyvinyl pyrrolidine (PVP) and 0.2% â-mercaptoethanol in 50 ml capacity polypropylene tubes and incubated at 65 ^{0}C in a water bath for 1 h with occasional, gentle swirling. Later, equal volumes of chloroform: isoamyl alcohol (24:1) was added to the samples. The tubes were gently shaken by inverting few times. Centrifugation was carried out at 10,000 rpm for 5 min and the aqueous phase was carefully transferred into a fresh centrifuge tube and mixed well with 0.6 volumes of ice coldisopropanol by gently inverting the tube. This mixture was incubated at -20 ^{0}C for 1 h to allow complete precipitation of DNA and then centrifuged at 12,000 rpm for 10 min to pellet the DNA. The pellet was washed with wash buffer (70% v/v ethanol) and air-dried. Finally, the DNA was dissolved in 100 µl of TE buffer. The quality and concentration of extracted DNA was determined by measuring the absorbance at 260 nm and 280 nm using UV visible spectrophotometer (Thermo scientific). Isolated genomic DNA was run on 1% agarose gel after treatment with RNase A for determining the quality and quantity of DNA.

***PCR and RAPD analysis*:** RAPD was performed for the amplification of target genomic DNA fragments. A total volume of 25 ìl of PCR mix was prepared in a sterile 0.2 ml eppendorf tube with 10 pmol/ìl each of both forward and reverse primers (Table 2), 1 ìl of DNA as a template, 50 µM of each dNTP, 1.5 mM $MgCl_2$ and 1 U of TaqDNA Polymerase (Bangalore Genei). Each PCR aliquot was mixed and the PCR reactions were performed. The standard reaction conditions carried out are given in Table 3. An aliquot from the amplified PCR product was used to analyze on 1% agarose gel and to check the amplification.

Data analysis: RAPD generated DNA banding patterns were converted to binary matrix by assigning 1 for presence of band and 0 for its absence by using multivariate analysis program NTSYS-PC (18). Genetic similarity was calculated with the help of Jaccard's coefficient

Vol. 10 (1) 5-12 January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

of similarity and a phylogenetic dendrogram was constructed by using unweighted pair group with arithmetic mean (UPGMA) taking the help of R-program.

Results

***Plant collection*:** Collected plants from different regions of Goa were identified as *T. indica* and *T. dalzelli*.*Tylophoraindica* (Burm.f.)Merr.Or Indian Ipecac commonly known as Antamul (Marathi), Pitvel (Konkani) is aperennial, woody liana, terrestrial and mesophytic in habitat. *Tylophora dalzellii* Hook.f. known as Dalzell Ipecac, commonly known as Lhan Pitmari in Marathi. It is a twine with terrestrial and mesophyte habitat. Out of six variants collected, four were identified as *Tylophora indica* obtained from Pernem, Margao,Valpoi and Rivona whereas two of them as *Tylophora dalzelli* collected from Rivona and Nuvem. Morphological variations were seen within *Tylophora indica* species obtained from Valpoi, with considerable differences in leaf morphology. They were labeled as *T. indica*

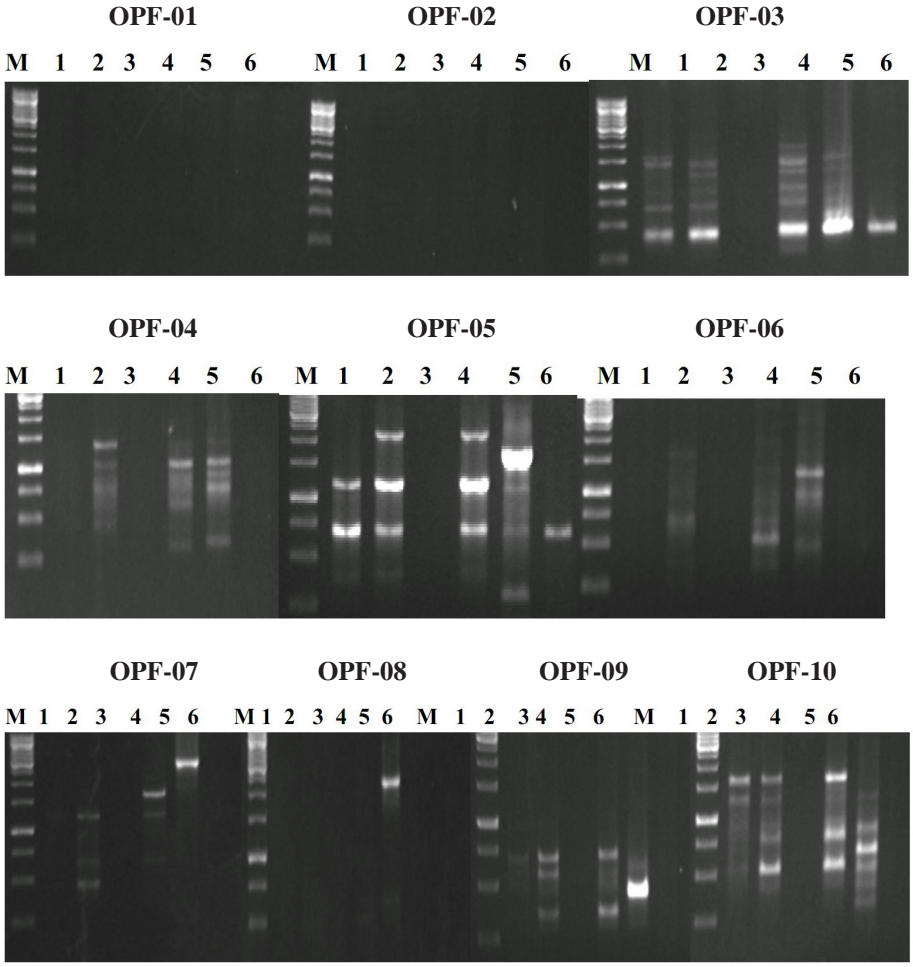

Fig. 1. RAPD PCR with OPF 1-10 primers. M = 1Kb DNA molecular weight marker, Lane 1 = TiP, Lane 2 = TiR, Lane 3 = TiV, Lane 4 = TiM, Lane 5 = TdR,Lane 6 = TdN.

(TiP); *T. indica* (TiM); *T. indica* (TiV); *T. indica* (TiR); *T. dalzelli* (TdR); *T. dalzelli* (TdN).

***PCR and RAPD analysis*:** Quality of genomic DNA was checked using UV-Visible spectrophotometer at 260/280 nm and electrophoresed on 1%agarose gel. Using genomic DNA as a template, amplification was carried out with different RAPD primers of OPF series. Amplified products were confirmed by running them on 1% agarose in 0.5X TAE at constant voltage. Out of 10 OPF primers, DNA of all the *Tylophora* variants was amplified with 8 OPF primers except TiV. OPF-1 and OPF-2 have not shown any bands; whereas OPF-3 to OPF-10 haveexhibited 39 bands varying from 40 to 3000 bp. On an average, each primer has generated 4.875 bands. However, OPF-10 has generated a maximum of 8 bands ranging from 350-1600 bp and OPF-8 has given minimum 1 band of 2700 bp (Table 3; Fig. 1). It is also observed that 100% bands generated by these primers are polymorphic. Similarity indices were developed on the basis of amplified products of 10 RAPD primers with the six variants (Table 4). Genetic similarities calculated using Jaccard's coefficient ranged from 0 to 0.40, its mean and standard deviation (SD) has been found to be 0.28 and 0.34 respectively. UPGMA cluster analysis for the study revealed similar results as that of similarity coefficients with TiM and TiR exhibiting 40% similarity. Variant TiV was the most diverse one among all the samples of *Tylophora*. UPGMA cluster analysis revealed a clear picture (Fig. 2) of all the samples.

Discussion

Molecular screening has become the mandatory technique for screening species/ variants to study their genetic differences, determine authenticity, and to find out phylogenetic relationship of many plants (19) differing in their geographical location. It is possible to resolve the genetic polymorphism if any among different species and variants using RAPD technique. Earlier,genetic diversity analysis among 15 barley landraces was carried out using the same technique (20). Also, cultivars of Tunisian pomegranate were investigated for genetic diversity using universal primers by RAPD (21). The technique is also used to resolve issues regarding medicinal plants. Cluster

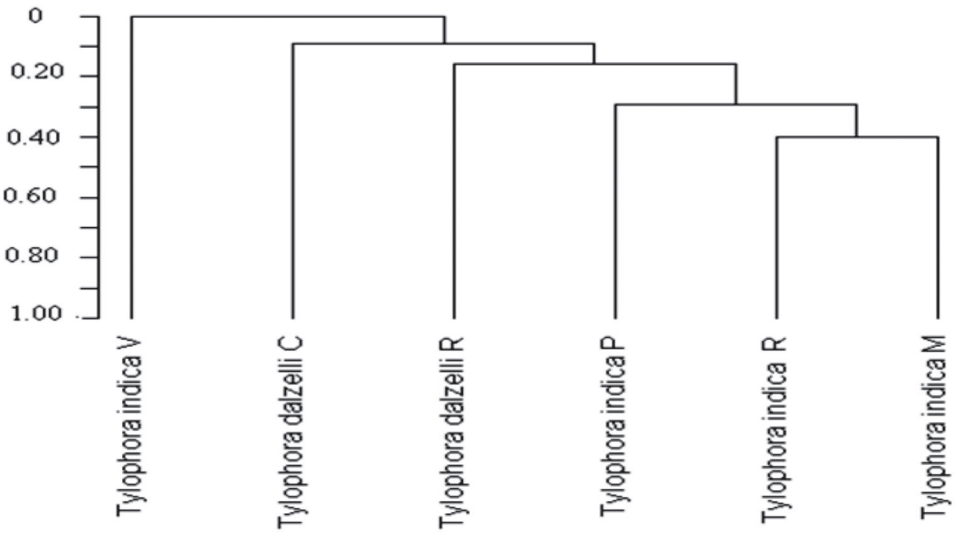

Fig. 2. UPGMA based dendrogram with genetic relationships among the six variants of Tylophora.

Table 1: Sampling sites of *Tylophora* species in Goa. NG- North Goa, SG- South Goa

Place	Sea level (m)	Geographical Location
Pernem (NG)-TiP	18	15.7169° N, 73.7978° E
Valpoi (NG)-TiV	42	15.5300° N, 74.1300° E
Rivona (SG)-TiR, TdR	31	15.1638° N, 74.1028° E
Nuvem (SG)-TdN	15	15.3089° N, 73.9461° E
Margao (SG)-TiM	31	15.2736° N, 73.9581° E

Table 2: RAPD PCR program conditions

Step	RAPD	
	Temp (^{0}C)	Time
Initial denaturation	94	5 min
Denaturation	94	1 min
Annealing	37	1 min
Elongation	72	2 min
Final Extension	72	10 min
Holding temperature	25	5 min

Table 3: RAPD analysis of *Tylophora* species and variants from Goa

Primer name	Primer sequence	Size of fragments	Number of bands	Polymorphic bands	Polymorphism %
OPF-1	ACG GAT CCT G	-	-	-	-
OPF-2	GAG GAT CCC T	-	-	-	-
OPF-3	CCT GAT CAC C	400-1700	7	7	100
OPF-4	GGT GAT CAG G	350-1900	6	6	100
OPF-5	CCG AAT TCC C	650-2300	5	5	100
OPF-6	GGG AAT TCG G	500-1400	2	2	100
OPF-7	CCG ATA TCC C	400-3000	6	6	100
OPF-8	GGG ATA TCG G	2700	1	1	100
OPF-9	CCA AGC TTC C	40-650	4	4	100
OPF-10	GGA AGC TTG G	350-1600	8	8	100
		Total	39	39	100

Table 4: Relationship among six variants of *Tylophora* as per Jaccard's similarity coefficient

TiP	TiR	TiV	TiM	TdR	TdN	
TiP	1					
TiR	0.38	1				
TiV	0	0	1			
TiM	0.29	0.40	0	1		
TdR	0.16	0.18	0	0.28	1	
TdN	0.22	0.1	0	0.09	0.10	1

analyses of hypericin content and RAPD markers grouped the clones of *Hypericum perforatum* L. in two major clusters and significant correlations were observed between them (22). *Tylophora indica* known for its exemplary medicinal uses faces exploitation and decline in wild populations (7). This also gives scope to use other related plants as adulterants in Ayurvedic preparations, which brings down the quality of drug and demand of herbal drugs in their natural form.

In the present investigation, two species of *Tylophora* known locally for treatment of asthma were used. RAPD was performed to assess the genetic polymorphism among the variants collected. The results showed that out of 10 primers used for amplification, 8 primers showed polymorphic nature of the variants. It was also seen that both the species showed 100% polymorphism. On the other hand, *T. rotundifolia* showed only 46% of polymorphism (23). Results from cluster analysis and linkage distance, indicated that the variant TiV is different from others used in this study. It appears that this plant has been introduced into the nurseries of Goa, but not a native to this place. Also, similarity between TdR and TdN was found to be only 10%. This gives scope to question the factors responsible for such a level of dissimilarity among the variants and species in such a close ecological zone of Goa, being only 3,702 square kilometers. Based on the molecular markers, it is inferred that the two species and the morphological variants collected from different locations are highly variable. This can be attributed to the area, size of species and higher cross pollination among them. A high genetic diversity further supports their diverse origin (24-25). Narrowing of gene pool and reduced genetic diversity pose challenges in the selection pressure brought in by environmental changes (23). The high genetic diversity observed in *Tylophora* species here may be reflecting that the species has adapted well to the environmental conditions (26). But, usage of *Tylophora dalzelli* as an antiasthamatic plant as a replacement of *T. indica* needs further studies through bioassays.

Acknowledgements

Authors wish to thank Prof. P. B. Kavi Kishor, Department of Genetics, Osmania University, Hyderabad, for providing laboratory facilities and guidance to carry out this research work.

Conflict of interest *:* The authors declare no financial or commercial conflict of interest.

References:

1. Shivpuri, D.N., Singhal, S.C. and Prakash, D. (1972). Treatment of asthma with an alcoholic extract of *Tylophora indica*: a crossover, double blind study. Annals of Allergy. 30, 407-412.

2. Gopalakrishnan, C., Shankaranarayan, D. and Nazimudeen, S.K. (1980). Effect of tylophorine, a major alkaloid of *Tylophora indica*, on immumopathological and

inflammatory reactions. Ind J Med Res. 71, 940-8.

3. Gallert, E. (1982). Theindolizidine alkaloids. J. Natural Prod. 45, 50-73.

4. Mujeeb, M., Aeri, V., Bagri, P. and Khan, S. A. (2009). Hepatoprotective activity of the methanolic extract of *Tylohora indica*(Burm f.) Merill Leaves. International J Green Pharmacy. 3, 125-127.

5. Ganguly, T., Badheka, L.P. and Sainis, K. B. (2001). Immunomodulatory effect of *Tylophora* on Con A induced lymphoproliferation. Phytomedicine. 8, 431-437.

6. Chitnis, M. P., Khandelkar, D.D., Adwankar, M.K. and Sahasrabudhe, M. B. (1972). Anti-cancer activity of the extracts of stem and leaf of *Tylophora indica*. Ind J Med Res. 60, 359-362.

7. Jayanthi, M., and Mandal, P.K. (2001). Plant regeneration through somatic embryogenesis and RAPD analysis of regenerated plants in *Tylophora indica* (Burm.F.Merril). In Vitro Cellular and Develop Biol – Plant. 37, 576-580.

8. Bhonsle, L. H. (1995). Alkaloids in tissue culture of *Heterostemma tanjorense* W. and A.(Asclepiadaceae). Thesis submitted to Goa university, Department of Biotechnology. Panaji, Goa. India.1-177.

9. Joshi, K., Chavan, P., Warude D. and Patwardhan B. (2004). Molecular markers in herbal drug technology. Current Sci. 87, 159-165.

10. Tariq, A., Mehmood, T., Ahmad, M., Sahar, U., Mushtaq, S. and Zafar, M. (2009). Comparative assessment of genetic diversity among the Asclepiadaceous species using randomly amplified polymorphic DNA (RAPD) markers and numerical taxonomy system (NTSYS) cluster analysis. JMed Plant Res. 8, 88-94.

11. Chaturvedi, P., Chowdhary, A. and Kawalkar, H. (2012). Characterization of *Tylophora* indica regenerated plants in vitro by RAPD and ISSR analysis. International Journal of Research in Phytochemistry Pharmacology. 2, 175-179.

12. Chopra, R. N., Nayar, S.L. and Chopra, I.C. (1956). Glossary of Indian Medicinal Plants, CSIR Pub, New Delhi, India: 94.

13. Najafi, S. and Deokule, S.S. (2010). Pharmacognostic study of *Tylophora dalzellii* Hook. F. J Med Plants Res. 4, 403-406.

14. Mapcoordinates (2015). Find Google Maps coordinates - fast and easy! Retrieved February 14, 2015, from Mapcoordinates. net: http://www.mapcoordinates.net/en.

15. Cooke, T. (1967). Asclepiadaceae. In T. Cooke, The Flora of the Presidency ofBombay; Vol II:Cornaceae-Chenopodiaceae (2nd Reprint Edition ed., pp. 226-228)., Calcutta: Botanical Survey of India.

16. Rao, R.S. (1985). Flora of Goa, Diu, Daman, Dadra and Nagarhaveli, Vol.II. Botanical Survey of India.

17. Doyle, J.J. and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bulletein. 19, 11-15.

18. Rohlf, F.J. (1993). NTSYS-pc: Numerical taxonomy and multivariate analysis system, Version 2.0, Exeter Software, Applied Biostatics Inc, New York.

19. Zybartaite, L., Zukauskiene, J., Jodinskiene, M., Janssens, S., Paulauskas, A. and Kupcinskiene, E. (2011). RAPD analysis of genetic diversity among Lithuanian populations of *Impatiens glandulifera*. Zembdirbyste Agriculture. 98, 391-398.

20. Abdellaoui, R., Kadri, K., Ben Naceur, M. and Bettaib Ben Kaab, L. (2010). Genetic

Diversity in Some Tunisian Barley Landraces Based On RAPD Markers. Pakistan J Bot. 42, 3775-3782.

21. Hasnaoui, N., Mars, M., Chibani, J. and Trifi, M. (2010). Molecular polymorphisms in Tunisian pomegranate (*Punicagranatum* L.) as revealed by RAPD fingerprints. Diversity. 2:107-114.

22. Tonk, F., Giachino, R., Sönmez, Ç., Yüce, S., Bayram, E. and Ýsa T. (2011). Characterization of various *Hypericum perforatum* clones by Hypericin and RAPD analysis. International J Agricult Biology.13, 31-37.

23. Sebastian, V. D.L., Subramanian, R. and Braganza, V. (2010). Assessment of genetic diversity within and among populations of *Tylophora rotundifolia*using RAPD markers. Gene Conserve. 9, 94-117.

24. Nagylaki, T. (1998). Fixation indices in subdivided populations. Genetics. 148, 1325-1332.

25. Anand, A., Rao, C. S., Eganathan, P., Kumar, N. A. and Swaminathan, M. S. (2004). Saving an endangered Taxon: *Syzygium travancoricum* Gamble: A case study focusing on its genetic diversity, and reintroduction. PhysiolMolBiol Plants. 10, 233-242.

26. Caro, T. M. and Laurenson, K. M. (1994). Ecological and genetic factors in conservation: Acautionary tale. Science. 263,485-486.