Octa Journal of Biosciences
International peer-reviewed journal
Octa. J. Biosci. Vol. 2(2):105-110

ISSN 2321 – 3663
Dec. 2014

Octa Journal of Biosciences

journal homepage: www.sciencebeingjournal.com

Determination of genetic relationship of some medicinally important *Phyllanthus* species by isozyme and nrITS-RFLP polymorphism markers

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ARTICLE INFO

Received 25 Sept. 2014
Revised 30 Oct. 2014
Accepted 30 Nov. 2014
Available online 25 Dec. 2014

Keywords: Isozymes, Phyllanthus, Dendrogram, Nuclear ribosomal Internal Transcribed Spacer sequence (nr-ITS), restriction fragment length polymorphism (RFLP) , Genetic Relationships

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ABSTRACT

Superoxidedismutase, Peroxidase and Esterase were used as biochemical markers to assess the genetic relationships between five species of the genus *Phyllanthus*. All three isozymes showed unique banding patterns for different species. A dendrogram was constructed depending upon these banding patterns, which assessed their genetic relationship properly. Nuclear ribosomal Internal Transcribed Spacer sequence based restriction fragment length polymorphism markers are also developed in this study. Among several restriction enzymes used in this study HpaII found to be the most helpful in distinguishing all the five species under study.

INTRODUCTION

The genus *Phyllanthus* is the largest and most diversified genus in the family Euphorbiaceae (Balakrishnan and Chakrabarty 2007). Plants of this genus have long been used in Ayurveda i.e. in Indian traditional medicines. Medicinal importance as well as range of diversity made the genus a very interesting system for scientific investigation. In the present study we have chosen five species namely *Phyllanthus emblica*, *Phyllanthus reticulatus*, *Phyllanthus amarus*, *Phyllanthus fraternus* and *Phyllanthus urinaria* that are therapeutically important, abundant locally and also vary in their habits. Fruits of P.emblica are a rich source of vitamin C; they are mainly used for their hepatoprotective and antioxidant activities (Bhattacharya et.al. 1999, Jose and Kuttan, 2000). P.reticulatus is claimed to have antidiabetic activity (Kumar et.al. 2007). P. amarus is famous for its hepatoprotective activity (Jain et.al. 2003). Aqueous extracts of P.fraternus has antioxidant properties (Sailaja and Setty 2006). P.urinaria is used as a preventive for urinary infection in different parts of India (Chaudhary & Rao 2002).

Biochemical and molecular marker based genetic diversity analysis among these species will be helpful in proper assessment of their genetic relationship and only then correct use of all these plants will be easier. Again this type of study can be used for refinement in their taxonomic status. Therefore the present investigation has focused on isozymes like Esterase (EST), Peroxidase (POD) and Superoxide Dismutase (SOD) as biochemical markers for assessing genetic relationships as well as their potentiality to serve as markers among the five species of *Phyllanthus* mentioned above. Isozymes refer to multiple molecular forms of an enzyme sharing a catalytic activity derived from a tissue of single organism (Markert and Moller 1959). As biochemical markers, isozymes have some advantages compared to morphological markers. The technique is non destructive since a small part of any tissue can be used for the purpose and a crude protein extract can be used without any purification step making it a simpler and less time consuming process. Although modern molecular techniques are considered to be more reliable but they demand sophisticated infrastructure and better expertise. Esterase, Peroxidase and Superoxide dismutase catalyze three different reactions in vivo. Esterase includes a group of isozymes consisting of a host of ester hydrolases. Peroxidases are a group of enzymes that catalyze oxidation-reduction reactions. They reduce H_2O_2 to water while oxidizing a variety of substrates. Thus, peroxidases are oxidoreductases, which use H_2O_2 as electron acceptor for catalyzing different oxidation reactions. Peroxidase is probably the most widely studied isozyme. Peroxidase can negatively influence food quality in raw or processed foods. Blanching inactivates the enzyme and is done prior to canning and freezing (Kermasha & Metche 1988). Peroxidase activity has been linked to disease resistance to anthracnose in beans (Okiror et al. 1982), anti-microbial activity (Albert el al.1986), fruit ripening (Prestamo & Manzano 1993), and lignin production in cell walls. Cipollini (1998) has even observed increased peroxidase activity in common beans due to wind-induced mechanical stress. Superoxide dismutases protect oxygen-utilizing species by dismuting the undesirable super oxide radicals into molecular oxygen and H_2O_2 (Mc Cord and Fridovich, 1969; McCord et.al. 1971). Variable responses of SOD to dehydration stress have been reported in literature including decreased activity (Quatracci et al.1994), lack of effect (Anderson 1995; Bertolli et al.1999) or increased activity (Srivali 2003) depending on plant species, tissue and stage of development. All these features make these enzymes very important biochemical markers. These three enzymes play very important roles in different metabolic processes in plants and therefore their presence is highly expected during scientific investigation. Again activity based staining of these isoenzymes are also very much established, hence these isoenzymes have been chosen to study the genetic relationships in the present study productionks were sterilized with equal volumes of zobell marine broth.

Internal Transcribed Spacer regions are sequences located in eukaryotic rRNA genes between the 18S and 5.8S rRNA coding regions (ITS1) and between the 5.8S and 25S(26S) rRNA coding regions (ITS2). Studies of restriction site variation in rDNA in populations of animals and plants have shown that while coding regions are conserved, these spacer regions are variable (Gerbi 1985). These spacer sequences have a high evolutionary rate and are present in all known nuclear rRNA genes of eucaryotes (White et.al.1991). They are useful for phylogenetic analysis among related species or among population within a species because these sequences evolve more rapidly than their flanking coding regions (Baldwin et al.1993). Bayer et.al (1996) enumerated several advantages of ITS that make it an ideal region to sequence for phylogenetic analysis of cogeneric species. The advantages of their use in phylogenetic analysis of co generic species are; I) its rate of evolution is appropriate for studies at the specific and generic levels; II) it is phylogenetically interpretable i.e., the sequences are relatively easy to align as there is very little length variation of this region at the generic level in flowering plants. III) it is large enough to offer potentially enough characters for phylogenetic reconstruction and IV) it is flanked by regions that are highly conserved within a genus, making polymerase chain reaction(PCR) amplification and sequencing straightforward. Here in this study we have attempted nuclear ribosomal internal transcribed spacer based restriction fragment length polymorphism markers to distinguish all five *Phyllanthus* species under study. RFLP or Restriction fragment length polymorphism is also a popular marker technology in which restriction patterns between genomes are compared to evaluate genetic differences. Ribosomal DNA spacer sequence RFLP has been applied in many plant species for their correct identification or some other purpose successfully (Ahmed et al. 2006, Kim et al.2007). Here we analyzed restriction profiles generated using different restriction enzymes of all the five species under study. So the present study is aimed to assess the genetic relationship in the five species of *Phyllanthus*

mentioned with the help of isozymes and nr-ITS markers.

MATERIALS AND METHODS

Plant leaves of all the five species were collected from different districts of West Bengal, India. The tissues were brought to the laboratory and stored in -70ºC until further analysis.

Isozyme extraction, gel electrophoresis and activity staining

Leaves (1g.) from different accessions (Table 2) of all five species were crushed with mortar and pestle in presence of 2ml extraction buffer (0.1M Tris, 0.25M Sucrose,1% PVP,0.1% Ascorbic acid,0.1%Cysteine Hydrochloride,1mM EDTA and magnesium chloride) of pH 6.8.The ground samples were centrifuged at 14000 rpm at 4ºC for 40 min to remove the debris. After decantation a second centrifugation at 40000 rpm at 4ºC for 1h was performed to clarify the extracts. All the protein samples were stored in -70C for isozyme analysis.

Vertical native polyacrylamide gel electrophoresis was performed in 16 cm x 14 cm slab gels of 1.5 mm thickness in a vertical gel electrophoresis unit. Approximately150 g of protein was loaded onto each well of the gel. The electrode buffer was Tris-glycine at pH8.3. The gel electrophoresis was performed at 4 °C for 4 h at 35 mA at constant current provided from a power supply unit. Activity staining for EST, POD and SOD were done according to the protocols of Shaw and Prasad (1970), Geburek and Wang (1990) and Roy S et al. (2006).

Gel electrophoresis experiments were done twice for each enzyme system to check the consistency in the appearance of bands. Isozymic variations were observed in the bands. For each accession, isozyme bands were scored. Loci were numbered consecutively, and alleles at each locus were labeled with Rf values, beginning from the most anodal form. Isozyme bands were scored by assigning 1 for the presence of a particular band and 0 for its absence, a binary data matrix was obtained which was used in constructing a dendrogram using the software package SPSS (version 9.0).

PCR amplification of total ITS region and RFLP

To amplify the total ITS region (ITS1- 5.8S - ITS2) from different Phyllanthus species two primers 17SE and 26SE (Sun et.al. 1994) were used and products were electrophoresed on a1.5% agarose gel. All the species showed a clear and distinct band slightly more than 900bp region. These PCR products were then subjected to restriction digestion in 20l volume using three different restriction enzymes EcoRV, HpaII & ClaI (Bangalore Genei, India). 200ng of PCR products were incubated at 37C for 4 hours with 5U of restriction enzymes each.

Digested products were then electrophoresed at a constant voltage of 62v. on a 1.5% agarose gel and visualized under UV light. A 100bp ladder (ranging 100bp-1000bp) was electrophoresed with the samples to determine the size of restricted fragments.

Table 1: Numbers of scorable isozyme bands for different accessions of the five species.

Accessions →. Scorable bands Isozyme →	*P. amarus* (var)				*P.fraternus* (var)		*P.urinaria* (var)				*P.emblica* (var)				*P.reticulatus* (var)	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SOD	6	6	6	6	8	8	8	8	8	8	4	4	4	4	3	3
POD	4	4	4	4	4	8	4	4	4	4	1	0	1	0	7	7
EST	1	2	2	1	3	3	1	0	1	0	0	0	0	0	2	2

Table 2: Morphological characters and collection sites of the five *Phyllanthus* species.

Name of species	Collection site	Habit
Phyllanthus emblica	North24parganas, Burdwan, South 24 parganas, Nadia.	Very tall tree. Normally reaching a height of 60 ft. (18m).
Phyllanthus reticulatus	North24parganas, Burdwan.	*Phyllanthus reticulatus* is a large straggling or climbing shrub growing from 8 to 10 ft in hei ght (Kritikar and Basu 2003).
Phyllanthus amarus	North24parganas, Burdwan.	Erect annual herb, 10 -90cm long, leaves oblong or sometimes oblong obovate with obtuse or rounded or occasionally cuneate base. (Chaudhary & Rao 2002).
Phyllanthus fraternus	North 24 parganas, kolkata.	Erect annual herb. 10 -60-cm.in heights, leaves elliptic - oblong with obtuse apex. (Chaudhary & Rao 2002).
Phyllanthus urinaria	North24parganas, Burdwan, Kolkata, South24parganas.	Erect or sometimes procumbent annual herb. 10 -70 cm.long leaves generally hispidulous along margins, oblique at base, distinctly mucronate at apex (Chaudhary & Rao 2002). .

RESULTS AND DISCUSSION

All the three isozymes produced different number of bands and their pattern was unique for different species (Figure 1 and 2). Several monomorphic as well as polymorphic bands were present there. Maximum numbers of bands i.e. 22 were observed for the enzyme peroxidase and minimum numbers of bands i.e. 6 were observed in case of esterase. For superoxidedismutase there were 20 bands. Accessions of Phyllanthus emblica showed very few numbers of bands in esterase and peroxidase systems but in case of SOD there were several bands. Total number of bands per accession for different isozymes is given in the Table 1. Unique isoforms are shown with arrows and Rf values and they can be treated as markers for species differentiation. *Phyllanthus fraternus* and *Phyllanthus amarus* showed almost same type of banding pattern.

Positions of clearly visible and scorable isozyme bands were transferred into a binary character matrix, with 1 for the presence and 0 for the absence of a band at a particular position. Proximity matrix was directly computed from Jaccard's coefficient using the software package SPSS version 9.0. A dendrogram (Fig.3) was obtained (using average linkage within groups) by hierarchical cluster analysis to establish the affinities between different accessions of the 5 species of *Phyllanthus*.Two main branches appeared in the dendrogram. First one consisted of *P.reticulates*, P.amarus and *P.fraternus*; the other one consisted of *P.urinaria and P.emblica*.

There are some reports involving isozymes in the genus *Phyllanthus* mainly from South India, but they are focused on one species at a time (Shaanker RU et al.1997, Geetha BS et al.2003, Marimuthu J et al.2007). In our study we tried to look differences in three isozymes within five species of this genus so that these isozymes can be used as markers to identify different species. Here intraspecific variations were found to be lower. Interspecific variations were present to sufficient extent as all the five species were different from each other. For *Phyllanthus emblica* there were very few bands in EST and POD systems, which could be possible due to lower expression of EST and POD in *Phyllanthus emblica* leaves. It is very clear from the table that among the three isozymes SOD is the most efficient marker as there is different number of isoforms (table 1) for this enzyme for different species whereas EST is the least efficient. Species-specific SOD bands are marked along with their Rf values in the figure 1. Based upon SOD banding pattern species differentiation can be done in future studies.

The dendrogram constructed based upon these data consisted of two main branches. First one consisted of P.reticulatus (var15,16), *P.amarus* (var1, 2,3,4) and *P.fraternus* (var5, 6); the other one consisted of P.urinaria (var7, 8,9,10) and P.emblica (var11, 12,13,14). Close genetic relationship between *P.amarus* (var1, 2,3,4) and *P.fraternus* (var5, 6) was again confirmed from the dendrogram as they positioned under the same branch. Although they are closely related but their separate identity is evident from their different clustering pattern. The shrub *P.reticulates* (var15, 16) formed an out-group here. *P.urinaria* and *P.emblica* showed closer genetic relationship as they clustered under the same branch.

Total ITS region was amplified for all the 5 species under study. All the species showed a clear and distinct band slightly more than 900bp region (Fig.4a). Restriction digestion profile with 3 different RE showed that only HpaII (Fig.4b) produced differential banding pattern for all the species whereas the other two RE produced similar kind of pattern for all the species. In recent years ITS based phylogenetic studies hugely contributed in the understanding of ancestral relationships. In the genus *Phyllanthus* there were some studies based on these sequences done in China (Lee SK et.al. 2006) and other countries (Kathriarachchi et.al. 2006). Present authors have also studied ITS1&2 regions from different accessions of all these five species and reported that data earlier (Bandyopadhyay and Sen Raychaudhuri 2011). A detailed report of all the scientific studies done on different species of this particular genus has been done by Sarin B et al. (2014). In the present study we found that HpaII produced differential banding pattern for all the five species under study and it could be used as a molecular marker in future studies.

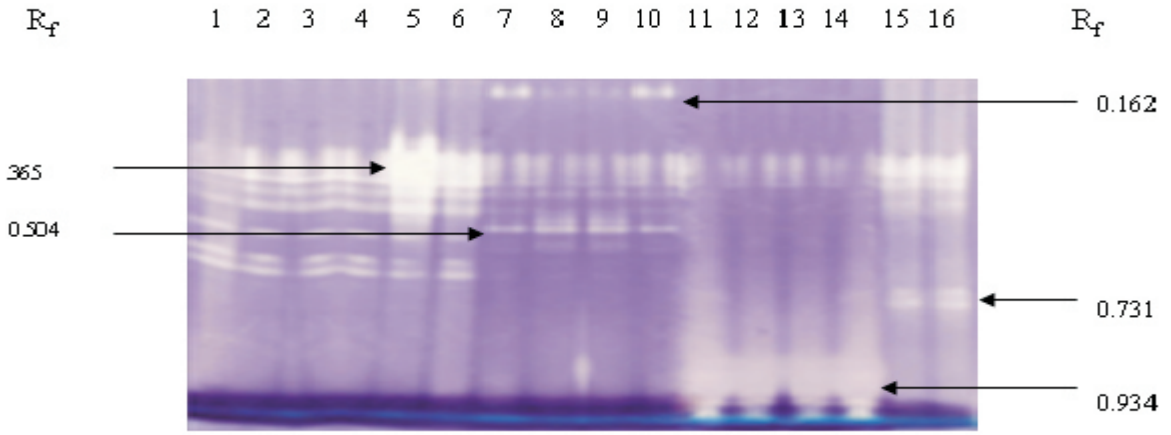


Figure 1

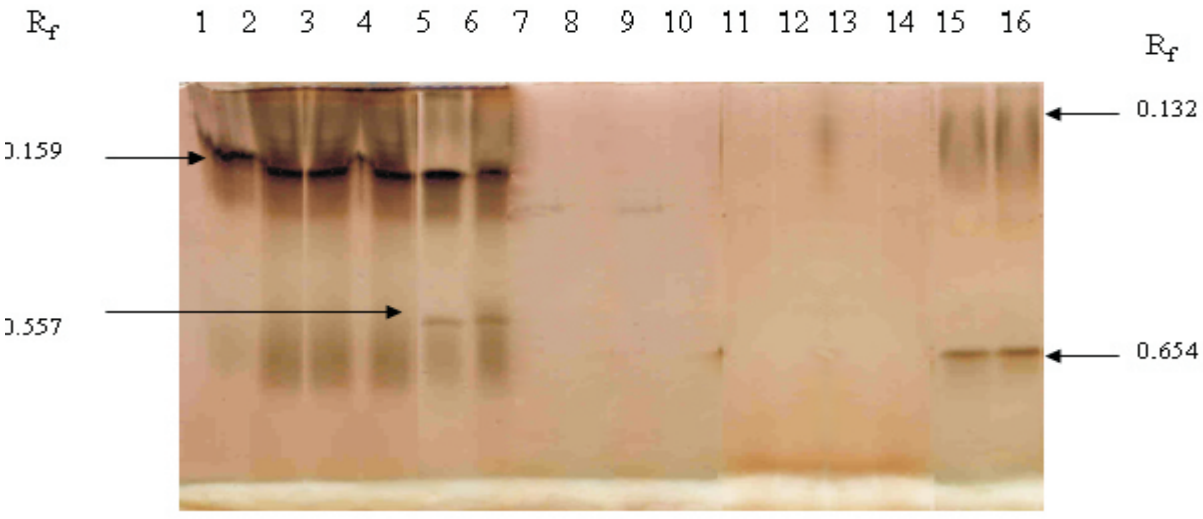


Figure 2

Fig1: SOD banding patterns in different species of *Phyllanthus.Lanes* 1-4 *P.amarus, Lanes* 5, 6 *P.fraternus*, Lanes7-10 *P.urinaria*, Lanes 11-14 *P.emblica,* Lanes15,16 *P. reticulates*

Fig.2: EST banding patterns in different species of *Phyllanthus*.Lanes1-4 *P.amarus*, Lanes5,6, *P.fraternus*,Lanes 7-10 *P.urinaria*, Lanes 11-14 *P.emblica*,Lanes15,16 *P. reticulates*

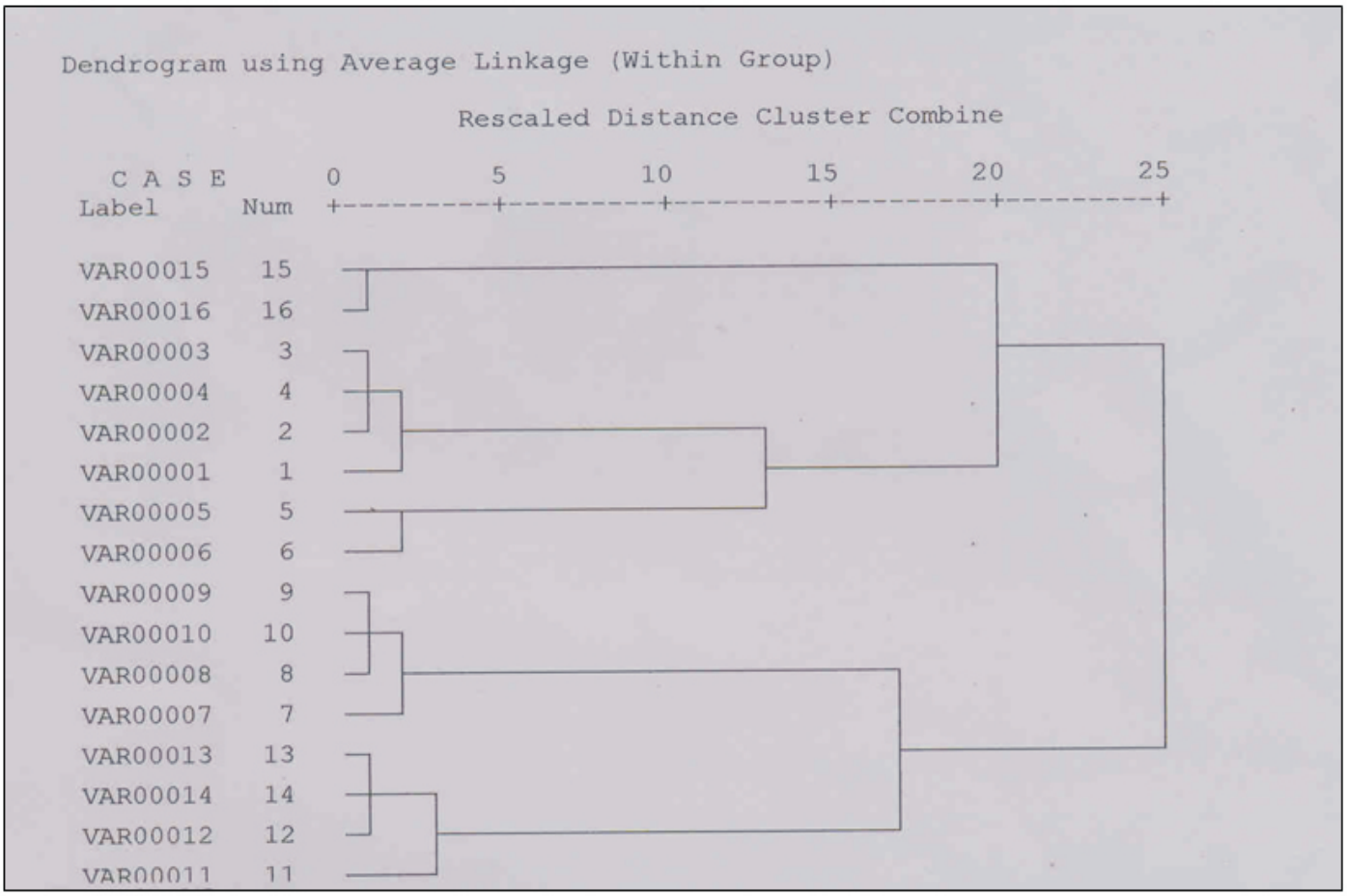
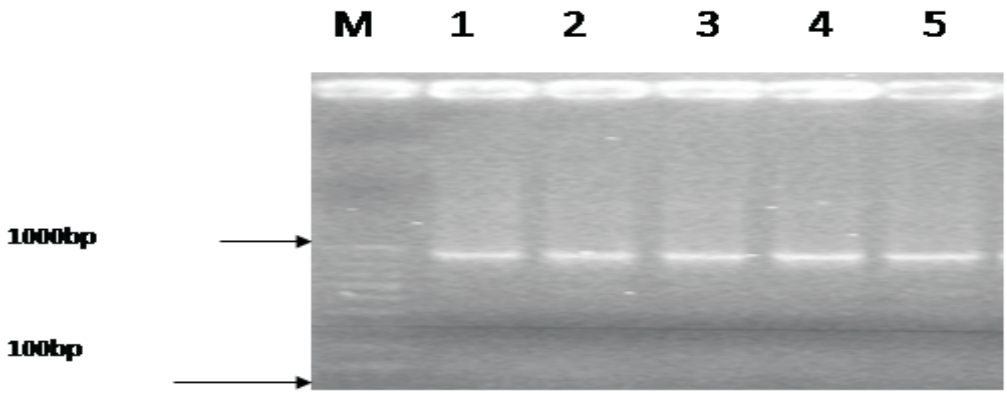
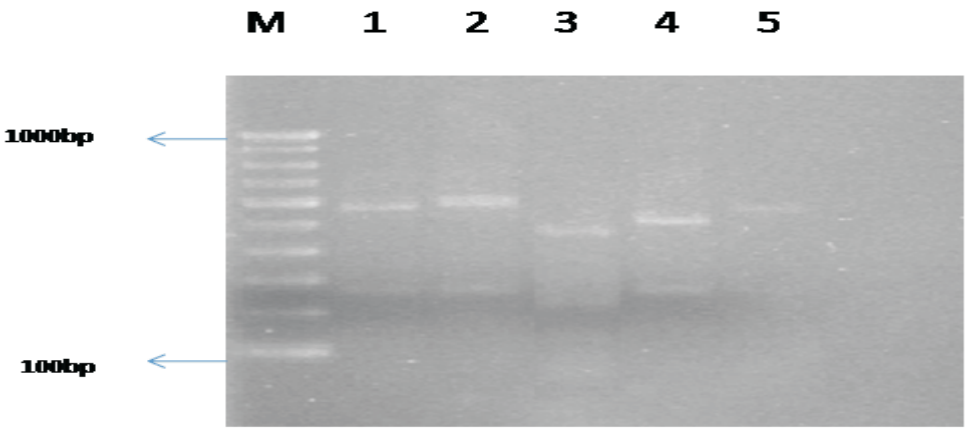


Fig.3: Dendrogram showing genetic relationships between different species of *Phyllathus. P.amarus* (var1, 2, 3,4), *P.fraternus* (var5, 6), *P.urinaria* (var7, 8,9,10), *P.emblica* (var11, 12,13,14) *P.reticulatus* (var15,16).



4a



4b

Fig. 4a: Total ITS region amplified from five species of *Phyllanthus*. Lanes M-5, 100bp marker, *P.emblica, P.reticulatus, P.amarus, P.fraternus* and *P.urinaria*.
4b: Restriction pattern generated by HpaII Lanes Lanes M-5, 100bp marker, *P.emblica, P.reticulatus, P.amarus, P.fraternus* and *P.urinaria*.

CONCLUSION

In conclusion, it can be said that the data obtained from these isozymes is very much helpful in determining the genetic relationships between five medicinally important species of the genus *Phyllanthus*. Besides, nr-ITS based RFLP also proved to be a molecular marker in different species under study. Therefore, the data could be used for identification and better management of our available plant resources in future.

Acknowledgments

Authors are very grateful to the Department of Biotechnology (DBT) and University Grants Commission (UGC), Govt. of India for financial assistance.

REFERENCES

1. Albert F.G., Bennett W., and Anderson, A. J (1986). Peroxidase associated with the root surface of *Phaseolus vulgaris*. *Can. J. Bot.* 64: 573-578.
2. Ahmed S.M, ,Verma V., Qazi P.H.,Ganaie M.M., Bakshi S.K and Qazi G.N(2005). Molecular phylogeny in Indian *Tinospora species* by DNA based molecular markers. *Pl Sys Evolution*. 256(1-4): 2005.
3. Anderson M.D., Prasad T.K., Stewart C.R (1995). Changes in isozyme profiles of catalase, peroxidase and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. *Plant Physio*l 109: 1247 - 1257.
4. Balakrishnan N.P and Chakrabarty T (2007). The family Euphorbiaceae in India: A synopsis of its profile, taxonomy and bibliography. 1st ed., Bishen Singh Mahendra Pal Singh, Dehra Dun.
5. Baldwin B.G., Sanderson M.J., Porter J.M., Wojciechowski M.F., Campbell C.S., Donoghue M.J (1995). The ITS region of nuclear ribosomal DNA a valuable source of evidence on angiosperms. *Mol.Bot.Genet* 82: 247-277.
6. Bartoli C.G., Simonthacchi M., Tambussi E.A., Beltrano J., Montaldi E.R., Puntarulo S(1999). Drought and watering dependent oxidative stress: Effect of antioxidant con-tent in Triticum aestivum L. leaves. J. Exp. Bot. 50: 375 – 383.
7. Bayer R. J., Soltis D. E., Soltis S. P (1996). Phylogenetic inferences in Antennaria (*Asteraceae: Gnaphalieae: Cassininae*) base on seqence from nuclear ribosomal DNA internal transcribed spacer ITS1. *Amer. J. Bot.* 83 (4):516–527.
8. Bhattacharya A., Chatterjee A., Ghosal S., and Bhattacharya S.K (1999). Antioxidant activity of tannoid principles of *Emblica officinalis* (Amla). *Indian J Exp Biol* 37:676-680.
9. Chaudhary L.B and Rao R.R (2002). Taxonomic study of herbaceous species of *Phyllanthus L. (Euphoebiaceae)* in India. *Phytotaxonomy* 2:143-162.
10. Cipollini D (1998). The induction of soluble peroxidase activity in bean leaves by wind-induced mechanical perturbation. *Am J Bot* 85,1586–1591.
11. Geburek T & Wang Q (1990). Inheritance of isozyme variants and their linkage relationships in Chinese fir (*Cunninghamia lanceolata Hook*). *Euphytica* 49: 193–201
12. Geetha B.S., Sabu K.K and Seeni S (2003). Genetic variation in south Indian population of *Phyllanthus amarus schum*.and *thonn. (Euphorbiaceae)*, assessed using isozymes. Proceedings of the Fifteenth Kerala Science Congress.29-31 January 196-201.
13. Gerbi S.A (1985). Evolution of ribosomal DNA. In:Molecular Evolutionary Genetics, Maclntyre, R.J.(Ed.). Plenum Press, New York: 419-517.
14. Jose K.J., Kuttan R (2000). Hepatoprotective activity of *Emblica officinalis* and Chyavanprash. *J. Ethnopharmacol*.72(1-2): 135-140.
15. Kermasha S and Metche M. (1988). Studies on seed peroxidase *Phaseolus vulgaris* cv, Haricot. *J. Food Sci*. 53: 247–252.
16. Kritikar K.R and Basu B.D (2003). Indian medicinal plants vol.9. 2nd edition. International Book Distributors, Dehradun, 3060.
17. Kumar S., Kumar D., Deshmukh R.R., Lokhand P.D., More S.N and Rangari V.D (2007). Antidiabetic potential of P.reticulates in alloxan induced diabetic mice. *Fitoterapia* 79(1): 21-23.
18. Kathriarachchi H., Samuelle R., Hofmann P., Mlinarec J., Wurdack K.J., Ralimanana H., Stussey T.F and Chase M.W (2006). Phylogenetics of tribe *Phyllantheae (Phyllanthaceae*; *Euphorbiaceae sensu lato*) based on nr ITS and plastid matK DNA sequence data. *Am. J. Bot*. 93:637-655.
19. Kim O.T., Bang K.H, In D.S., Lee J.W., Kim Y.C., Shin Y.S et al. (2007). Molecular authentication of ginseng cultivars by comparison of internal transcribed spacer and 5.8S rDNA sequences. *Plant Biotechnol Rep*. (1): 163-167.
20. Lee S.K., Li P.T., Lau D.T., Yung P.P., Kong R.Y., Fong W.F (2006). Phylogeny of medicinal *Phyllanthus species* in China based on nuclear ITS and chloroplast atpB-rbcL sequences and multiplex PCR detection analysis. *Planta Med*.72: 721-726.
21. Market C.L and Moller F (1959). Multiple Forms of Enzymes, Tissue, Ontogenetic and Species Specific pattern. *Proc Natl Acad Sci U S A* 45: 753-763.
22. McCord J.M., Fridovich I (1969). Superoxide dismutase: An enzymic function *forerythrocuprein* (Homocuprein). *J.Biol.Chem*. 244: 6049-6055.
23. Marimuthu J., Antonsamy A (2007). Somaclonal variation Somoclonal variation studies on *Phyllanthus amarus Schum* & *Thonn*. IJB.5(4):240-245.
24. McCord J.M., Keele B.B., Fridovich I (1971). An enzyme based theory of obligate anaerobiosis: The physiological function of superoxidedismutase. *Proc.Natl.Acad.U.S.A*.68: 1024-1027.
25. Jain N., Shasany A.K., Sundaresan V., Rajkumar S., Darokar M.P., Bagchi G.D., Gupta A.K., Kumar S., Khanuja S.P.S (2003). Molecular diversity in *Phyllanthus amarus* assessed through RAPD analysis. Curr. Sc., 85(10): 1454-1458.
26. Jain N., Shasany A.K., Sundaresan V., Rajkumar S., Darokar M.P., Bagchi G.D., Gupta A.K., Kumar S., Khanuja S.P.S (2003). Molecular diversity in *Phyllanthus amarus* assessed through RAPD analysis. Curr. Sc., 85(10): 1454-1458.
27. Prestamo G and Manzano P (1993). Peroxidases of selected fruits and vegetables and the possible use of ascorbic acid as an antioxidant. Hort. Science., 28:48–50.
28. Quatracci M.F., Sgherri C.M., Pinzono C and Navari F (1994). Superoxide radical pro-duction in wheat plants differently sensitive to drought. Proc. R. Soc. Edinb., 102B: 287 – 290.
29. Roy S., Begum Y., Chakraborty A., Sen Raychaudhuri S (2006). Radiation-induced phenotypic alterations in relation to isozymes and RAPD markers in Vigna radiata (L.) Wilczek. *Int. J. Rad. Biol*., 82: 823-32.
30. Sailaja R and Setty O.H (2006).Protective effect of *Phyllanthus fraternus* against allyl alcohol induced oxidative stress in liver mitochondria. *Jr. Ethnopharmacol*., 105: 201-209.
31. Sarin B., Verma N., Martin J. P. and Mohanty A (2014). An Overview of Important Ethnomedicinal Herbs of *Phyllanthus* Species: Present Status and Future Prospects. The Scientific World Journal, Volume 2014, Article ID 839172, 12 pages http://dx.doi.org/10.1155/2014/839172.
32. Shaw C.R and Prasad R (1970). Starch gel electrophoresis of enzymes. A compilation of recipes. Biochem Genet., 4: 297–320.
33. Srivalli B., Sharma G., Khanna Chopra R (2003). Antioxidative defense system in an upland rice cultivar subjected to increasing intensity of water stress followed by recovery. *Physiol. Plant.*, 119: 503 - 512.
34. Shaanker R.U and Ganeshaiah K.N (1997). Mapping genetic diversity of *Phyllanthus emblica*: Forest gene banks as a new approach for in situ conservation of genetic resources. *Current SC*., 73(2):165-168.
35.White T.J., Bruns T., Lee S., Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics .In M.Innis, D.Gelfand,J.Snisky,T White (eds) PCR protocols: a guide to methods and applications. Academic Press , San Diego, California,USA,315-322.