ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 9, Issue 5, 2016

Online - 2455-3891
Print - 0974-2441

Research Article

GENETIC DIVERSITY AND RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS OF *PESTALOTIA* SP. ISOLATES OF ENDOPHYTES FROM DIFFERENT HOST

RAJASEKAR ARVINDGANTH, GOVINDARAJAN KATHIRAVAN*, THIRUGNANASAMBANDAM THILAGAVATHI, VARSHA B

Department of Biotechnology, Vels University, Pallavaram, Chennai - 600 117, Tamil Nadu, India. Email: kathir14journals@gmail.com

Received: 07 May 2016, Revised and Accepted: 18 May 2016

ABSTRACT

Objective: The genetic diversity of fungal endophytes medicinally important plant leaves. Isolates fungi were genotypically compared by random amplified polymorphic DNA (RAPD) techniques. The results indicate that RAPD can be employed for detecting genetic diversity of *Pestalotia* species from medicinal plants and for pre-selection of these isolates for bioactive screening program.

Methods: Using different instrumental methods for isolation and identification of bioactive compounds and RAPD molecular method for detection of taxol producing fungi.

Results: Our studies also suggest diversity of endophytes as it differs in plant diversity. *Pestalotia* spp. are of considerable interest to researchers and pharmacists due to their capabilities of synthesizing a wide range of economically important bioactive molecules.

Conclusion: RAPD markers did not differentiate and place the isolates into respective host or locations from which they were isolated. The relationship between species isolated from the same or different hosts do not support phylogenetic analysis and also morphologically similar species form close relationships rather than the isolates of the same host.

Keywords: Genetic diversity, Endhophytic fungi, *Pestslotia* sp., Random amplified polymorphic DNA.

INTRODUCTION

Among the various anticancer drugs of plant origin, taxol is considered as the most important chemotherapeutic agent discovered so far. Taxol was approved in 1992 by the US Food and Drug Administration for the treatment of refractory ovarian and breast cancer. It was shown to be active against a variety of cancers such as lung, gastrointestinal, neck and head as well as malignant melanoma [1]. The most important member of the clinically useful natural anticancer agent is paclitaxel (taxol), which was first extracted from the bark of western yew (*Taxus brevifolia*) [2]. This compound is the world's first billion-dollar anticancer drug, and it is used to treat a number of other human tissue-proliferating diseases as well.

By the early 1990's, however, fungi had been isolated from many of the world's representative yew species. After several years of effort, the search for novel sources of taxol has led to the isolation of a novel endophytic fungus (*Taxomyces andreanae*) colonizing the inner bark of the yew tree (*T. brevifolia*) which is capable of producing taxol and other taxanes *de novo* when grown in semi-synthetic liquid medium [3,4]. However, the yield of taxoids from *T. andreanae* is very low (24-50 ng/L). This study also suggests that improved culture techniques, addition of activators/elicitors, and application of genetic engineering methods may result in enhanced production of taxoids by this endophytic microbe and permit commercialization of *T. andreanae* for taxol production.

Strobel *et al*. (1996b) studied taxol production from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*, and reported that some of the most commonly found endophytes of the world's yews are *Pestalotia* spp. Li *et al*. (1996) also reported that one of the most commonly isolated endophytic species is *Pestalotia microspora*. Strobel *et al*. (1997) isolated *Pestalotia guepini* from extremely rare and previously thought to be extinct, Wollemi pine (*Wollemia nobilis*), which has been proved to produce taxol. Subsequently, Li *et al*. (1998) isolated an endophytic fungus *Periconia* sp. from *Torreya grandifolia*.

The production of taxanes in a few cases reached 50-100 ng/L. Wang *et al*. (2000) studied taxol production from *Tubercularia* sp. isolated from Southern Chinese yew (*Taxus mairei*) in the Fujian province of southeastern China. In addition to that, Shrestha *et al*. (2001) showed a new report in the production of taxol from three different endophytic fungi isolated from the Himalayan yew *T. wallachiana,* namely, *Sporormia minima*, *Trichothecium* sp., and an unidentified dimorphic fungus were confirmed by different analytical and immunoassay methods. It can be inferred that fungi more commonly produce greater quantity of taxol than higher plants, and the distribution of those fungi producing taxol is worldwide and not confined to endophytes specific host are from specific geographic location. Thus, it may be thought that taxol had its origin in certain fungi and ultimately, if there is any lateral gene transfer, it may have been in the direction of the microbe to the higher plants (Strobel and Daisy, 2003) [5]. *In vitro* taxol production from *Pestalotia breviseta*, a coelomycetous fungi, first reported by Kathiravan and Sriraman (2010) [6], was confirmed by ultra violet (UV), infrared, high performance liquid chromatography, nuclear magnetic resonance, and liquid chromatography mass spectroscopy. The presence of taxol was identical with that of authentic taxol. Similar work was carried out by Kathiravan and Muthumary, 2009 [7]; Kathiravan *et al.,* 2013).

Random amplified polymorphic DNA (RAPD) analysis

Prakash *et al*., 2007 [8], the genetic diversity of fungal endophytic in root, bark, and twigs of four medicinally important plants, *Azadirachta*, *Holarrhena antidysenterica*, *Terminalia arjuna,* and *Terminalia chebula* were examined. Thirty isolates of *Pestalotia* and two isolates of *Brataliniarobiliardoides* were genotypically compared by RAPD techniques polymorphic bands were obtained using 23 random primers. The data were subjected to un weighted pair group method with arithmetic mean cluster analysis. Finally, the results were indicate that RAPD can be employed for detecting genetic diversity of medicinal plants.

Assigbetse *et al*. [9], in this investigation, he was used pathogenicity and RAPD markers to assess genetic diversity among 46 isolates of

Fusarium oxysporumf. sp. *vasinfectum* of worldwide origin. Based on pathogenicity testes on five differential cotton cultivars and species, isolates were differentiated in to three races (A, 3 and 4), restricted to defined geographic areas. The amount of genetic variation was evaluated by polymerase chain reaction (PCR) amplification with a set of 11 random 10-mer primers. All amplifications revealed scorable polymorphisms among the isolates, and a total of 83 bands positions were scored (1/10) for the 11 primers tested. Genetic distances between each of the isolates were calculated and cluster analysis was used to generate a dendrogram showing relationships between them. Isolates clustered in to three groups corresponding to their pathological reactions. He suggests that RAPD markers can be a quick and reliable alternative for differentiating isolates of *F. oxysporum*, *F. vasinfectum* into their respective pathogenicity group.

METHODS

The general laboratory techniques followed in the course of the present investigation were as outlined by Booth (1971). The test fungi used in this study were grown in 2L Erlenmeyer flasks containing 500 ml mid medium supplemented with 1 g soytone L^{-18} for taxol production. Three mycelial agar plugs (0.5 cm) were used as inoculum. The organisms were grown at 24°C±2°C under static condition for 3 and 4 weeks (Strobel *et al.,* 1990) [10].

Culture media

Mid medium was supplemented with soytone - 1.0 g, sucrose - 30.0 g/L, ammonium tartrate - 5.0 g, yeast extract - 0.5 g, $Ca_2 (NO)_3$ - 280 mg, KNO_3 - 80 mg, KCl - 60 mg, $MgSO_4$ - 360 mg, NaH_2PO_4 - 20 mg, H_3BO_3 - 1.4 mg, $MnSO_4$ - 5.0 mg, $ZnSO_4$ - 2.5 mg, and KI - 0.7 mg.

Extraction of taxol

Extraction of taxol was performed according to Strobel *et al* (1990) [10]. After incubating the culture for 3 and 4 weeks, the culture filtrate was passed through four-layered cheesecloth. To avoid fatty acid contamination of taxol, 0.25 g of $NaCO_3$ was added to the filtrate. The culture fluid was extracted with two equal volumes of methylene chloride and the organic phase was evaporated to dryness under reduced pressure at 35°C.

Column chromatography

A 1.5×30 cm column of silica gel was loaded with the crude sample dissolved in methylene chloride. Elution of the sample was done in a stepwise manner with solvent system as 70 ml of 100% methylene chloride, 20:1 v/v methylene chloride:ethylacetate, 10:1 v/v methylene chloride:ethylacetate, 6:1 v/v methylene chloride:ethylacetate, 3:1 v/v methylene chloride:ethylacetate, and 1:1 v/v methylene chloride:ethylacetate. Fractions having the same mobility as that of the authentic taxol were combined and evaporated to dryness. The residue was subjected to thin layer chromatography (TLC).

TLC analysis

TLC analysis was carried out on Merck 1 mm (20×20 cm) silica gel plate developed with solvent A (chloroform:methanol, 7:1 v/v) followed by solvent B (chloroform:acetonitrile, 7:3 v/v), solvent C (ethyl acetate:2-propanol, 95:5, v/v), solvent D (methylene chloride:tetrahydrofuran, 6:2 v/v), and solvent E (methylene chloride:methanol: dimethylformamide, 90:9:1, v/v/v) respectively. The area of the plate containing putative taxol was carefully removed by scraping off the silica at the appropriate Rf and eluted with acetonitrile. Taxol was detected with 1% w/v vanillin/sulfuric acid reagent after gentle heating 2. It appeared as a bluish spot that faded to dark gray after 24 hrs.

UV spectroscopic analysis of taxol

The purified sample of taxol was analyzed by UV absorption, dissolved in 100% methanol at 273 λ_{max} [19], and compared with authentic taxol.

DNA extraction

DNA extraction was performed using phenol chloroform extraction method. The total DNA extraction method was performed by Chomczynski and Sacchi (1987) [11].

RAPD analysis

Genetic diversity among isolates of *Pestalotia* from plants was assessed using RAPD. How many primers were used for reproducible banding patterns in this study. Primers were selected based on a preliminary study. PCR amplification was carried out with a total reaction volume of 50 µl containing 10× buffer, 2 mM of each dNTP, 2 µM/µl of each primer, 5 U/µl Taq DNA polymerase, and 50 ng of template DNA.

PCR amplification condition

Amplification was carried out with Eppendorf Mastercycler® ep94°C for 5 minutes, 34 cycles of 94°C for 40 seconds, 36°C for 30 seconds, 72°C for 90 seconds, and a final extension at 72°C for 10 minutes. The amplified products were separated on a 2.0% agarose gel in 1× TBE at 75 V for 3 hrs. The gel was stained with ethidium bromide and the amplified product was visualized under a UV transillumination.

RESULTS AND DISCUSSION

The endophytic fungi are one of the most unexplored and diverse group of organism that makes symbiotic associations with higher life forms and may produce beneficial substances for host [12] fungi have been widely investigated as a source of bioalive compounds. An excellent example of this is the anticancer drug, taxol which had been previously supposed to occur only in the plants [5].

Endophytic organism has received considerable attention after they were found to protect their host against insect pests, pathogens, and even domestic herbivorous [12]. However, only a few plants were studied for their endophyte biodiversity and their potential to produce bioactive compounds. Recently, studied were carried out about the endophytic biodiversity, taxonomy, reproduction, host ecology, and their effort on host [13-16]. Endophytes are now considered as an outstanding source of bioactive natural products because they occupy unique biological niches as they grow in so many unusual environments (Strobel and Daisy [5]; Strobel *et al*., 2009).

CONCLUSION

Genetic distances between each of the isolates were calculated, and cluster analysis was used to generate a dendrogram showing relationships between them. Isolates clustered in to three groups corresponding to their pathological reactions. He suggests that RAPD markers can be a quick and reliable alternative for differentiating isolates of *Fusarium oxysporum, F. vasinfectum* into their respective pathogenicity group.

DESCRIPTION OF THE FUNGI IN CULTURE (PLATE 1)

***Pestalotia acaciae* thuem**

Pustules, hemispherical or globose to lenticular, black, 150 µm diam. Conidiomataeustromatic, cupulate, separate or confluent, dark brown, at first immersed, then erumpent, thick walled, dehiscence irregular. Conidiogenous cells holoblastic, annellidic. Indeterminate, integrated, cylindrical, hyline, smooth, with one to three percurrent proliferations. Conidia 5 celled, elliptic to ovate, hardly constricted at septa, straight, 19-23×7-8.5 µm intermediate colored cells olivaceous, 14-19 µm long, the upper two umber, lowest olivaceous, apical hyline cells hidden, basal hyline cells obtuse, setulae 3, 21-32 µm long, and pedicels 3-6 µm long.

***Pestalotia canangae* Koord., Verh. Kakad**

Pustules, distributed without order, subglobose, subepidermal, erumpent on maturity, sooty, 75-150 µm in diam. Conidiomataeustromatic, cupulate, separate or confluent, dark brown, at first immersed, then erumpent, thick walled, dehiscence irregular. Conidiogenous cells holoblastic, annellidic. Indeterminate, integrated, cylindrical, hyline, smooth, with one to three percurret proliferations. Conidia 5 celled, clavate to fusiform slightly constricted at the septa, apical cells broad conic, short, turbinate, basal cells acute or long coniod, 5 celled 18-26×6.5-8.5 µm intermediate colored cells 14×16 µm, upper two of them umber or fuliginous: Setulae 3 or rarely branched, 17-31 µm widely divergent, pedicels 4-7 µm long.

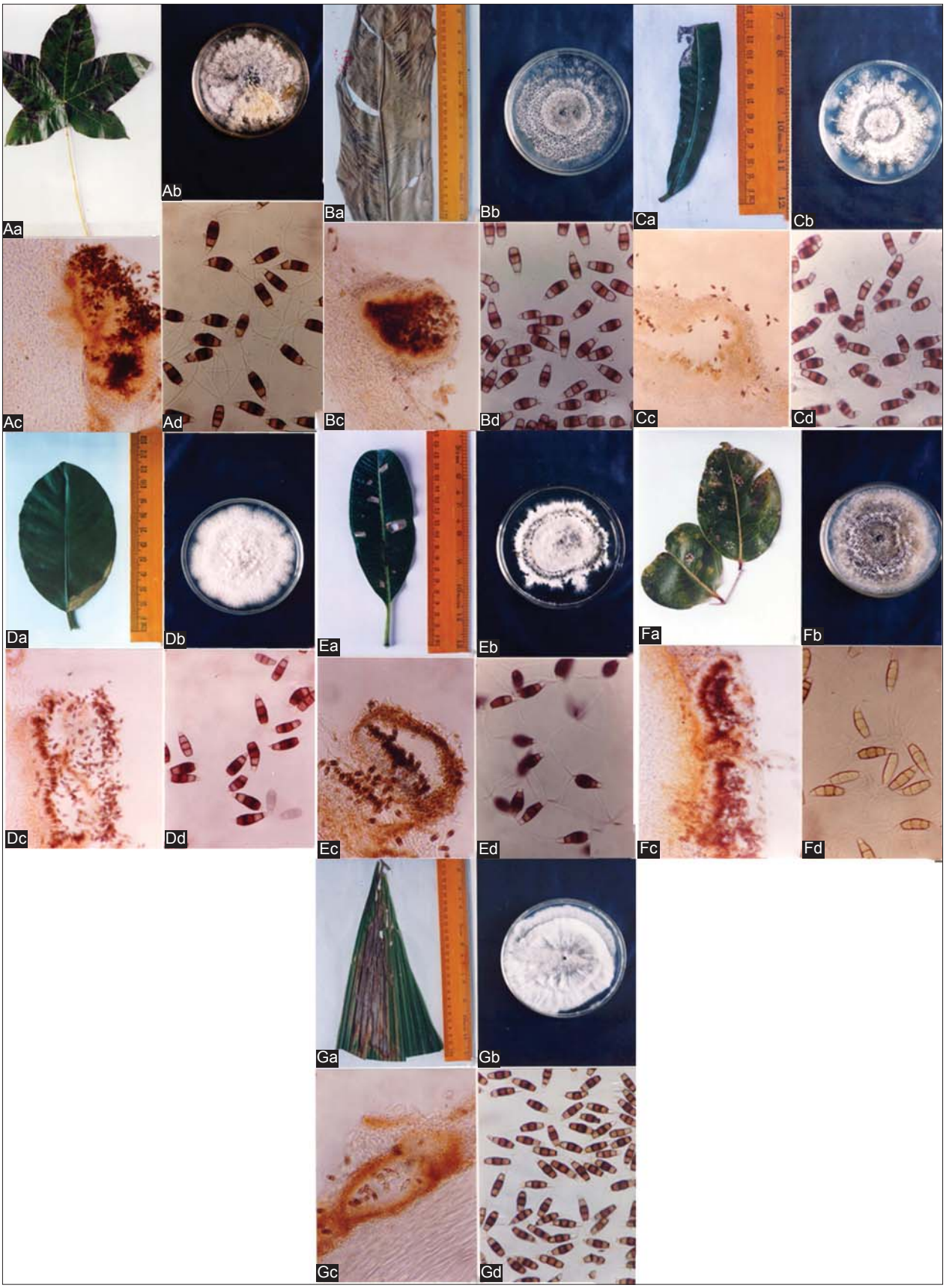

Plate 1: Different host, cultural morphology, vertical section of pycnidiaum and conidial morphology. Infected Leaf of (A) *Ailanthus malabarica*, (B) *Canna indica*, (C) *Codieum* sp., (D) *Artocarpus heterophyllus,* (E) *Plumeria alba,* (F) *Ficus glomerata,* (G) *Oredoxa* sp. (a) Infected leaf. (b) Culture, (c) Vertical section of the pycnidium ×320, (d) mature conidia (aqueous mount) ×700

***Pestalotia clavispora*., Atk.**
Pustules scattered 150-275 µm diam. Convex, prominent above, visible below. Conidiomataeustromatic, cupulate, separate or confluent, dark brown, at first immersed, then erumpent, thick walled, dehiscence irregular. Conidiogenous cells holoblastic, annellidic. Indeterminate, integrated, cylindrical, hyline, smooth, with one to three percurret proliferations. Conidia 5 celled, clavate to fusiform slightly constricted at the septa, apical cells broad conic, short, turbinate, basal cells acute or long coniod, 5 celled 18-26×6.5-8.5 µm intermediate colored cells 14×16 µm, upper two of them umber or fuliginous: Setulae 3 or rarely branched, 17-31 µm widely divergent, pedicels 4-7 µm long.

***Pestalotia conigena*., Lev**
Pustules, globose to lenticular, black innate to erumpent. Conidiomataeustromatic, cupulate, separate or confluent, dark brown, at first immersed, then erumpent, thick walled, dehiscence irregular. Conidiogenous cells holoblastic, annellidic. Indeterminate, integrated, cylindrical, hyline, smooth, with one to three percurret proliferations. Conidia 5 - celled, equilateral, slender fusiform, slightly constricted at septa, 19-26×7-8.5 µm intermediate colored cells 14×18 µm, long the upper two umber, lowest olivaceous, apical hyline cells conic to cylindrical, basal hyline cells long, acute or conic, setulae 2-4, 12-42 µm long, pedicels 9-16 µm long.

***Pestalotia eriobotrifolia*., Guba**
Pustules, globose to lenticular, subepidermal, erumpent, punctiform, 150-300 µm. Conidiomataeustromatic, cupulate, separate or confluent, dark brown, at first immersed, then erumpent, thick walled, dehiscence irregular. Conidiogenous cells holoblastic, annellidic. Indeterminate, integrated, cylindrical, hyline, smooth, with 1-3 percurret proliferations. Conidia 5 - celled, straight, broad fusiod, slightly constricted at septa, 19.5-25×7.5-9.5 µm intermediate colored cells olivaceous 13×16 µm, long apical hyaline cells short conic to cylindric, basal hyaline cells the upper two umber, lowest olivaceous, apical hyline cells conic, setulae 2-3, 17-27 µm long, pedicels 7 µm long.

***Pestalotia fibricola*., Grove**
Pustules, oblong - elliptic black 125-450×125-225 µm. Conidiomataeustromatic, cupulate, separate or confluent, dark brown, at first immersed, then erumpent, thick walled, dehiscence irregular. Conidiogenous cells holoblastic, annellidic. Indeterminate, integrated, cylindrical, hyline, smooth, with 1-3 percurret proliferations. Conidia 5-celled, elliptic, narrow fusiform, 15-18×4.5-5.5 µm intermediate colored cells olivaceous the upper two umber, lowest olivaceous, apical hyaline cells elongate 4-5 µm long, basal hyaline cells short, broad conic to acute, setulae 2-3, 11-19 µm long, pedicels 3-5 µm long.

***Pestalotia foedaris*., Sacc. and Ell**
Pustules, densely, gregarious, innate - reumpent, 200-425×170-340 µm. Conidiomataeustromatic, cupulate, separate or confluent, dark brown, at first immersed, then erumpent, thick walled, dehiscence irregular. Conidiogenous cells holoblastic, annellidic. Indeterminate, integrated, cylindrical, hyline, smooth, with 1-3 percurret proliferations. Conidia 5 - celled, rather slender to fusiform, tapering toward the base, erect, 19-24×5.5-7 µm, slightly or hardly constructed at septa. Intermediate-colored cells guttulate, 14-17 µm long the upper two umber, lowest olivaceous, walls darker, apical hyaline cells acute, setulae 3 rarely 4, divergent, slender, 4-18 µm long, pedicels 4-9 µm long.

The results here in indicate that *Pestalotia* spp. Despite being isolated from different host and possess considerable diversity in morphology and isolates grouped together based on similarities in conidal morphology as elaborated by Jeewon *et al*. [17,18]. Our studies also suggest diversity of endophytes as it differs in plant diversity. *Pestalotia* spp. are of considerable interest to researchers and pharmacists due to their capabilities of synthesizing a wide range of economically important bioactive molecules (Strobel, 2002; Tomita, 2003). *Pestalotia* as possible for screening for bioactive if the taxonomic identification is made only to genus level (Jeewon *et al.,* 2004).

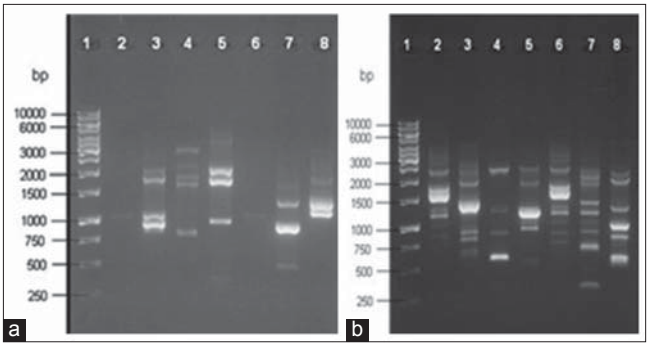

Fig. 1: (a and b) Agarose gel (1%) showing random amplified polymorphic DNA profile analysis of polymerase chain reaction amplification with primer PGF 01 and PGF 02 Lane 1: Marker, Lane 2: *Pestalotia acaciae*, Lane 3: *Pestalotia canangae,* Lane 4: *Pestalotia clavispora*. Lane 5: *Pestalotia conigena*, Lane 6: *Pestalotia eriobotrifolia*, Lane 7: *Pestalotia fibricola*, Lane 8: *Pestalotia foedaris*

In the present investigation, the isolates from different host (PGF: 01 PGF: 02) grouped together, the isolates were distinguishable from each other. RAPD techniques can be employed to detect genetic diversity by checking for different activities. RAPD markers did not differentiate and place the isolates into respective host or locations from which they were isolated. The relationship between species isolated from the same or different hosts do not support phylogenetic analysis and also morphologically similar species form close relationships rather than the isolates of the same host (Fig. 1).

REFERENCES

1. Suffness M, Wall ME. Discovery and development of taxol. In: Suffness M, editor. Taxol – Science and Applications. Boca Raton, USA: CRC Press; 1995. p. 3-25.
2. Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. *J Am Chem Soc* 1971;93(3):2325-7.
3. Stierle A, Strobel G, Stierle D. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 1993;260(5105):214-6.
4. Stierle A, Strobel G, Stierle D, Grothaus P, Bignami G. The search for a taxol-producing microorganism among the endophytic fungi of the Pacific yew, *Taxus brevifolia*. J Nat Prod 1995;58(9):1315-24.
5. Strobel G, Daisy B. Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 2003;67(4):491-502.
6. Kathiravan G, Sri Raman V. *In vitro* TAXOL production, by *Pestalotiopsis breviseta* – A first report. Fitoterapia 2010;81(6):557-64.
7. Kathiravan, G, Muthumary J. Extraction of taxol, an anticancer drug from coelomycetous fungi *Pestalotiopsis versicolor* and *Phyllosticta murrayicola*. Mycol Balcanica 2009;6(1-2):55-60.
8. Tejesvi MV, Kini KR, Prakash HS, Subbiah V, Shetty HS. Genetic diversity and antifungal activity of species of *Pestalotia* isolated as endophytes from medicinal plants. Fungal Divers 2007;24:37-54.
9. Assigbetse KB, Fernandez D, Dubois MP, Geiger JP. Differentiation of *Fusarium oxysporum* f. Sp. Vasinfectum races on cotton by random amplified polymorphic DNA (RAPD) analysis. Phytopathology 1994;84(6):622-6.
10. Strobel GA, Hess WM, Ford E, Sidhu RS, Yang X. TAXOL from fungal endophytes and the issue of biodiversity. J Int Microbiol Biotechnol 1990;179:417-23.
11. Chomczynski P, Sacchi N. Single-step method of RNA isolation by guanidinumthiocyanate-phenol: Chloroform extraction. *Anal Biochem* 1987;162(1):156-9.
12. Weber J. A natural control of Dutch elm disease. Nature (Lond) 1981;292:449-51.
13. Petrini O. Taxonomy of endophytic fungi of aerial plant tissues. In: Fokkema NJ, van den Heuvel J, editors. In: Microbiology of the Phyllosphere. Cambridge: Cambridge University Press; 1986. P. 175-87.
14. Arnold AE, Maynard Z, Gilbert GS. Fungal endophyte in dicotyledonous

neotropical trees: Patterns of abundance and diversity. Mycol Res 2001;105(2):1502-7.
15. Clay K, Schardl C. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. Am Nat 2002;160 Suppl 4:S99-127.
16. Selosse MA, Schardl CL. Fungal endophytes of grass: Hybrids rescued by vertical transmission? An evolutionary vertical perspective. New Phytol 2007;173(3):452-8.
17. Jeewon R, Liew EC, Simpson JA, Hodgkiss IJ, Hyde KD. Phylogenetic significance of morphological characters in the taxonomy *Pestalotiopsis* species. Mol Phylogenet Evol 2003;27(3):372-83.
18. Jeewon R, Liew EC, Hyde KD. Phylogenetic relationships of *Pestalotiopsis* and allied genera inferred from ribosomal DNA sequences and morphological characters. Mol Phylogenet Evol 2002;25(3):378-92.