รายงานฉบับสมบูรณ์
เรื่อง

การนำวิธีทางอณูชีววิทยามาใช้ในการจำแนก และการตรวจวินิจฉัยโรคฉี่หนูในคนและสัตว์

Application of molecular biological methods for differentiation and detection of leptospirosis in human and animals

โดย

ผศ.ดร.วาสนา สุขุมศิริชาติ
ภาควิชาชีวเคมี คณะแพทยศาสตร์
มหาวิทยาลัยศรีนครินทรวิโรฒ

Output

Presentations

1. Petchuensakul A, Saengjaruk P, Chasiri K, and **Sukhumsirichart W.** Identification of leptospires using rep-PCR. The 29th Congress on Science and Technology of Thailand, Golden Jubilee Convention Hall, Khon Kean University, Thailand, October, 20-22, 2003, p. 89.
2. Petchuensakul A, Saengjaruk P, Chasiri K, and **Sukhumsirichart W.** Discrimination of pathogenic and non-pathogenic *Leptospira* spp. by PCR. The 17th FAOBMB symposium/2nd IUBMB Special Meeting/7th A-IMBN Conference, which will be held in Bangkok, Thailand during November 22-26, 2004.
3. Petchchuensakul A., Saengjaruk P., Ssantiwatanakul S., **Sukhumsirichart W.** Molecular phylogenetic analysis of *Leptospira* spp. using band character of PCR-RFLP. Joint international Tropical Medicine Meeting 2004, 29 November-1 December, Ambassador Hotel, Bangkok, Thailand. p. 251.

Publication

1. Petcheunsakul A., Yasawong M., Saengjaruk P., Santiwatanakul S., Chansiri K., and Sukhumsirichart W. Identification of *Leptospira* spp. based on phylogenetic tree inferring from rep-PCR profile. Molecular and Cellular Probes (submitted), 2006.

IDENTIFICATION OF LEPTOSPIRA BASED ON PHYLOGENETIC TREE INFERRING FROM REP-PCR

A. Petcheunsakul,[1] M. Yasawong,[1] P. Saengjaruk,[2] S. Santiwatanakul,[2] K. Chansiri,[1] and W. Sukhumsirichart[1]

[1] Department of Biochemistry, [2] Department of Pathology, Faculty of Medicine, Srinakharinwirot University, Sukhumvit 23, Bangkok, 10110, Thailand

ABSTRACT

Leptospirosis is a zoonotic disease and remain health problem worldwide. This disease is caused by pathogenic bacteria in the genus *Letospira* which have been classified into more than 250 serovars. Because of rapid identification and differentiation of leptospiral serovars are critical in preventing high mortality associated with certain serogroups. Herein, twenty reference strains of letospires were analyzed by using rep-PCR technique. A single primer, iRep1, was used for generating fingerprinting patterns on agarose gel electrophoresis. The sizes of the amplified DNA fragments were estimated by comparison to standard molecular weight markers and scored as discrete variables. The phylogenetic tree was constructed by the use of neighbor-joining method which created the leptospiral serogroup into three different genetic clusters, A, B, and C. Firstly, cluster A contained djasiman, pyrogenes, icterohaemorrhageni, patoc, saigon, javanica, and sejroe. Secondly, cluster B consisted of autumnalis, cynopteri, hebdomadis, hyos, copenhageni and rachmati. Lastly, cluster C was comprised of canicola, bangkok, bratisiava, andamana CH 11, andamana. The data indicated that rep-PCR profiles could be used for discrimination of *Leptospira* serogroup from different animal reservoirs as well as dynamic study of the disease. Moreover, the method is simple, rapid and reliable which could be applied for laboratory use with basic equipment.

Keywords: rep-PCR, Fingerprinting, Leptospira, Differentiation, Phylogenetic tree.

Corresponding author: Dr. Wasana Sukhumsirichart
Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University, Sukhumvit 23, Bangkok 10110, Thailand.
Tel: +66-2-664100 ext. 4601 Fax: +66-2-2600125
E-mail: wasanas@swu.ac.th

INTRODUCTION

Leptospiral strains are being classified as serovars and serogroups on the basis of their antigenic relatedness. The strains within *Leptospira* species were differentiated based on genomic DNA [1-2]. A gold standard of molecular typing method is Pulse Field Gel Electrophoresis (PFGE) [3] but it is time consuming [4]. Several PCR-based DNA–fingerprinting methods have been introduced for genetic characterization of leptospires. Arbitrarily primed polymerase chain reaction (AP-PCR) has been used to determine the taxonomic relationship between leptospiral serovars [5-7], to rapidly identify leptospires [8], and to examine genetic variability among strains of serovar Hardjo [9]. However, this method lack of reproducibility and standardization. Amplified fragment length polymorphism (AFLP) is a highly sensitive method for DNA fingerprinting within any organism [10]. It is reproducible assay for differentiation clonally derived strains. However, this method is labor-intensive [11]. Repetitive element sequence-based PCR (rep-PCR) is a typing method that enables the generation of DNA fingerprinting that discriminates bacterial strains [12, 17-21]. This method relies on the uses primers complementary to interspersed repetitive consensus sequence that enhances the amplification of diverse-sized DNA fragments consisting of sequences between the repetitive elements [13]. Repetitive DNA sequences present in multiple copies in the genome of most gram-negative and gram-positive bacteria Three families of them have been identified, the 35-40 bp repetitive extragenic palindromic (REP) sequence [14], the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence [15], and the 154 bp (BOX) element [16]. These sequences appear to be located in distinct, intergenic positions around the genome. Rep-PCR is become the most widely used method of DNA typing in *Bacillus subtlis* [17], *Citrobacter diversus* [18], *Staphylococcus aureus* [19], *Enterobacter aerogenes* [20] and *Leptospira interrogans* [21]. The comparison studies of rep-PCR to other typing methods such as multi-locus enzyme electrophoresis [18], biochemical characterizations [22], or ribotyping [23-24], revealed that rep-PCR is more superior than the other methods. It provide a good correlation with PFGE results [25]. Rep-PCR exhibit high discrimination power, moderate reproducibility, low cost and rapid. Therefore, in this studies, rep-PCR was used to discriminate the twenty reference strains of leptospires. The phylogenetic trees were constructed to evaluate the reliability of this method.

MATERIALS AND METHODS

Leptospiral strains

Twenty reference strains of leptospires found in human and animals were obtained from National Institute of Health, Thailand, during the year 2001 to 2003. Details of the strains were given in Table 1.

Table 1 List of leptospiral serovars used in this study

Sample No.	Serovar	Strain	Serogroup	Species
1	ratislava	Jez Bratislava	Australis	*L. interrogan*
2	bangkok	BD92	Australis	*L. interrogan*
3	bataviae	Swart	Bataviae	*L. interrogan*
4	canicola	Hond Utrecht IV	Canicola	*L. interrogan*
5	copenhageni	M20	Icterohaemorrhagiae	*L. interrogan*
6	Icterohaemorrhagiae	M20	Icterohaemorrhagiae	*L. interrogan*
7	pyrogenes	Salinem	Pyrogenes	*L. interrogan*
8	hebdomadis	Hebdomadis	Hebdomadis	*L. interrogan*
9	Autumnalis new	Akiyami A	Autumnalis	*L. interrogan*
10	rachmati	Rachmat	Autumnalis	*L. interrogan*
11	patoc	Patoc 1	Semaranga	*L. biflexa*
12	andamana	CH11	Andaman	*L. biflexa*
13	CH11	CH11	Andaman	*L. biflexa*
14	djasiman	Djasiman	Djasiman	*L. kirschneri*
15	cynopteri	3522C	Cynopteri	*L. kirschneri*
16	saigon	L79	Louisiana	*L. noguchuii*
17	javanica	Veldrat Bat.46	Javanica	*L. borgpetersenii*
18	poi	Poi	Javanica	*L. borgpetersenii*
19	sejroe	M84	Sejroe	*L. borgpetersenii*
20	Hyros/tarassovi	Perepelitsin	Tarassovi	*L. borgpetersenii*

Preparation of genomic DNA

Genomic DNA of the leptospiral strains were extracted and purified using DNAzol reagent (Gibco ^{TM}). The purified DNA was dissolved in sterile-distilled water.

Rep-PCR analysis

The twenty DNA samples were subjected to rep-PCR amplification. The PCR processes were performed for at least three times in thermal cycle (MJ PTC 200, MJ Research Inc., USA) for confirming the results. The reaction was carried out in a 10 µl reaction mixture containing 50 ng of purified DNA, 2.5 µM primer iRep1 (5'-GCGGACTCATACCCGCT-3'), 300 µM of each dNTPs, 2.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase. The temperature profile for amplification consisted of one cycle of 2 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 50°C and 1.5 min at 72°C and one cycle of 2 min at 72°C. PCR products were electrophoresed in 1.5% agarose gels in TBE buffer at 100 volt for approximately 60 min prior to ethidium bromide staining.

Data Analysis

The size of all amplified DNA fragments were estimated from the agarose gel by comparison the size with standard molecular weight marker (100 bp DNA ladder). The data were scored as discrete variables, using "1" and '0' to indicate the presence and absence of a band. Only those fragments with medium to high intensity were taken into account. A pair-wises matrix of distances were computed using Jaccard's algorithm [26] in the FreeTree program [27]. From this matrices, the phylogenetic tree was constructed by using neighbor-joining method according to Saitou and Nei [28]. Robustness of the nodes in the dendrogram was tested by bootstrap analysis using 10,000 re-samplings. The tree was viewed, annotated and printed using TreeView [29].

RESULTS

The DNA fingerprinting resulted from iRep1 primer amplification of repetitive element of twenty reference strains leptospire serogroup were electrophoresed on agarose gel. The DNA pattern was shown in Fig. 1.

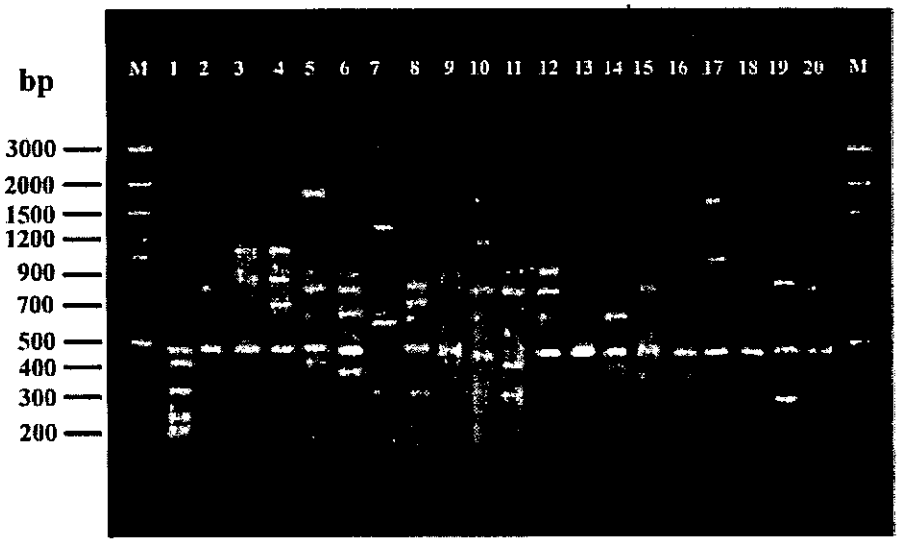

Fig. 1 Rep-PCR fingerprinting profile of twenty *Leptospira* reference strains using iRep1 primer. Lane M represents 100 bp ladder DNA; lane 1, bataviae; lane 2, copenhageni; lane 3, bangkok; lane 4, bratislava; lane 5, poi; lane 6, pyrogenes; lane 7, cynopteri; lane 8, hebdomadis; lane 9, sejroe; lane 10, rachmati; lane 11, saigon; lane 12, javanica; lane 13, canicola; lane 14, icterohaemorrhagiae; lane 15, djasiman; lane 16, patoc; lane 17, andamana; lane 18, CH11; lane 19, autumnalis; and lane 20, hyos/tarassovi .

Phylogenetic tree construction

The phylogenetic tree constructed by using neighbor-joining method according to Saitou and Nei was shown in Fig. 2. Twenty serogroup of *Leptospira* spp. scan be categorized into three different genetic clusters, A, B, and C. Cluster A contained djasiman, pyrogenes, icterohaemorrhageni, patoc, saigon, javanica, and sejroe. Cluster B consisted of autumnalis, cynopteri, hebdomadis, hyos, copenhageni and rachmati. Cluster C, comprised of canicola, bangkok, bratisiava, andamana CH 11, andamana.

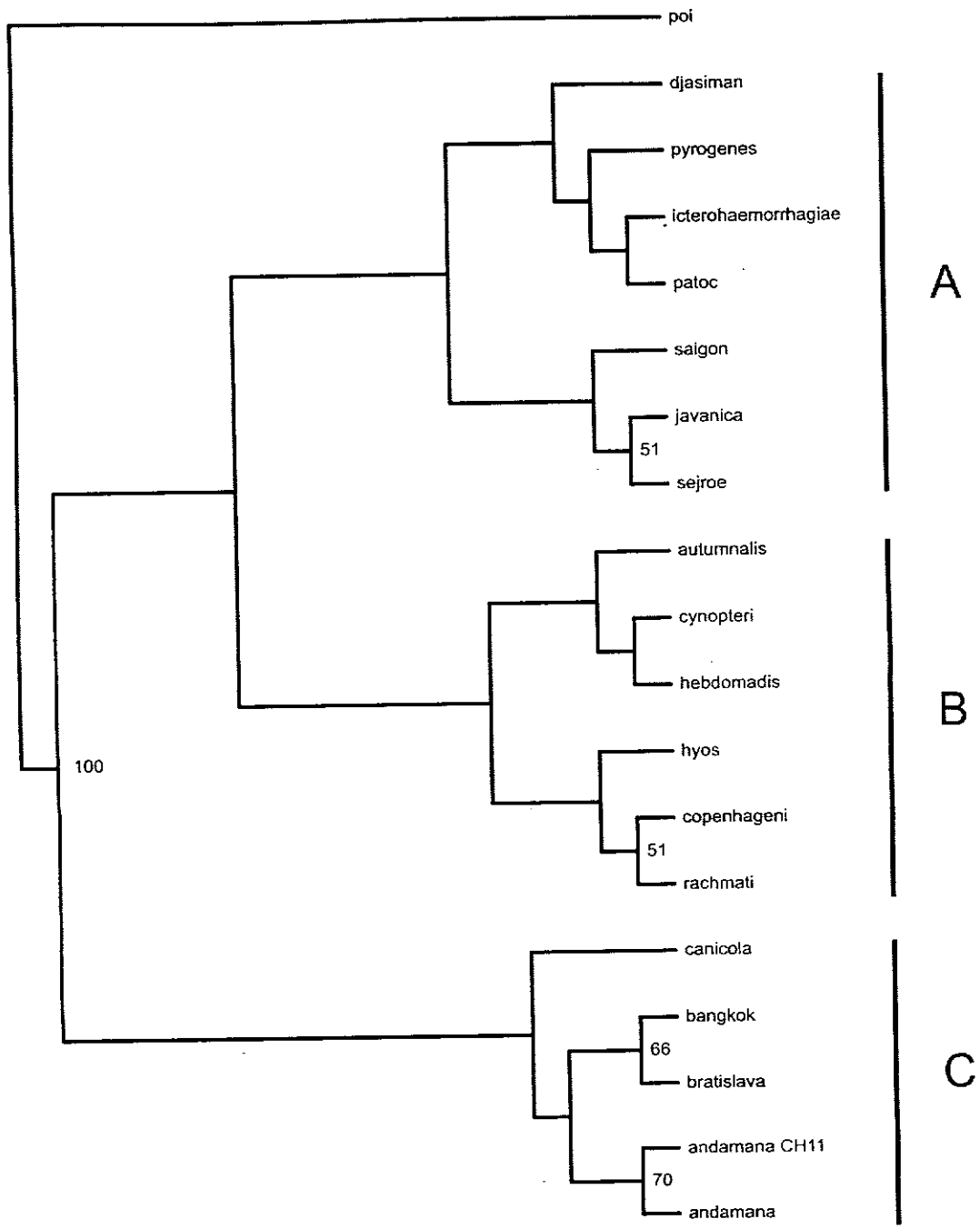

Fig. 2 Dendrogram generated for twenty serovars of *Leptospira* using neighbor-joining cluster analysis based on Jaccard's similarity estimated from rep-PCR data in FREE TREE program. Robustness of the nodes was tested by bootstrap analysis using 10,000 re-samplings. The numbers at the nodes on the tree are the bootstrap percent values (only values greater than 50 percent are shown).

DISCUSSION AND CONCLUSION

This studies describes the identification of *Leptospira* serogroup by using rep-PCR technique. This method is rapid and specific for detecting leptospiral DNA which contaminated with other bacteria due to the use of single primer designed from repetitive sequence of *L. interrogans* [21]. This technique provides reliable result and need only a common and inexpensive equipment [21], in contrast to fluorescent amplified fragment lenghtpolymorphism (FAFLP), a powerful genotyping technique which need a complicate and expensive instrument, therefore, it is suitable only for a highly equipped-laboratory [30].

. The iRep1 primer amplification yielded DNA fingerprinting that could be distinguished leptospires among twenty serogroups. The size of amplified fragments were in the range 200 bp to 2,000 bp, but major visible bands were appeared at 500 bp, 800 bp, and 900 bp. The DNA patterns were completely the same after three times repeating the experiment indicated of high reproducibility of the iRep1 primer. This method provides low cost per test and high discrimination power.

The phylogenetic tree constructed from the data of scored bands separated the leptospires into 3 clusters; A, B and C which included the members from 7, 6 and 4 serogroups, respectively. This data demonstrated that the *Leptospira* spp. belonging to different serogoups may contain similar repetitive DNA sequence in their genome.

In conclusion, the typing by using rep-PCR could be applicable for rapid discrimination of *Leptospira* spp. from human and animals. The technique is specific and reliable which is suitable for laboratory with basic molecular instrument.

ACKNOWLEDGMENT

This research was supported by Srinakhrinwirot University under the budget year 2003 (Project no. 45/2545 and 46/2545).

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PO-1.23

DISCRIMINATION OF PATHOGENIC AND NON-PATHOGENIC *LEPTOSPIRA* SPP. BY USING PCR

Ancharee Petchchuensakul[1], Patchairn Saengjaruk[2], Somchai Santiwatanakul[2], Wasana Sukhumsirichart[1]

[1]Department of Biochemistry, [2]Department of Pathology, Faculty of Medicine, Srinakharinwirot University, Tel. +66-2-2602122-4 ext. 4601; Fax. +66-2-2600125; E-mail: tuinoi@hotmail.com

Leptospirosis is an emerging infectious disease caused by *Leptospira* spp. Differential diagnosis of leptospires is difficult due to the variation and often "flu like" symptoms which may result in missed or delayed diagnosis. Confirmatory serological diagnosis of leptospires is usually made using microscopic agglutination test (MAT). Other techniques, such as the enzyme linked immunosorbent assay (ELISA) and slide agglutination test (SAT), can detect different classes of antibody but may subject to false positive reactions and require confirmation of these results by the MAT. In this study, PCR technique was applied for discrimination of *L. interrogans* from *L. interrogans* related bacteria such as non-pathogenic *L. biflexa* (serovar andamana and patoc) by using two sets of primers. The first primer set, inHF/inHR, was specific for *L. interrogans*; no PCR amplification product from bacteria related to *L. interrogans* and the DNA from *E. coli* was observed. The second set, LepA/LepB primers could detect both of pathogenic and non-pathogenic leptospires. These results revealed that PCR method can be used for simultaneous discrimination of pathogenic and non-pathogenic leptospires in one reaction tube.

Abstract: Leptospirosis is a zoonosis, which remains health problem worldwide. The classification is based on pairwise comparison of the extent of cross-absorption of pathogen against with specific antibody. In Thailand, the control of leptospires outbreaks is difficult due to the lack of epidemiological data in detecting and identifying the pathogen in epidemic areas. In this study the Repetitive-element primed PCR (Rep-PCR) technique were used for the identification of *Leptospira* spp. In twelve serovars were identified using six primers designed from repetitive sequence of bacteria. The DNA pattern from Box1 primer showed the most polymorphism between twelve serovars. The Rep-PCR technique was useful for the identification of *Leptospira* spp. in Thailand and increase the understanding of epidemiology of *Leptospira* spp.

SB-278P: Biotechnology of Phytoestrogen-rich; *P. mirifica*: III. Comparative proximate analysis

Wichai Cherdshewasart
Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand; e-mail address: cwichai@sc.chula.ac.th

Abstract: Proximate analysis of Kwao Krua plants revealed that M. collettii contained the highest amount of starch (32.59%) as compared with P. mirifica (27.50%) and B. superba (16.59%). M. collettii contained the highest amount of protein (8.42%) as compared with B. superba (7.35%) and P. mirifica (7.31%). B. superba contained the highest amount of fat (0.94%) as compared with P. mirifica (0.57%) and M. collettii (0.44%) M. collettii contained the highest amount of fiber (33.69%) as compared with B. superba (26.16%) and P. mirifica (10.92%). P. mirifica contained the highest amount of ash (3.69%) as compared with B. superba (1.36%) and M. collettii (0.57%) P. mirifica contained the highest amount of carbohydrate (77.51%) as compared with B. superba (64.19%) and M. collettii (56.88%). The results demonstrated clearly that the 3 species of Kwao Krua plants contained different amount of key parameters for food analysis.

SB-279P: Biotechnology of Phytoestrogen-rich; *P. mirifica*: IV. Mutagenicity and antimutagenicity by Ames

Malin Julsiri[1] and Wichai Cherdshewasart[2]
[1]Department of Microbiology, Faculty of Pharmacy, Mahidol University, Sriayudhya Road, Bangkok 10400; [2]Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand; e-mail address: cwichai@sc.chula.ac.th

Abstract: Mutagenic test and antimutagenic test by preincubation method with *S. typhimurium* strain TA 98 and TA 100 in the presence of AF2 was applied to the White (*P. mirifica*) Kwao Krua plants in parallel with kudzu (*P. lobata*). The mutagenicity test revealed that all the test plant extracts exhibited no mutagenicity. *P. mirifica* exhibited low number of His^+ revertant in the test with TA 98 at the extract concentration of 2.5, 5, 10 and 20 mg/plate. *P. mirifica* and *M. collettii* showed low number of His^+ revertant. *P. lobata* exhibited 1.45, 1.30, 1.46 and 1.17 times higher at the extract concentration of 2.5, 5, 10 and 20 mg/plate, respectively in the test with TA 100. The antimutagenicity test with TA 98 revealed that *P. mirifica* and *P. lobata* showed antimutagenicity. The survival test between *P. mirifica* and TA100 confirmed that the plant exhibited no cytotoxicity to the cells. The mutagenic and antimutagenic test of *P. mirifica* was confirmed in rec test. The result demonstrated clearly that the plant exhibited only antimutagenicity.

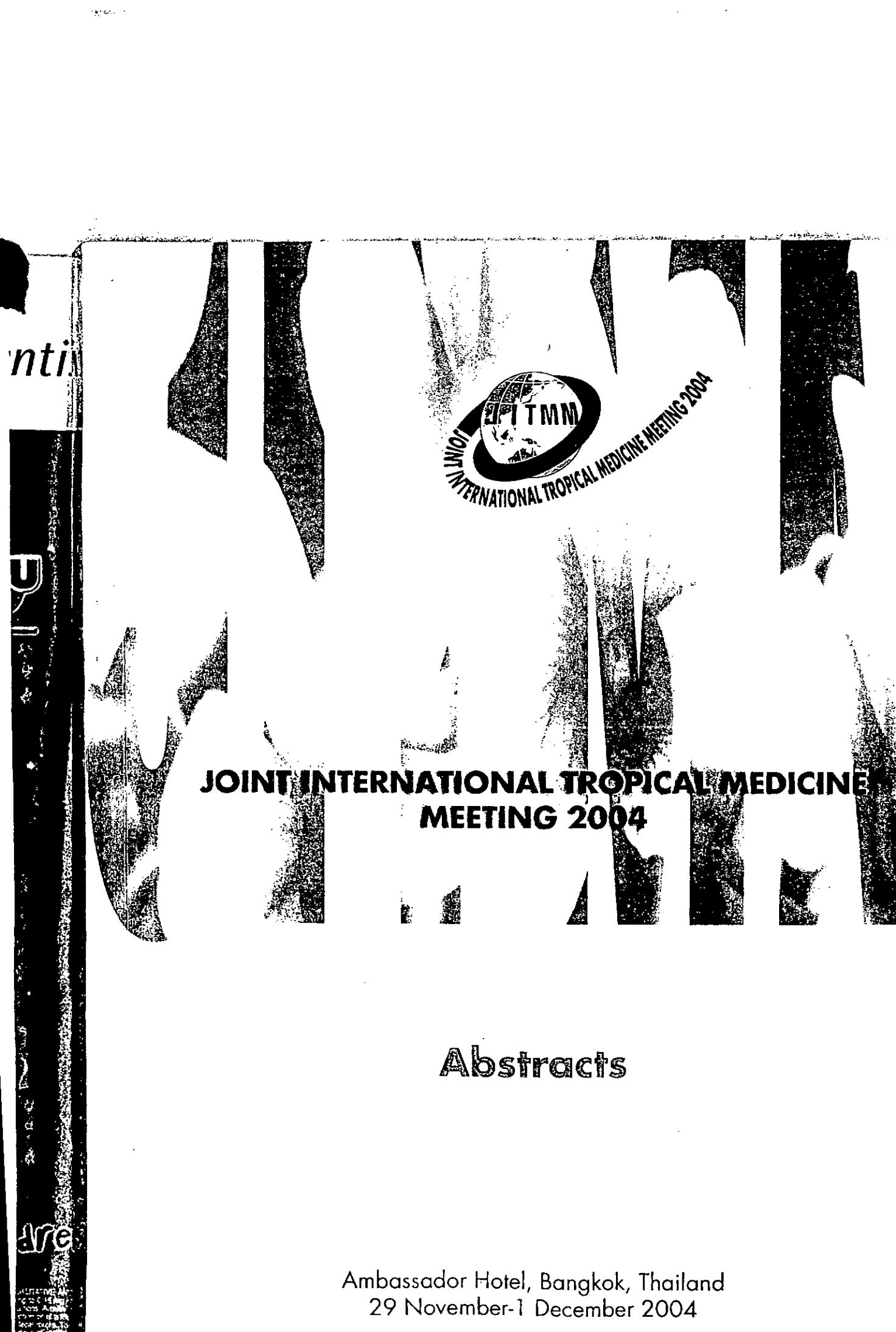
SB-280P: Biotechnology of Phytoestrogen-rich; *P. mirifica*: XVI. Micro-propagation and field trials

Wichai Cherdshewasart[1], Kanokporn Sompornpailin[2] and Surisa Reecharoen[1]
[1]Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand; e-mail address: cwichai@sc.chula.ac.th; [2]Department of Applied Biology, Faculty of Science, King Mongkut's Institute of Technology, Ladkrabang, Bangkok 10520, Thailand

Abstract: Micro-propagation and field trials were carried out for the phytoestrogen-rich herb *P. mirifica* with the aim of establishing the raw material supply for the rapidly increasing global phytoestrogen demand. Callus induction from *P. mirifica* seeds was successful in MS medium containing 0.5 mg / l NAA. The shoot induction was subsequently induced in MS medium containing 0.5 mg / l BAP. The root induction was effective in WPM medium supplemented with 1.0 mg / l NAA and 2 % sucrose. Multiple shoots emerged from the seedling nodal tissue on MS medium supplemented with 0.5 mg / l BAP. Root culture derived from the excised seedling roots showed a maximal mass yield in liquid MS medium containing 0.5-1.0 mg / l NAA. Field trials of the *in vitro*-derived plants showed numerous tubers with a similar pattern of structure and cellular organization as that of the naturally grown tubers. TLC analysis showed a similar pattern in the field trial derived tubers and the naturally grown tubers as well as root cultures whereas weaker chemical profile was present in the callus.

SB-281P: Biotechnology of Phytoestrogen-rich; *P. mirifica*: XIV. Microbial elimination in Kwao Krua herbs

Wannipa Phianphak[1], Sirirat Rengpipat[2] and Wichai Cherdshewasart[3]



JOINT INTERNATIONAL TROPICAL MEDICINE MEETING 2004

Abstracts

Ambassador Hotel, Bangkok, Thailand
29 November-1 December 2004

MOLECULAR PHYLOGENETIC ANALYSIS OF *LEPTOSPIRA* SPP. USING BAND CHARACTERS OF PCR-RFLP

Anchalee Petchchuensakul[1], Patcharin Saengjaruk[2], Somchai Santiwatanakul[2], Wasana Sukhumsirichart[1]

[1]Department of Biochemistry, [2]Department of Pathology, Faculty of Medicine, Srinakharinwirot University, Thailand

Leptospirosis is a zoonosis caused by *Leptospira* spp. These bacteria comprise of several species which are found as a free living and a causative agent. The objective of this study was to evaluate the genetic relationship between *Leptospira* serovars by using PCR-RFLP technique. Twenty reference strains of leptospires were amplified using primers designated from 16S rDNA gene (nucleotide positions 46 to 375). The PCR product of 331 bp in length was separately digested with *Hha* I, *Dde* I, *Sau* 3AI, and *Alu* I. The digested DNA was eletrophoresed on 1.8% agarose gel and their band patterns were analyzed by computer program analysis. Six individual strains were clearly distinguished. Non-pathogenic and pathogenic strains can also be differentiated. Phylogenetic tree constructed by comparing band characters placed the leptospires into 10 clades. From this data suggest that PCR-RFLP is a simple method for studying the genetic relationship of leptospires. · · · · ·