

## 16S rRNA-Based Metagenomic Analysis of Endophytic Actinomycetes Diversity from *Tinospora crispa* L. Miers

MONA PRIMANITA<sup>1</sup>, ARIS TRI WAHYUDI<sup>2</sup>, AND YULIN LESTARI<sup>2,3\*</sup>

<sup>1</sup>Microbiology Study Programme, Graduate School, Institut Pertanian Bogor, Darmaga Campus, Bogor 16680, Indonesia;

<sup>2</sup>Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor, Darmaga Campus, Bogor 16680, Indonesia;

<sup>3</sup>Biopharmaca Research Center, Institut Pertanian Bogor, Taman Kencana Campus, Bogor 16151, Indonesia

Endophytic actinomycetes associated with medicinal plants is very important as source of various bioactive compounds. The fact that more than 99% of microbes that exist in nature may have the potency but still unexplored. Published data regarding diversity of endophytic actinomycetes from *Tinospora crispa* is mainly based on a culturable approach. This paper describes the first reported data regarding metagenomic analysis on the diversity of endophytic actinomycetes from *T. crispa* based on 16S rRNA gene using PCR-DGGE. There were some similarities amongst endophytic actinomycetes found in stems, roots, and leaves with soil actinomycetes community in the rhizosphere of *T. crispa*. There were a total of 21 bands found from the DGGE analysis which were interpreted using Phoretix 1D software. Diversity of actinomycetes in the stems, leaves, roots were represented by 17, 16, and 14 bands, respectively. Whereas only 10 bands represented diversity of actinomycetes in the soil rhizosphere. The 12 dominant and or different bands with 180 bp in size were molecularly sequenced. The A4 and A9 bands have 95% and 86% similarities with *Williamsia* and *Streptomyces*, respectively. These similarities were less than 97% thus may indicate novel actinomycetes. The other 10 sequenced bands have closed similarity ranging from 97-100% and they were closely related to the genus *Streptomyces*, *Microbacterium*, *Amycolatopsis*, *Actinomadura*, *Actinoplanes*, *Actinokineospora*, *Kibdelosporangium*, *Williamsia*, and *Kocuria*. These findings indicate that diversity of actinomycetes can be found associated with *T. crispa*.

Key words: 16S rRNA, DGGE, endophytic actinomycetes, metagenomic, *Tinospora crispa*

Aktinomiset endofitik yang berasosiasi dengan tanaman obat berperan penting sebagai sumber berbagai senyawa bioaktif. Fakta mengindikasikan bahwa lebih dari 99% mikrob yang ada di alam diduga memiliki potensi, tetapi belum banyak dieksplorasi. Keragaman aktinomiset endofit yang telah dilaporkan masih terbatas pada pendekatan *culturable*. Data hasil penelitian ini merupakan informasi baru tentang keragaman aktinomiset endofit *T. crispa* yang dianalisis status metagenomiknya berdasarkan gen 16S rRNA menggunakan PCR-DGGE. Terdapat beberapa kesamaan antara aktinomiset endofit yang ditemukan di batang, akar, dan daun dengan komunitas aktinomiset tanah di rhizosfer *T. crispa*. Total pita yang ditemukan dari analisis DGGE yang menggunakan perangkat lunak *Phoretix 1D* yaitu sebanyak 21 pita. Keanekaragaman aktinomiset di batang, daun dan akar yaitu 17, 16 dan 14 pita, sementara hanya 10 pita ditemukan di tanah rhizosfer. Berdasarkan profil DGGE, diperoleh 12 pita yang dominan dengan panjang pita 180 bp yang kemudian disekuensing. Pita A4 dan A9 masing-masing memiliki kesamaan 95% dan 86% dengan *Williamsia* dan *Streptomyces*. Kesamaan sekuen tersebut kurang dari 97% sehingga diduga aktinomiset tersebut tergolong baru. Terdapat 10 pita lainnya yang memiliki kesamaan berkisar 97-100% dan berkekerabatan dekat dengan genus *Streptomyces*, *Microbacterium*, *Amycolatopsis*, *Actinomadura*, *Actinoplanes*, *Actinokineospora*, *Kibdelosporangium*, *Williamsia*, dan *Kocuria*. Data tersebut menunjukkan bahwa beragam aktinomiset dapat ditemukan berasosiasi dengan *T. crispa*.

Kata kunci : 16S rRNA, aktinomiset endofit, DGGE, metagenomik, *Tinospora crispa*

*Tinospora crispa* L. Miers is a medicinal plant traditionally utilized by Asian people as an antidiabetic agent (Puranik *et al.* 2010; Patela and Mishrab 2012). Endophytic actinomycetes from *T. crispa* are capable of producing secondary metabolites which can also

function as an antidiabetic agent (Pujiyanto *et al.* 2012). This phenomenon is presumably the result of genetic exchange and evolution between endophytes and their host (Tan and Zou 2001). The biological function of *T. crispa* as an antidiabetic agent may be associated with the presence and diversity of actinomycetes in the host plant tissue. Actinomycetes are very important microbes as they have an ability to

\*Corresponding author; Phone: +62-251-8622833, Fax: +62-251-8622833, E-mail: [yulinlestari@gmail.com](mailto:yulinlestari@gmail.com)

produce various metabolite compounds. Previous study done by Pujiyanto *et al.* (2012) showed that among several medicinal plants which were traditionally used as antidiabetics possessed endophytic actinomycetes which may function as source of antidiabetics. Amongst the 65 culturable endophytic actinomycetes isolated from those antidiabetic plants, 32 isolates were found from *T. crisper*.

Diversity of actinomycetes in *T. crisper* can be explored using culture dependent and or independent approaches. Isolation of actinomycetes using a culture-dependent method has faced several constraints such as cultivation conditions, techniques used, isolation media and cultivation time (Qin *et al.* 2012). These partly because many members of the actinomycetes genus are relatively slow-growing and some genera are often difficult to be cultured (Nimnoi *et al.* 2010). Moreover, it is only 0.1-10% of all microbes in nature can be cultured in the laboratory (Zeyauallah *et al.* 2009). Culturable microbes can grow rapidly in nutrient-rich media, aerobic conditions and at moderate temperatures (Thontowi 2009), however, more than 99% of microbes that exist in nature, which may have a very useful potential, are still not known nor utilized (Zeyauallah *et al.* 2009). The suitable method, i.e. a culture-independent, needs to be used to explore whole diversity of endophytic actinomycetes. For that purpose, a metagenomic approach, which does not require culture, is used for genome analysis of bacterial communities (Thontowi 2009).

The DGGE (Denaturing Gradient Gel Electrophoresis) technique is used to study microbial communities in situ. PCR-DGGE result can be used to observe metagenomic data, especially from the dominant endophytic actinomycete species that colonizes plant organs and the rhizosphere (Nimnoi *et al.* 2010). Information on endophytic actinomycete diversity from *T. crisper* based on 16S rRNA gene using a culture-independent technique has remained obscure. This research aimed to explore diversity of endophytic actinomycetes in *T. crisper* based on 16S rRNA-based metagenomic analysis using PCR-DGGE. For data comparison, diversity of actinomycetes in the rhizosphere of *T. crisper* was also assessed. Metagenomic information on endophytic actinomycetes from *T. crisper* can be further used for searching of their functional consequences.

## MATERIALS AND METHODS

**Sample Extraction and Surface Sterilization.** *T. crisper* plants were obtained from Medicinal Plants Collection Garden, Biopharmaca Research Center, Institut Pertanian Bogor. The leaves, stems and roots of plants were cleaned and cut into pieces, then their surfaces were sterilized sequentially by soaking in 70% alcohol for 1 min, then in 1% sodium hypochlorite (NaOCl) for 5 min, followed by soaking in 70% alcohol for 1 min. In the final step, samples were rinsed 3 times with sterile distilled water. To prove that only endophytic actinomycetes genome were extracted, the last soaking water during surface sterilization process was spreaded on Humic Acid Vitamin B (HV) medium and checked for their grown colonies (Combs and Franco 2003) used as negative control test. Soil samples were taken from rhizosphere of *T. crisper* at a depth of approximately 1-5 cm from the soil surface.

**Genomic DNA Extraction from Endophytic Actinomycetes and Soil Actinomycetes.** Genomic DNA of endophytic actinomycetes in plant samples was extracted using Genomic DNA Mini Kit (Plant) Geneaid. Soil DNA was extracted using the Power Soil DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA). The used procedures followed the standard protocol recommended by the manufacturer. The extraction results were then quantitatively checked using Nano Drop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

**PCR for DGGE.** Amplification was done using nested PCR technique which was conducted to obtain propagation of 16S rRNA gene using actinomycetes specific primers (Table 1). Step 1 amplification was conducted using Promega GoTaq Green Master Mix with the mixed composition consisted of 12.5 µL of master mix 27f dan 16Sact1114r 60 pmol primers at 0.25 µL each, 5 µL of DNA template and added with nuclease free water to a volume of 25 µL. PCR conditions used were pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 60 s, annealing at 65 °C for 45 s (a decrease at 0.5 °C per cycle was set for the first 20 cycles, the last decrease was at 55 °C), elongation at 72 °C for 120 s for 30 cycles and final elongation at 72 °C for 7 min (Zhang *et al.* 2013). Step 2 amplification also used Promega GoTaq Green Master Mix with the mixed composition of 25 µL of Promega GoTaq Green, P338f-GC dan P518r 100

Table 1 Primer sequences

Primer	Sequences	Reference
27f	5'-AGAPTTTGATCCTGGCTCAG-3'	Bruce <i>et al.</i> 1992; Martina <i>et al.</i> 2008
16Sact1114r	5'-GAGTTGACCCCGGCRGT-3'	Bruce <i>et al.</i> 1992; Martina <i>et al.</i> 2008
P338f	5'-ACTCCTACGGGAGGCAGCAG-3'	Ovreas <i>et al.</i> 1997
P518r	5'-ATTACCGCGGCTGCTGG-3'	Ovreas <i>et al.</i> 1997
GC clamp	5'CGCCCGCCGCGCGCGGGCG GGGCGGGGCACGGGGG	Ovreas <i>et al.</i> 1997

\* Description: F / f, forward primer; R / r, reverse primer, GC clamp added at the end 5'primer P338f

pmol primers at 0.25  $\mu\text{L}$  each, 1  $\mu\text{L}$  of step 1 PCR products was used as a DNA template and added with nuclease free water to a volume of 50  $\mu\text{L}$ . Amplification was conducted using T1-thermocycler (Biometra, Goettingen, Germany) with these following optimizations: pre-denaturation at 94  $^{\circ}\text{C}$  for 5 min, denaturation at 94  $^{\circ}\text{C}$  for 60 s, annealing at 55  $^{\circ}\text{C}$  for 45 s, elongation at 72  $^{\circ}\text{C}$  for 60 s for 30 cycles and final elongation at 72  $^{\circ}\text{C}$  for 5 min (Zhang *et al.* 2013).

The amplification result was then observed by migrating 5  $\mu\text{L}$  of the amplicon on 1% agarose gel, 80 volts for 45 min. The migration result was then stained with 0.1% ethidium bromide for 15 min and then viewed under UV transilluminator and documented using Gel Doc. The remaining PCR products were stored at -20  $^{\circ}\text{C}$  prior to be analysed using DGGE.

**DGGE (Denaturing Gradient Gel Electrophoresis).** The DGGE was conducted using the D Code Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). The amount of 30  $\mu\text{L}$  template (25  $\mu\text{L}$  of PCR products + 5  $\mu\text{L}$  of Loading Dye) was migrated at 1 mm 8% polyacrylamide gel (acrylamide-bisacrylamide [37.5:1]) in a 7 L 1X TAE (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) using urea as a denaturant. The denaturant gradient of 30% -70% (100% denaturant was made with 8.4 g of 7 M urea, 8 ml of formamide, 0.4 mL of 50x TAE, 4 mL of acrylamide-bisacrylamide and added with sterile distilled water to a volume of 20 mL). Migration was conducted at 60  $^{\circ}\text{C}$  and 150 volts for 5 h. Once the migration was completed, the gel was soaked and stained with SYBR Safe (Invitrogen-Molecular Probes, Carlsbad, CA, USA) for 1 h. Gel visualization was conducted using the G:BOX (Syngene, Frederick, MD, USA). The selected dominant and or different bands were cut and each

band was put into a microtube containing 100  $\mu\text{L}$  of ddH<sub>2</sub>O and stored for 24 hours at 4  $^{\circ}\text{C}$ . Acrylamide gel image results with G:BOX were analyzed using 1D Phoretix software to estimate the total band appeared. DGGE products was settled for 24 h, then the products were vortexed and amplified in PCR instrument using primers without GC Clamp with similar PCR conditions.

**Sequencing of 16S rRNA Gene and Construction of Phylogenetic Tree.** The PCR products were then sent to the sequencing service company (1stBASE Malaysia). Sequencing was performed using double stranded PCR-DGGE product. Sequencing results were compared to the data base from the NCBI website (<http://www.ncbi.nlm.nih.gov>) with the Basic Local Alignment Search Tool (BLAST). Nucleotide sequence alignment and phylogenetic tree construction were conducted using MEGA 5.2 software. Construction of a phylogenetic tree was conducted using the Neighbour Joining method.

## RESULTS

**PCR-DGGE Profile and Phylogenetic Tree of Actinomycetes Based on 16SrRNA Genes.** The last soaking water which was placed in HV test medium showed no growth, proving that only genomic DNA of endophytic actinomycetes were extracted. The genomic DNA concentrations of 35.1 ng  $\mu\text{L}^{-1}$ , 37.2 ng  $\mu\text{L}^{-1}$ , and 30.9 ng  $\mu\text{L}^{-1}$  were obtained from the roots, stems and leaves, respectively. The soil genomic DNA concentration obtained was 3.7 ng  $\mu\text{L}^{-1}$ . The purity of DNA obtained from this extraction with the A260/A280 parameter ranged from 1.6 to 1.7 which slightly lower than the recommended value (1.8-2.0) (Sambrook and Russell 2001), indicating some

impurities. However, this impurity seem did not interfere during the DNA replication process.

Amplification with nested PCR was conducted in 2 steps using actinomycetes specific primers i.e. 27f/16Sact1114r and P338fGC/P518r. Step 1 PCR was successfully amplifying the band with a length of ~1087 bp (Fig 1a), while step 2 PCR was at ~180 bp length (Fig 1b). In this study, 12 dominant and or different bands were obtained from DGGE profile (Fig 2a). There were some similarities between endophytic actinomycetes community found in stems, roots, leaves and soil. Interpretation using Phoretix 1D software showed that diversity of actinomycetes community were represented by 17, 16, 14 and 10 bands in the stems and leaves, roots and soil, respectively. Total number of bands obtained with this software was 21 bands (Fig 2b). The 12 dominant sequenced bands revealed their genetic relationship based on their phylogenetic tree (Fig 2c). Analysis of phylogenetic tree based on genetic distance matrix (p-distance) showed nucleotide sequence similarity in the stems and leaves, but there were several differences from the roots and soil (Fig 2d). Almost all endophytic actinomycetes who reside in leaves have also been found in the stems, while for roots and soil, their actinomycetes were more varied.

The alignment results of 12 partial sequences (~180 bp) of endophytic actinomycetes from *T. crispata* plant and soil actinomycetes in the rhizosphere was analyzed using the BLAST program (Table 2). The A2 and A10 sequences have 100% similarity with *Actinokineospora auranticolor* strain IFO 16518 and *Amycolatopsis orientalis* strain HCCB10007,

respectively. A1, A3, A5, A6, A7, A8, A11 and A12 sequences have  $\geq 97\%$  similarity with *Streptomyces acidiscabies* strain ATCC 49003, *Williamsia marianensis* strain DSM 44944, *Kocuria halotolerans* strain YIM 90716, *Microbacterium ginsengisoli* strain Gsoil 259, *Kibdelosporangium philippinense* strain A 80407, *Actinomadura rifamycinii* strain JCM 3309 and *Actinoplanes* sp., respectively. A relatively new sequence was also found based on the percentage of a maximum identity from the BLAST results showing the data base sequence similarity at 95% and 86 % of the ~180bp (A4 and A9 sequences). These sequences were closely related with *Williamsia marianensis* strain DSM 44944 and *Streptomyces javensis* strain B22P3, respectively. The phylogenetic relatedness of endophytic actinomycetes 16S rRNA gene sequences from *T. crispata* and rhizosphere soil was compared with closely related strains sequenced from database (Fig 3).

**Abundance of Endophytic Actinomycetes in Plants and Soil Actinomycetes.** Based on the nucleotide partial sequenced at ~180 bp length, there were 12 species of endophytic and soil actinomycetes dominant in *T. crispata* plant. Among the 12 species obtained, one species found in the soil has a closely related with *Streptomyces acidiscabies* strain ATCC 49003 (1). Meanwhile, six endophytes and soil actinomycetes have a close relationship to *Actinokineospora auranticolor* strain IFO 16518 (2), *Kocuria halotolerans* strain YIM 90716 (5), *Microbacterium ginsengisoli* strain Gsoil 259 (6), *Kibdelosporangium philippinense* strain A 80407 (8), *Streptomyces javensis* strain B22P3 (9), and *Acti-*

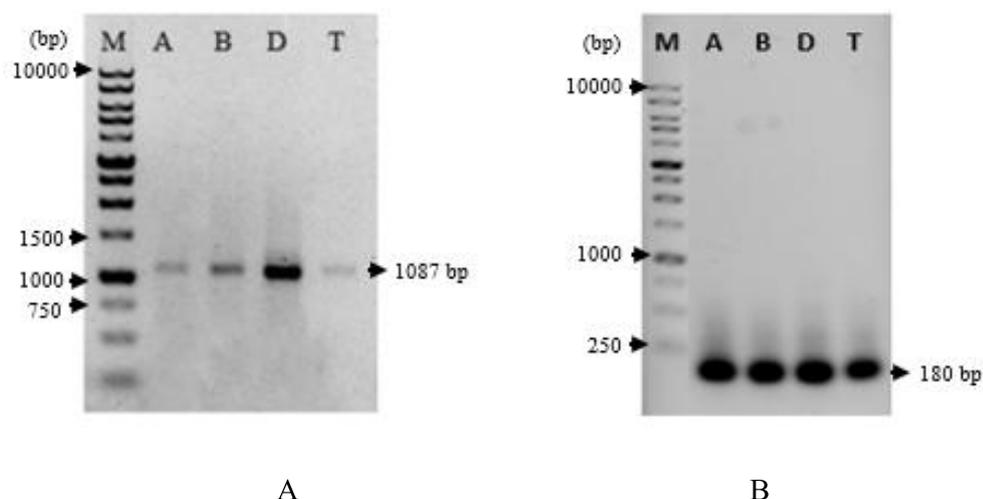


Fig 1 (A) Step 1 amplification results of 16S rRNA gene of endophytic actinomycetes in roots, stems, leaves of *T. crispata* plant (A), (B), (D) and soil (T) (~1087bp) and (B) Step 2 with length of ~180bp.

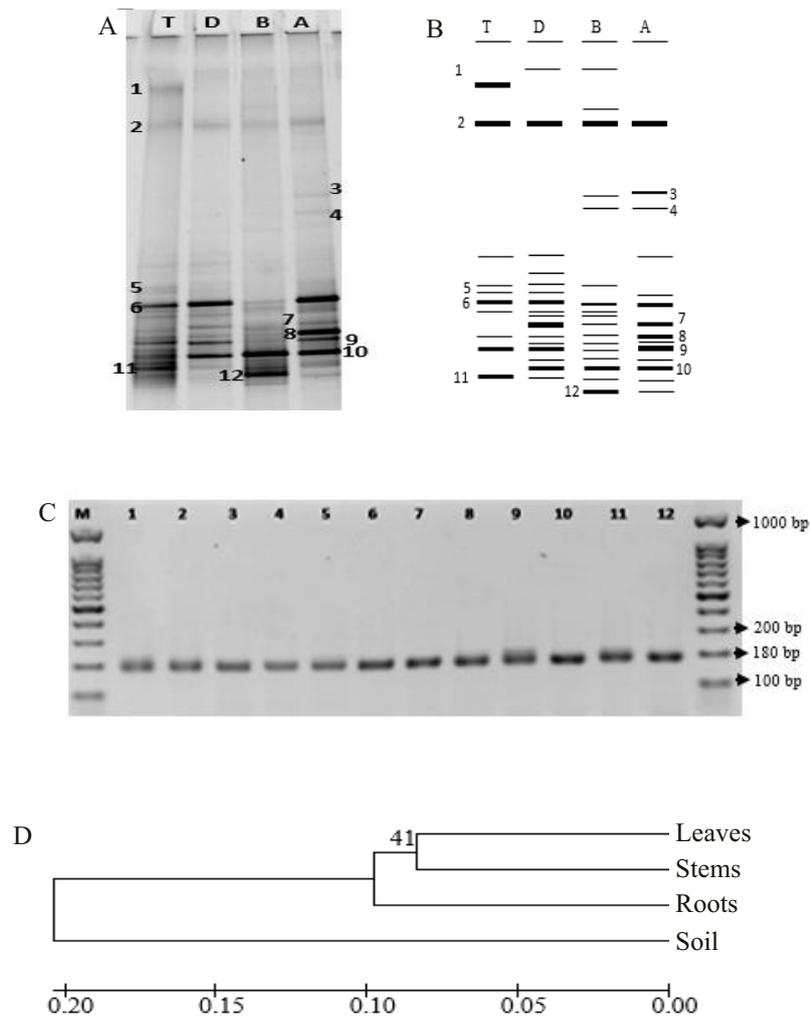


Fig2 (A) DGGE profile 16S rRNA gene of endophytic actinomycetes from *T. crispata* plant and soil actinomycetes; (B) Interpretation from Phoretix 1D software. Numbers next to bands indicate the cut/splitted bands for re-amplification. Wells from left to right: (T) soil, (D) leaves, (B) stems, and (A) roots; (C) Re-amplification DGGE bands; (D) p-distance analysis.

Table2 Sequence similarity of 16S rRNA gene of endophytic actinomycetes from medicinal plant *T. crispata* and soil actinomycetes with reference strain (GenBank)

Comparison sequence from database	Band	Maximum identity	E-Value	Accession
<i>Streptomyces acidiscabies</i> strain ATCC 49003	A1	97%	1e-81	NR116534.1
<i>Streptomyces javensis</i> strain B22P3	A9	86%	6e-46	NR028958.1
<i>Williamsia marianensis</i> strain DSM 44944	A3	97%	2e-80	NR043263.1
<i>Williamsia marianensis</i> strain DSM 44944	A4	95%	1e-76	NR043263.1
<i>Kocuria halotolerans</i> strain YIM 90716	A5	99%	8e-89	NR044025.1
<i>Kocuria halotolerans</i> strain YIM 90716	A7	99%	1e-83	NR044025.1
<i>Actinokineospora auranticolor</i> strain IFO 16518	A2	100%	2e-89	NR040873.1
<i>Microbacterium ginsengisoli</i> strain Gsoil 259	A6	97%	1e-81	NR041516.1
<i>Kibdelosporangium philippinense</i> strain A 80407	A8	98%	2e-85	NR025572.1
<i>Amycolatopsis orientalis</i> strain HCCB10007	A10	100%	3e-89	NR103940.1
<i>Actinomadura rifamycinini</i> strain JCM 3309	A11	99%	3e-88	NR113155.1
<i>Actinoplanes</i> sp. SE 50/110 strain SE 50/110	A12	98%	2e-84	NR074431.1

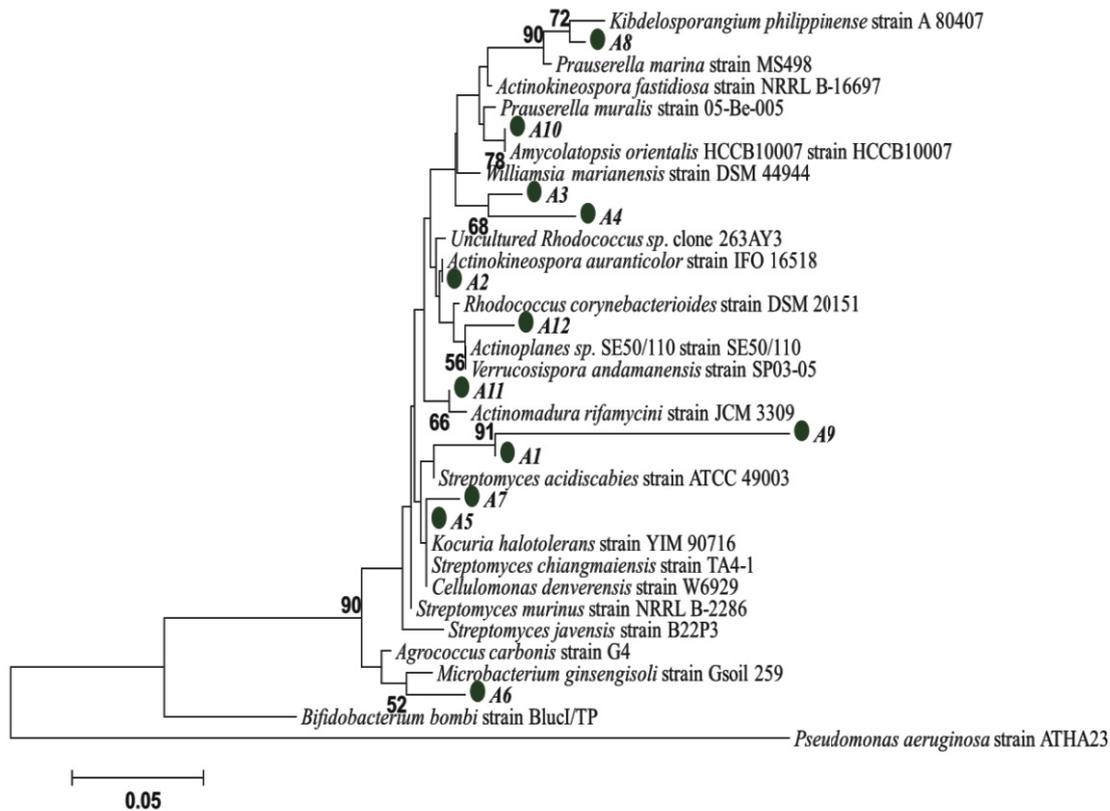


Fig 3 Phylogenetic relatedness of 16S rRNA gene sequences of endophytic actinomycetes from *T. crisper* and soil actinomycetes of DGGE product (analysis with 1000 bootstrap replicated).

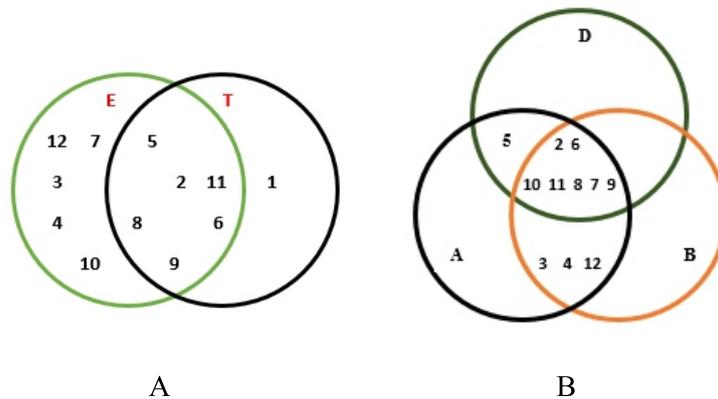


Fig 4 (A) Abundance of soil actinomycetes (T) and endophytic actinomycetes (E) (B) endophytic actinomycetes in roots (A), stems (B) and leaves (D).

*nomadura rifamycini* strain JCM 3309 (11). There were 5 species which found only as endophytes which were closely related with *Williamsia marianensis* strain DSM 44944 (3,4), *Amycolatopsis orientalis* strain HCCB10007 (10), *Kocuria halotolerans* strain YIM 90716 (7), *Actinoplane* sp. strain SE 50/110 (12) (Fig 4a).

There were several similar endophytic actinomycetes found in different plant organs (Figure 4b). Actinomycetes that have a close relationship with *Williamsia marianensis* strain DSM 44944 (3,4) and *Actinoplanes* sp. strain SE 50/110 (12) were found in

roots and stems, while *Kocuria halotolerans* strain YIM 90716 (5) were found in roots and leaves. The actinomycetes which were found in roots, stems and leaves were closely related with *Actinokineospora auranticolor* strain IFO 16518 (2), *Microbacterium ginsengisoli* strain Gsoil 259 (6), *Amycolatopsis orientalis* strain HCCB10007 (10) and *Actinomadura rifamycini* strain JCM 3309 (11), *Streptomyces javensis* strain B22P3 (9), *Kibdelosporangium philippinense* strain A 80407 (8) and *Kocuria halotolerans* strain YIM 90716 (7).

## DISCUSSION

As the first published report, this study describes metagenomic diversity of endophytic actinomycetes in roots, stems, leaves of *T. crispera* plant and actinomycetes from rhizosphere soil based on 16S rRNA-gene using PCR-DGGE technique. DGGE profiles shows each separate band which representing one of its own species community. The dominant species and the intensity of each band indicates their relative abundance (Nimnoi *et al.* 2010). Amplification with nested PCR and DGGE was also successfully detects actinomycetes community in the soil, plant rhizosphere of *Artemisia tridentata* and from roots of *Aquilaria crassna* (Heuer *et al.* 1997; Franco *et al.* 2009; Nimnoi *et al.* 2010). Genetic distance (p-distance) analysis of 16S rRNA gene sequences of the selected bands indicates similarity of the actinomycetes community between stems and leaves, but has several differences from roots and soil. This phenomenon is predicted due to the migration of actinomycetes from stems to the leaves, soil and roots. Differences in the nucleotide sequences based on the p-distance matrix indicates species diversity between each sample. The differences are thought to be influenced by differences in isolated parts of plant organs and the effect of actinomycetes found in phyllosphere.

Endophytic actinomycetes community was found to be more diverse than rhizosphere soil actinomycetes. Relatively low actinomycetes community in the soil was allegedly due to the high competitive niches in the soil (Hibbing *et al.* 2010). Endophytes are sheltered from environmental stresses and microbial competition by the host plant and they seem to be ubiquitous in plant tissue (Nimnoi *et al.* 2010). Endophytic actinomycetes population in the stems and leaves exhibit a more diverse than in the roots. While relatively low actinomycetes community in the roots was allegedly due to the migration of roots endophytic actinomycetes to other plant organs as a result of the old plants used in this research work.

Association between actinomycetes in the soil with endophytic actinomycetes occur as it is seen in Figure 4a. Several endophytic actinomycetes in *T. crispera* plant organs are also found in the soil rhizosphere. Actinomycetes which abundantly live in the rhizosphere soil can colonize and penetrate into plant roots (Sardi *et al.* 1992; Nimnoi *et al.* 2010). With a passive penetration, microbial endophytic can also

move in the open space or cracks in the root tip (Nimnoi *et al.* 2010). Actinomycetes in roots migrate to other plant organs through the intercellular and vascular system (Feng *et al.* 2004). Similar results were also reported by Qin *et al.* (2012) that the abundance of endophytic actinomycetes on the stems and leaves of medicinal plant *Maytenus austroyunnanensis* showed greater diversity than the roots. These phenomenon may affects the production cycle of the active compounds in the leaves and stems of *T. crispera*, which often used for medicinal treatment. Microbial endophytics of medicinal plants have a role in the biochemical cycles and production of bioactive compounds in their host (Zhao *et al.* 2011).

The study shows that several similar endophytic actinomycetes are found in different plant organs (Fig 4b), which is presumably due to the migration of the endophytic actinomycetes between plant organs (Tian *et al.* 2007). The endophytes can come from the surrounding environments, such as rhizosphere and phyllosphere of plants which can enter the plant tissue through stomata, lenticel and physical injuries (broken trachoma) or area in which the lateral roots emerged (Susilowati 2010). Interaction between endophytic microbes and plants is regarded as symbiosis mutualism. Under this type of interaction, endophytic microbes obtains nutrients and protection from the host plant, but they produce compounds that can trigger plant growth and suppress pathogens, phosphate solvent, assimilation of nitrogen for plants (Rosenblueth and Romeo 2006) and able to produce enzyme inhibitor (Khamna *et al.* 2009; Pujiyanto *et al.* 2012).

Based on the partial sequences similarity and phylogenetic relatedness of 16S rRNA gene, there are two spesies which were closely related to the genus *Streptomyces*. *Streptomyces* is the dominant actinomycetes isolated from the soil rhizosphere (Khamna *et al.* 2009). The structure of microbial community in the soil is determined by environmental factors such as soil characteristics, type of plants and cultivation that can cause the changes in the structure of soil microbes (Zhao *et al.* 2012). Plant roots exudates stimulate the growth of actinomycetes in the rhizosphere and the synthesis of antimicrobial compounds. The diversity of endophytic actinomycetes species in roots and stems of potato samples which were analyzed with DGGE showed to have a high similarity with *Streptomyces* (Sessitsch *et al.* 2002). On the agar medium (a culture-dependent), *Streptomyces* are the more frequently found genus.

*Streptomyces* and some other genera such as *Micromonospora* and *Streptosporangium* are reported as the most dominating genera isolated from wheat, potatoes and several medicinal plants (Sessitsch *et al.* 2002; Coombs and Franco 2003; Zhao *et al.* 2011). Previous research work on the isolation of endophytic actinomycetes from *T. crispata* using a culture dependent technique based on 16S rDNA-gene, found that amongst the 32 isolates of actinomycetes, the majority were closely related with *Streptomyces* (Pujiyanto *et al.* 2012). In this paper, the diversity of endophytic actinomycetes are described based on metagenomic analysis which considered as a culture-independent technique. There are two sequences which are closely related with *Streptomyces*, while others are more closely related to rare actinomycetes. Based on a culture independent approach, this work succeeds to obtain 21 dominant sequences. The relatively low number of sequences obtained may be due to the distribution of each band using DGGE results represent one species in a community. In this case, different strains in one species is only represented by one band. PCR-DGGE is able to separate the analyzed samples into several separate bands. Each separate band represents one of its own species. The DGGE profile indicates dominant species and the intensity of each band shows their relative abundance (Nimnoi *et al.* 2010). Meanwhile, in a culture-dependent technique, isolates can be derived from a different strain belongs to one species.

A culture-independent technique based on 16S rRNA gene using PCR-DGGE are able to explore metagenomics diversity of endophytic actinomycetes. This approach able to explore diversity of actinomycetes which are relatively difficult to be cultured or rare and even undiscovered or novel endophytic actinomycetes from *T. crispata*. This study found a species which was closely related to *Actinokineospora auranticolor* strain IFO 16518, a rare actinomycetes. *Actinokineospora* is a rare endophytic actinomycetes which is found with culture-independent on the leaves of medicinal plant *Maytenus austroyunnanensis* (Qin *et al.* 2012). *Actinokineospora* has an ability to produce antimycoplasm (Hasegawa 1991) and antitrypanosomally actinosporin A and B (Harjes *et al.* 2014; Abdelmohsen *et al.* 2014). Analysis of soil actinomycetes community in Xinjiang, China using 16S rRNA based PCR-DGGE discovered species with a closely related with *Microbacterium* (Zhang *et al.* 2013). *Microbacterium* was reported to have antibacterial and anti-fungal

activity (Qin *et al.* 2009) and producing  $\beta$ -glucosidase (Park *et al.* 2008). Using a similar approach, endophytic *Actinomadura* was isolated from roots of *Aquilaria crassna* (Nimnoi *et al.* 2010). *Actinomadura* has been reported to produce Indole Acetic Acid (IAA) as a growth hormone and produce siderophore (Khamna *et al.* 2009) as well as antibacterial compounds (Qin *et al.* 2009). This work found a band which has sequence similarity with *Kocuria*. *Kocuria halotolerans* strain YIM 90716 is rarely found as endophytic actinomycetes. This genus is reported to have been found in seawater, marine sediment, water and desert soil (Kaur *et al.* 2011). It has the ability to produce IAA and siderophore (Khamna *et al.* 2009) as well as biosurfactant (Sarafin *et al.* 2014). For *Williamsia* which are found in this study as endophyte, this genus has also been found in roots of *Eucalyptus microcarpa* (Kaewkla & Franco 2012). It has an ability to produce RDX (Hexahydro-1,3,5-Trinitro-1,3,5-Triazine) bioremediation (Thompson *et al.* 2005). *Kibdelosporangium* is also a rare genus reported as an endophytic (Janso & Carter 2010). It has a capability in producing antiviral cycloviracins (Tomita *et al.* 1993) and kibdelomycin (Philips *et al.* 2011). As endophytic actinomycetes, *Amycolatopsis* and *Actinoplanes* were isolated from uncultivated rice paddy roots (Tian *et al.* 2007). *Amycolatopsis* was found to have antibacterial and anti-fungal activity (Qin *et al.* 2009), whereas *Actinoplanes* produced Teicoplanin, a glycopeptide antibiotic for the treatment of multiresistant gram-positive infection (Taurino *et al.* 2011) and  $\alpha$ -glucosidase inhibitors (Zhang *et al.* 2003). Meanwhile the  $\alpha$ -glucosidase inhibitors can also be produced by *Streptomyces* isolated from *T. crispata* (Pujiyanto *et al.* 2012). It is clearly described that actinomycetes able to produce various bioactive compounds thus have open the potency to discover new bioactive compounds.

## ACKNOWLEDGEMENT

This research work was partly supported by scholarship from Directorate of Higher Education awarded to Mona Primanita and also partly funded by University Priority Research Grant (Decentralisation scheme) contract number 02/01/2014/SP DIPA-023.04.2.189772/2014 given to Yulin Lestari.

## REFERENCES

Abdelmohsen UR, Cheng C, Viegelmann C, Zhang T,

- Grkovic T, Ahmed S, Quinn RJ, Hentschel U, Ebel RE. 2014. Dereplication strategies for targeted isolation of new antitrypanosomal actinosporins A and B from a marine sponge associated-*Actinokineospora* sp. EG49. *Mar Drugs*. 12 (3): 1220-1244. doi: 10.3390/md12031220.
- Bruce KD, Hiorns WD, Hobman JL, Osborn AM, Strike P, Ritchie DA. 1992. Amplification of DNA from native populations of soil bacteria by using the polymerase chain reaction. *Appl Environ Microb*. 58: 3413-3416. doi: 10.1128/AEM.71.12.8265-8272.2005.
- Coombs JT, Franco CMM. 2003. Isolation and identification of actinobacteria from surface sterilized wheat roots. *Appl Environ Microbiol*. 69 (9): 5603-5608. doi: 10.1128/AEM.69.9.5603-5608.2003.
- Feng C, Hua SS, Feng CS, Xian JY. 2004. Migration of *Azospirillum brasilense* YU62 from roots to stem and leaves inside rice and tobacco plants. *Act Botanica Sinica*. 46: 1065-1070.
- Franco G, Hernandez R, Barrios N, Strapz JL, Crawford DL. 2009. Molecular and cultural analysis of seasonal actinomycetes in soil from *Artemisia tridentata* habitat. *Phyton Int J Exp Botany*. 78: 83-90.
- Harjes J, Ryu T, Abdelmohsen UR, Silva LM, Horn H, Ravasi T, Hentschel U. 2014. Draft genome sequence of the antitrypanosomally active sponge-associated bacterium *Actinokineospora* sp. strain EG49. 2014. *Genome Announc*. 2 (2): 160-165. doi: 10.1128/genomeA.00160-14.
- Hasegawa T. 1991. Studies on motile arthrospore-bearing rare actinomycetes. *Actinomycetol*. 5(2): 64-71. doi: 10.3209/saj.9\_98.
- Heuer H, Krsek M, Baker P, Smalla K, Wellington EMH. 1997. Analysis of actinomycete communities by specific amplification of gen encoding 16S rRNA and gel electrophoretic separation in denaturing gradients. *Appl Environ Microbiol*. 63 (8): 3233-3241. doi: 10.1128/AEM.71.12.8265-8272.2005.
- Hibbing ME, Fuqua C, Parsek MR, Peterson SB. 2010. Bacterial competition; surviving and thriving in the microbial jungle. *Nat Rev Microbiol*. 8 (1): 15-25 doi: 10.1038/nrmicro2259.
- Janso JE, Carter GT. 2010. Biosynthetic potential of phylogenetically unique endophytic *Actinomycetes* from tropical plants. *Appl Environ Microbiol*. 76 (3): 4377-4386. doi: 10.1128/AEM.02959-09.
- Kaur C, Kaur I, Raichand R, Bora TC, Mayilraj S. 2011. Description of a novel actinobacterium *Kocuria assamensis* sp. nov. isolated from a water sample collected from the river Brahmaputra, Assam, India. *A Van Leeuw*. 99: 721-726. doi: 10.1007/s10482-010-9547-9
- Kaewkla O, Franco CMM. 2012. Rational approaches to improving the isolation of endophytic actinobacteria from Australian native trees. *Microb Ecol*. 65 (2): 384-393. doi: 10.1007/s00248-012-0113-z.
- Khamna S, Yokota A, Lumyong S. 2009. Actinomycetes isolated from medicinal plant rhizosphere soils: diversity and screening of antifungal compounds, indole-3-acetic acid and siderophore production. *J Microbiol Biotechnol*. 25 (4): 649-655. doi: 10.1007/s11274-008-9933-x.
- Martina K, Jan K, Tamas F, Ladislav C, Marek O, Genevieve LG, Yvan ML, Marketa SM. 2008. Development of a 16S rRNA gene based prototype microarray for the detection of selected actinomycetes genera. *A Van Leeuw*. 94 (3): 439-453. doi: 10.1007/s10482-008-9261-z.
- Nimnoi P, Pongsilp N, Lumyong S. 2010. Genetic diversity and community of endophytic actinomycetes within the roots of *Aquilaria crassna* Pierre ex Lex assessed by actinomycetes-specific PCR and PCR-DGGE of rRNA gene. *Biochem Syst Ecol*. 38 (4): 595-601. doi:10.1016/j.bse.2010.07.005.
- Overeas L, Forney L, Daae FL, Torsvik V. 1997. Distribution of bacterioplankton in Meromictic lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl Environ Microbiol*. 63 (9): 3367-3373. doi: 10.1128/AEM.71.12.8265-8272.2005.
- Park JM, Kim MK, Kim HB, Im WT, Yi TH, Kim SY, Soung NK, Yang DC. 2008. *Microbacterium ginsengisoli* sp. Nov a beta-glucosidase producing bacterium isolated from soil of a ginseng field. *Int J Syst Evol Microbiol*. 58 (2): 429-433. doi: 10.1099/ij.s.0.65226-0.
- Pillips JW, Goetz MA, Smith SK, Zink DL, Polishook J, Onishi R, Salowe S, Wiltsie J, Allocco J, Sigmund J, Dorso K, Lee S, Cruz AMDL, Martin J, Vicente F, Genilloud O, Lu J, Painter RE, Young K, Overbye K, Donald RGK, Sing SB. 2011. Discovery of kibelomycin, a potent new class of bacterial type II topoisomerase inhibitor by chemical-genetic profiling in *Staphylococcus aureus*. *Chem Biol* 18: 955-965. doi: 10.1016/j.chembiol.2011.06.011.
- Patela MB, Mishrab SM. 2012. Magnoflorine from *Tinospora cordifolia* stem inhibits  $\alpha$ -glucosidase and is antiglycemic in rats. *J Func Food*. 4: 78-86. doi: 10.1016/j.jff.2011.08.002.
- Pujiyanto S, Lestari Y, Suwanto A, Budiarti S, Darusman LK. 2012. Alpha-glucosidase inhibitor activity and characterization of endophytic actinomycetes isolated from some Indonesian diabetic Medicinal Plants. *Int J Pharm Pharm Sci*. 4 (1): 327-333.
- Puranik N, Kammar KF, Devi S. 2010. Anti-diabetic activity of *Tinospora cordifolia* (Willd) in Streptozotocin diabetic rats; does it act like sulfonylureas? *Turk J Med*

- Sci. 40 (2): 265-270. doi:10.3906/sag-0802-40.
- Qin S, Chen HH, Zhao GZ, Li J, Zhu WY, Xu LH, Jiang JH, Li WJ. 2012. Abundant and diverse endophytic actinobacteria associated with medicinal plant *Maytenus austroyunnanensis* in Xishuangbanna tropical rain forest revealed by culture dependent and culture independent methods. *Environ Microbiol Rep.* 4 (5): 522-531. doi: 10.1111/j.1758-2229.2012.00357.x.
- Rosenblueth M, Romeo EM. 2006. Bacterial endophytics and their interactions with host. *Am Phyt Soc.* 19 (8):827-837. doi: 10.1094/MPMI-19-0827.
- Sambrook J, Russell. 2001. *Molecular Cloning: A Laboratory Manual*. 3<sup>rd</sup> Edition. New York (US): Cold Spring Harbor Laboratory Pr.
- Sarafin Y, Donio MBS, Velmurugan S, Michaelbabu M, Citarasu T. 2014. *Kocuria marina* BS-15 a biosurfactant producing halophilic bacteria isolated from solar salt works in India. *Saudi J Biol Sci.* 21 (6):511-519. doi:10.1016/j.sjbs.2014.01.001.
- Sardi P, Saracchi M, Quaroni S, Petrolini B, Borgonovi GE, Merli S. 1992. Isolation of endophytic *Streptomyces* strains from surface-sterilized roots. *Appl Environ Microbiol.* 58 (8): 2691-2693. doi: 10.1128/AEM.71.12.8265-8272.2005.
- Sessitsch A, Reiter B, Pfeifer U, Wilhelm E. 2002. Cultivation independent population analysis of bacterial endophytics in tree potato varieties based on eubacterial and actinomycetes specific PCR of 16S rRNA genes. *FEMS Microbiol Ecol.* 39 : 23-32. doi: 10.1111/j.1574-6941.2002.tb00903.x
- Susilowati DN, Hidayatun N, Tasliah, Mulya K. 2010. Keragaman bakteri endofitik diisolasi dari empat varietas padi dengan Metode ARDRA [The diversity of endophytic bacteria isolated from four varieties of rice with ARDRA method]. *Berita Biologi.* 10: 241-248.
- Tan RX, Zou WX. 2001. Endophytics: a rich source of functional metabolites. *Nat Prod Rep.* 18:448-459. doi: 10.1039/B100918O.
- Taurino C, Frattini L, Marcone GL, Gastaido L, Marinelli F. 2011. *Actinoplanes teichomyceticus* ATCC 31121 as a cell factory for producing teicoplanin. *Microbial Cell Fact.* 10: 1-13. doi:10.1186/1475-2859-10-82.
- Thompson KT, Crocker FH, Fredricksom HL. 2005. Mineralization of the cyclic nitramine explosive Hexahydro-1,3,5-Trinitro-1,3,5-Triazine by *Gordonia* and *Williamsia* spp. *Appl Environ Microbiol.* 71 (12): 8265-8272. doi: 10.1128/AEM.71.12.8265-8272.2005.
- Thontowi A. 2009. Pendekatan metagenomik dan bioinformatika untuk menganalisis komunitas mikroba laut Indonesia [Metagenomik and bioinformatics approach to analyzing the Indonesian marine microbial communities]. *Sigma.* 12: 15-22.
- Tian X, Cao L, Tan H, Han W, Chen M, Liu Y, Zhou S. 2007. Diversity of cultivated and uncultivated actinobacterial endophytics in the stems and roots of rice. *Microbiol Ecol.* 53 (4): 700-707. doi 10.1007/s00248-006-9163-4.
- Tomita K, Hoshino Y, Miyaki T. 1993. *Kibdelosporangium albatum* sp. nov., producer of the antiviral antibiotics cycloviracins. *Int J Sys Bacteriol.* 43: 297-301. 0020-7713/93/020297-05\$02.00
- Zhang CS, Podeschwa M, Block O, Altenbach HJ, Piepersberg W, Wehmeier UF. 2003. Identification of a 1-epi-valienol 7-kinase activity in the producer of acarbose, *Actinoplanes* sp. SE50/110. *FEBS Lett.* 540: 53-57. doi: 10.1016/S0014-5793(03)00222-9.
- Zhang W, Long XQ, Huo XD, Chen YF, Lou K. 2013. 16S rRNA-based PCR-DGGE analysis of actinomycete communities in fields with continuous cotton cropping in Xinjiang, China. *Microb Ecol.* 66 (2): 385-393. doi: 10.1007/s00248-012-0160-5.
- Zhao K, Penttinen P, Guan T, Xiao J, Chen Q, Xu J, Lindstrom K, Zhang L, Zhang X, Strobel GA. 2011. The diversity and anti-microbial activity of endophytic actinomycetes isolated from medicinal plants in Panxi Plateau, China. *Curr Microbiol.* 62 (1): 182-190. doi: 10.1007/s00284-010-9685-3.
- Zhao K, Penttinen P, Chen Q, Guan T, Lindstrom K, Ao X, Zhang L, Zhang X. 2012. The rhizosphere of traditional medicinal plants in Panxi China, host a diverse selection of actinobacteria with antimicrobial properties. *Appl Microbiol Biotechnol.* 94 (5): 1321-1335. doi: 10.1007/s00253-011-3862-6.
- Zeyauallah, Ramli MR, Islam B, Atif M, Benkhayal FA, Nehal M, Rizvi MA, Ali A. 2009. Metagenomics-an advanced approach for non-cultivable microorganisms. *J Biotechnol Mol Biol Rev.* 4(3): 049-054.