ACTINOMYCETES ISOLATED FROM WETLAND AND HILL PADDY DURING THE WARM AND COOL SEASONS IN SARAWAK, EAST MALAYSIA

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ABSTRACT

As part of the Natural Product Discovery programme at Sarawak Biodiversity Centre (SBC), our study targeted isolation and evaluation of actinomycetes diversity from paddy rice fields. Samples from two types of paddy farming system practiced in Sarawak, wet land and hill paddy, were collected and processed leading to the selection of 578 strains distributed among 24 genera and 10 families. Analysis using phylogenetic clustering indicated a total of 159 taxonomic units (TU). The taxonomic position and the ranking of the TU allowed their classification in 4 novel species, 61 putative novel species and 94 known species or species of uncertain position. The high genus diversity and percentage of novel or putative novel species demonstrate the biodiversity potential of Sarawak ecosystems, even in man-managed ecosystems.

Keywords: paddy field, actinomycetes, ranking, taxonomic unit

INTRODUCTION

Isolation of rare actinomycetes from paddy rice (*Oryza sativa* L.) field in the Kuching Division, Sarawak were made to evaluate their diversity and distribution. Paddy is the third most widely planted crop in Malaysia (6880 km^2) after oil palm and rubber (**DOA, 2011**), with Sarawak being the nation's fourth largest rice producer, after Kedah, Perak and Kelantan (**Sarawak Energy, 2013**). Rice is cultivated either in marshy, lowland areas, periodically flooded or hilly regions where natural rainfall provides adequate amount of water (**Joshi, 2002**). Wetland paddy represents an intermediate system between terrestrial ecosystems and aquatic ecosystems (**Liesack *et al.,* 2000; Kazutake, 2007; Ding *et al.,* 2014**). In hill paddy, slash-and-burn crop cycle is practiced with 3-30 years long fallow period which allows reconstitution of dense secondary formations, a biomass source of fertility for the next planting cycle (**MARDI, 2011**). The uniqueness of paddy environment have led to the isolation of novel genera such as *Oryzihumus* (**Kageyama *et al.,* 2005**), *Humihabitans* (**Kageyama *et al.,* 2007**) *Humibacillus* (**Kageyama *et al.,* 2008**) and *Actinophytocola* (**Indananda *et al.,* 2010**).

In response to the ecological stress and survival mechanisms, soil microorganisms are induced to release bioactive compounds. Secondary metabolites obtained from Actinomycetes provide a potential source of many novel compounds with antibacterial, antitumour, antifungal, antiviral, antiparasitic and other properties (**Genilloud *et al.,* 2011; Omura, 2011; Solecka *et al.,* 2012**). For instance, a novel antibacterial compound Clostomicin was isolated from *Micromonospora echinospora subsp. Arminiaca* KMR593 isolated from paddy soil (**Takahashi *et al.,* 1986**). Since then, the biotechnological potential of actinomycetes from paddies have not been efficiently exploited.

Soils in managed agricultural systems often differ from the unmanaged systems (**Jangid *et al.,* 2008; Wu *et al.,* 2008; Lauber *et al.,* 2013**). Actinomycetes have beneficial roles in soil nutrients cycling and agricultural productivity which have led to reports on higher population of actinomycetes in agricultural land compared to forest soils (**Burck *et al.,* 1989; Elliot and Lynch, 1995; Lopes *et al.,* 2011**). Soil microbial ecosystem also function as a sensitive biological marker, useful to identify ecosystem condition, disturbance and damage (**Roper and Ophel-Keller, 1997**).

In this paper we propose an approach allowing better evaluation of the isolated strains diversity and distribution through ranking and clustering analysis. A prokaryotic species is considered to be a group of strains that are characterized by a certain degree of phenotypic consistency, showing over 70 % of DNA–DNA binding and traditionally over 97 % of 16S ribosomal RNA (rRNA) gene-sequence identity (**Gevers *et al.,* 2005**). However, species can be differentiated at a level of 98.2 – 99 % 16S rRNA similarity (**Kim *et al.,* 2014**).

Apart from the commonly collected soil samples, rhizospheric soil and roots were also included for the isolation of actinomycetes in this project. Rhizospheric soil is the ecological zone surrounding the roots of growing plants and are affected by the plant root activities (**Plaster, 2002; Doi *et al.,* 2010**). Rhizospheric soils also have high nutrient content leading to higher microbial load (**Adegboye and Babalola, 2012**). Rice roots provide polysaccharides, amino acids and organic acids (**Rovira, 1969; Kimura, 1977; 1983**). Actinomycetes in the rhizosphere produce antibiotics in the vast majority of cases protecting the plants (**Compant *et al.,* 2010**). As a result, the community structures of soil microorganisms in the rice rhizosphere are expected to differ significantly from the same paddy field bulk soil (**Doi *et al.,* 2010**).

Endophytes are common in plants, with an estimated of 1 million species yet to be discovered (**Joseph and Priya, 2011**). Interest in investigating endophytic actinomycetes are due to the microbes' ability to produce anti-microbials, extra-cellular enzymes and their involvement with bioremediation and biocontrol (**Inderiati and Franco, 2008; Gangwar *et al.,* 2012; Chankhamhaengdecha *et al.,* 2013**). Therefore, such samples were also taken into consideration.

MATERIALS AND METHODS

Site Description

Both study sites are located in the Kuching Division, Sarawak, East Malaysia. The study area has a tropical climate with uniform temperatures through the year, ranging from 32 °C during the day to 28 °C at night (**Dominick *et al.,* 2012**). Rainfall is common throughout the year averaging 4 000 mm per annum (**Gandaseca, 2014**). Conditions are drier between April and June, while the wettest months are between December to February. Relative humidity ranges from 57 % to 100 % over the course of the year, rarely dropping below 47 % (WeatherSpark 2013).

Hill Paddy

Hill paddy field in this study is located at Padawan (N01° 13.248' E 110° 18.680'). The half hectare paddy field was planted along a hill slope. The site was a secondary forest before it was cut and burn for paddy planting. Paddy were planted using seeds, once or twice in a year with a growth period of five months before they can be harvested. Other food crops were planted at areas having high ash content among the paddy plants like maize, vegetables, and short-life fruit

trees such as bananas and papayas. A total of 500 kg hectare^{-1} fertiliser (NPK 12:12:17) is applied twice, one month after planting and two to three months before harvesting. The area will then be left to recover for at least a year for the next cut and burn cycle.

Wetland Paddy

The one-hectare wetland paddy field located in Serian (N01° 08.880' E 110° 29.053') is part of the 10 hectares paddy plots that are farmed for generations. This field plot is irrigated by canals from a nearby river. A total of 500 kg hectare^{-1} fertiliser (NPK 12:12:17) are applied evenly throughout the field twice (one week after planting the seedlings and two months before harvesting).

Sample Collection

The samples (soil, rhizospheric soil and rice roots) were collected in December (high rainfall, 500-700 mm) and February/March (low rainfall, 200-300 mm) 2011 and 2012 from wetland and hill paddy field plots. Second sample collection was conducted prior to the harvest.

Uneven distribution of microorganisms is often a limiting factor during ecological studies (**Girvan et al., 2004; Schwarzenbach et al., 2007; Wolfe et al., 2007**). Potential bias is minimised by collecting samples at different spots in each study area and mixing them into a composite sample (**Silva et al., 2013**). For this study, four composite samples from each paddy field environment were collected. Composite soil samples from each plot were mixtures of three sub-samples. Soil samples were collected at regular grid spacing from the upper (0 – 10 cm) soil layers. For wetland paddy, subsamples were taken at 2 m apart. As for hill paddy, subsamples were taken from two different sites based on elevation. Sampling apparatus were cleaned with 70 % ethanol for each collection. Rhizospheric soil were taken from soil attached to the rice plant roots.

Inappropriate storage conditions of the soil samples can adversely affect microbial communities (**Trabue et al., 2006; Zornoza et al., 2006**). To avoid overgrowth of fast-growing microorganisms and maintaining the microbial composition, soil samples were kept in zip lock bags/ bottles. Root samples were kept in tubes with 40 % glycerol. Collected samples were then placed in cool box during the expedition.

Soil Sample Analysis

The subsamples for each paddy site were thoroughly mixed, air-dried for 2 weeks at room temperature and then stored in plastic containers prior to the analysis. Air dried soil sample analysis, excluding pH, were conducted by the Department of Agriculture, Kuching, Sarawak. Soil pH was measured using a 1:5 (w/v) ratio of freshly collected soil to water (**Ellis and Foth, 1996**). CEC was measured using distillation method (**Malaysia Standard, 1980**). Total organic carbon (TOC) was determined using instrumental dry combustion method using Multiphase Organic Carbon Analyzer. Available phosphorus (P) by method based on **Bray and Kurtz (1945)**. Exchangeable cations analysis was carried out by leaching the soil with neutral ammonium acetate (**Malaysia Standard, 1980**). The concentrations of the base cations in the leachate are determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) 7300 DV.

Actinomycetes Isolation

Pre-treatment of Samples

Rhizospheric soil was taken from soil still attached to the paddy plant roots. Rhizospheric soil and soil samples collected were air-dried at room temperature for 7 days, ground and sieved. With exception of roots, the samples were then processed with various pre-treatments and enrichment techniques namely:

- **AD**, Air Drying: 1 g of soil, air dried for 7 days (**Labeda, 1990**)
- **HT**, Heat Treatment: 1 g of air-dried soil were heated at 120 ºC for 30 min (**Kim et al., 1995**)
- **FM**, Flooding Method: 1 g of air dried and heat-treated samples was suspended in a flooding solution (0.1 % skim milk 0.1M, potassium phosphate buffer pH 7, 10 % soil extract) in 50 mL centrifuge tube and incubated at 30 ºC for 90 min, centrifuged at 4000 rpm for 10 min and incubated again at 30 ºC for 30 min (**modified from Suzuki et al., 1999**)
- **PH**, Phenol treatment: 1 g of heat-treated samples were suspended in 1.5 % phenol solution and incubated for 10 min at room temperature (**Panthier et al., 1979**).

In addition to AD, soil free roots were subjected to separate pre-treatments:
- **FD**, freeze dry: freeze dried roots were rehydrated in sterile water for 3 min, cleaned using 0.1 % Tween 20 (15 sec) and sterile water (1 min) twice and finally with sterile water supplemented with Terbinafin (1 mg/L) for 5 min.

- **AD+S**, air dry with sterilisation: AD roots were rehydrated in sterile water for 3 min prior to gentle sterilisation using 0.1 % Tween 20 (30 sec), 75 % ethanol (15 sec), 1 % H$_2$O$_2$ (15 sec), 75 % ethanol (15 sec), 0.2 % SDS (3 min) and sterile water (1 min) with a final rinse using sterile water supplemented with Terbinafin (1 mg/L) for 5 min.

Pre-treated roots were then pounded in the presence of 0.2 % SDS. The slurry filtered using sterile gauze to eliminate large roots pieces and supernatant used for dilution. All treated samples were serially diluted to 10^{-2}, 10^{-3} and 10^{-4} using 0.2 % SDS solution.

Isolation Media

Pre-treated sample were inoculated onto the following media, with three replicates each:

- **SEA,** Soil Extract Agar: 250 mL soil extract, 0.1 % D-glucose, 0.05 % yeast extract, 0.05 % K$_2$HPO$_4$ and 1.5 % agar (**modified from Hamaki et al., 2005**)
- **HSV,** Humic Acid Vitamin Agar: humic acid 1.0 g, Na$_2$HPO$_4$ 0.5 g, KCl 1.7 g, MgSO$_4$.7H2O 0.05 g, FeSO$_4$.7H2O 0.01 g, CaCl$_2$.2H2O 0.735 g, CaCO$_3$ 0.02 g, agar 16 g, trace element, vitamin mixture (**modified from Hayakawa and Nonomura, 1987**)
- **APA,** Modified Arginine Vitamin Agar: L-Arginine 0.1 g, L-Asparagine 0.1 g, Glutamic acid 0.1 g, Histidine 0.1 g, D-glucose 0.25 g, Glycerol 0.25 g, Yeast extract 0.1 g, N-Z amine 0.1 g, KH$_2$PO$_4$ 50 mg, MgSO$_4$.7H2O 50 mg, Cellulose 0.5 g, trace element 0.5 mL, Rice extract 25 mL , agar 15 g, vitamin mixture 2 mL (**modified from Shirling and Gottlied, 1966**)
- **REA,** Rice Extract Agar: 2.5 g cooked rice filtrate, KNO$_3$ 0.5 g, trace element mix 0.5 mL, KH$_2$PO$_4$ 0.1 g, NaCl 0.1 g, Mg$_2$SO4. 7H$_2$O 0.005 g, yeast extract 0.005 g, Vitamin mixture 1.0 mL, Agar 15.0 g

with
- **Trace element mix** per litre, CaCl$_2$.2H$_2$O 4 g, ZnSO$_4$.7H$_2$O 2 g, N$_2$B$_4$O$_7$.10H$_2$O 0.1 g, FeSO$_4$. 7H$_2$O 5 g, KJ 0.05 g, CoCl$_2$.6H$_2$O 0.5 g, CuSO$_4$.5$_2$O 0.12 mg, MnCl$_2$.4H$_2$O 2 g, Na$_2$MoO$_4$.2H$_2$O 0.05 g, H$_2$SO 1- 2 mL
- **Vitamin mixture** per 100 mL, p-aminobenzoic acid 50 mg, calcium pantothenate 50 mg, inositol 50 mg, niacin 50 mg, pyridoxin HCl 50 mg, riboflavin 50 mg, thiamine HCl 50 mg, biotin 25 mg, water up to100 mL. Sterilisation by filtration.

To suppress the overgrowth of unwanted filamentous fungi and bacterial population, the isolation agar media were supplemented with 1 ppm Terbinafin, 25 ppm Nalidixic Acid, and 25 ppm Cycloheximide. Inoculated agar plates were incubated at 28 ºC for 2 weeks before the total number of colonies for actinomycetes, bacteria and fungi were counted. Further incubation for up to six weeks was necessary for the isolation of slow-growing strains.

Colonies Selection and Purification

After at least two weeks of incubation, typical actinomycetes colonies that grew on isolation agar were thoroughly observed under light microscope with a 50-x long distance objective (Olympus LMPLFLN; Olympus, Tokyo, Japan). Observation and selection were based on morphological characteristics of spore-forming structures. Colonies with spore chain structures tentatively belonging to rare actinomycetes were purified onto yeast extract-malt extract agar (ISP2) (**Shirling and Gottlieb, 1966**) without antibiotics to obtain pure cultures.

Representatives of common genera within the family *Streptomycetaceae* and *Micromonosporaceae* were also selected and purified with severe dereplication. Sporulation and other features of purified cultures were characterised on SEA agar, while colonial morphology was observed on ISP2 agar plates. Spore chain structures and colonial morphology were recorded in a database and photo documented.

Actinomycetes Identification

Preliminary identification of pure cultures was based on morphological observations, mainly sporulation type, presence of aerial mycelium and hyphae fragmentation, allowing a tentative assignment to a family or a genus (**Shirling and Gottlieb, 1966; Cross, 1989; Bergey's Manual, 2012**). Obvious duplicates from one subsample were eliminated. The colonies on SEA were directly observed using 50 x long distance objective (Olympus LMPLFLN; Olympus, Tokyo, Japan), further identification was conducted using molecular methods.

DNA was obtained by the "freeze-thaw" modified method by Muramatsu *et al.* 2003. Master mix (30 µl) containing 2 µl of the DNA extract, 20 mM each primer SRR181F (5"- GTT TGA TCC TGG CTC AGG AC - 3") and SRR182R (5"-GGT GTT CCT CMH GAT ATC TG - 3"), 10 mM dNTP, 10 x buffer (without MgCl$_2$) and 1 Unit Taq DNA polymerase (New England Biolabs) was used to amplify the 16S rRNA genes with the following parameters: 1 min at

96°C, 30 cycles of 45 sec at 96 °C, 1 min at 53 °C, 2 min at 72 °C and the final annealing for 7 min at 72 °C. Amplified products were purified (GFX PCR DNA GE Healthcare) and sequenced with primer pair SRR178F (5"- GAA CGC TGG CGG CGT GCT -3") & SRR192R' (5"- GCA TTY CAC CGC TAC ACC -3") using *BigDye®* Terminator v3.1 Cycle Sequencing Kits based on Sanger's dideoxy sequencing method.

Phylogenetic Clustering

Phylogenetic trees were then constructed for each genus based on the 16S rRNA identification. Sequence alignment was done using BioEdit (**Hall, 1999**). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (**Tamura *et al.*, 2011**). The evolutionary history was inferred using the Neighbour-Joining method (**Saitaou and Nei, 1987**). The evolutionary distances were computed using the Maximum Composite Likelihood method (**Tamura *et al.*, 2004**). For genera with only one strain or one cluster, phylogenetic trees were constructed by adding in sequences from the top 10 16S rRNA BLAST results. This is to ensure that the isolated strain clusters within the identified genus.

Ranking

Sequence-similarity search for all sequences were done using BLAST programme against 16S rRNA database from GenBank (**Benson *et al.*, 2012**). Taxonomic positions of the strains were made based on the maximum identity (MI) score. Individual strains were assigned into one of the five categories below:

Category	Blast homology (MI)	Taxonomic Position
Category 1	96.9 % and below	Novel genus
Category 2	97 - 98.65 %	Novel species
Category 3	98.7 - 99.5 %	Putative novel species
Category 4	99.6 - 99.8 %	Putative known species
Category 5	99.9 % and above	Identical or closely related species

RESULTS AND DISCUSSION

All 578 sequences were deposited in Genbank (KY052189 - KY052766). In this study, the results are only tendencies as the number of strains isolated was limited. *In vitro* cultivation covers about 0.1 to 10% of total soil microbiota (**Kell *et al.*, 1998; Torsvik and Ovreas, 2002**).

Phylogenetic Clustering

Overall, 159 distinct phylogenetic clusters or TU from the total of 578 strains were delineated. *Micromonospora*, having the highest number of strain isolated (257 strains) were represented by 40 TU similar with *Streptomyces* (120 strains, 40 TU). An average of 6 strains per TU were selected for *Micromonospora*, compared to *Streptomyces* with average of 3 strains per TU. Analytical approach using TU effectively eliminate over-representation of strains. *Streptacidiphilus*, *Mycobacterium* and *Dactylosporangium* showed highest species diversity with the average number of strains per TU of 1.17, 1.20 and 1.30, respectively. This was not taking into account genus with only one representative species or strain isolated. Figure 1 shows phylogenetic clusters of strains within a genus *Actinoallumurus* while Figure 2 shows phylogenetic position of a strain with top 10 blast result.
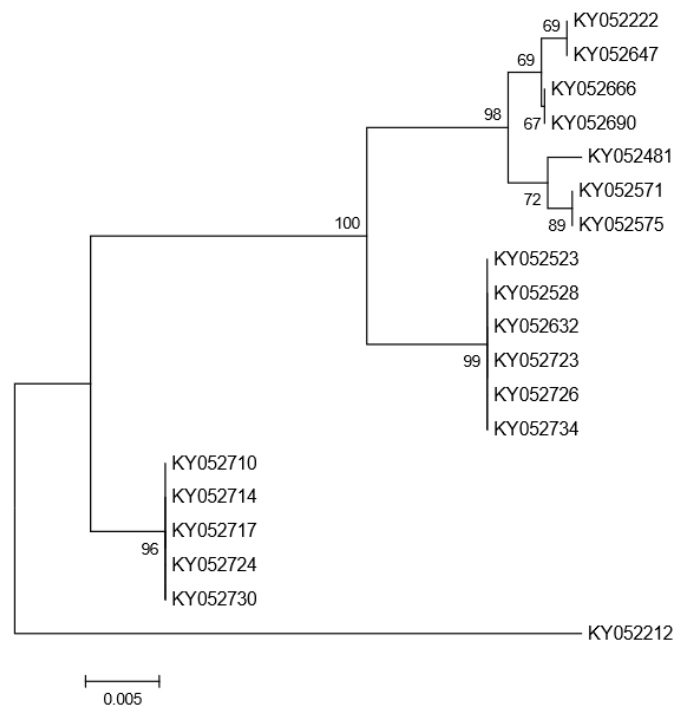

Figure 1 Phylogenetic tree of all strains isolated by under the genera *Actinoallumurus*
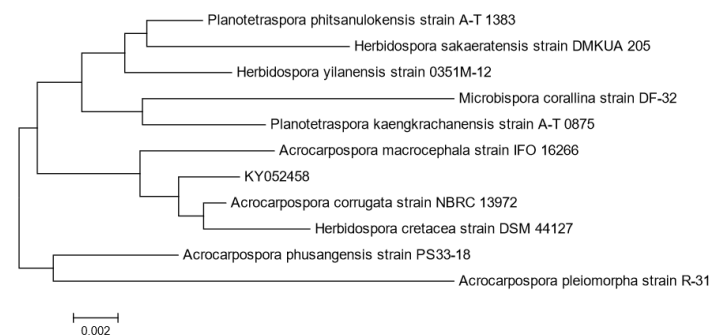

Figure 2 Phylogenetic tree for strain KY052458 (*Acrocarpospora* sp.) with sequences from top 10 16S rRNA database

Ranking

For this project, TU were divided into five categories based on their 16S sequencing and phylogenetic analysis (see Table 1).

Table 1 Taxonomic unit by Ranking and Category

No.	Family	Genus	No. of strains	TU	TU%	Category	No. of TU/Category					
							1	2	3	4	5	TOTAL
1	*Micromonosporaceae*	*Micromonospora*	257	40	25	C1, C3, C4, C5	1	0	11	20	8	**40**
2		*Dactylosporangium*	13	10	6	C2, C3, C4, C5	0	1	1	6	2	**10**
3		*Planosporangium*	8	3	2	C3, C4	0	0	1	2	0	**3**
4		*Verrucosispora*	1	1	1	C3	0	0	1	0	0	**1**
5		*Polymorphospora*	1	1	1	C2	0	1	0	0	0	**1**
6	*Streptomycetaceae*	*Streptomyces*	120	40	25	C2, C3, C4, C5	0	3	12	14	11	**40**
7		*Streptacidiphilus*	7	6	4	C3, C4, C5	0	0	2	3	1	**6**
8		*Kitasotospora*	2	2	1	C2, C5	0	1	0	0	1	**2**
9	*Streptosporangiaceae*	*Astrosporangium*	1	1	1	C2	0	1	0	0	0	**1**
10		*Planotetraspora*	3	1	1	C5	0	0	0	0	1	**1**
11		*Streptosporangium*	17	5	3	C3, C4, C5	0	0	1	2	2	**5**
12		*Nonomuraea*	12	3	2	C1, C4, C5	1	0	0	1	1	**3**
13		*Microtetraspora*	8	1	1	C5	0	0	0	0	1	**1**

14		*Microbispora*	35	5	3	C3, C4, C5	0	0	1	3	1	**5**
15		*Sphaerisporangium*	19	9	6	C1, C2, C3	1	7	1	0	0	**9**
16		*Acrocarpospora*	1	1	1	C5	0	0	0	0	1	**1**
17	*Nocardioidaceae*	*Nocardia*	41	12	8	C2, C3, C4, C5	0	1	3	5	3	**12**
18	*Thermomonosporaceae*	*Actinoallomurus*	19	7	4	C1, C2, C3, C4	1	3	2	1	0	**7**
19		*Actinomadura*	1	1	1	C4	0	0	0	1	0	**1**
20	*Mycobacteriaceae*	*Mycobacterium*	6	5	3	C2, C5	0	4	0	0	1	**5**
21	*Tsukamurellaceae*	*Tsukamurella*	3	1	1	C5	0	0	0	0	1	**1**
22	*Geodermatophilaceae*	*Blastococcus*	1	1	1	C2	0	1	0	0	0	**1**
23	*Micrococcaceae*	*Kocuria*	1	1	1	C5	0	0	0	0	1	**1**
24	*Thermoactinomycetaceae*	*Shimazuella*	1	1	1	C3	0	0	1	0	0	**1**
		Subtotal	578	158	100		4	23	37	58	36	**158**

Legend: TU -Taxonomic unit, C - Category

Novel Species or Genus

Under Category 1 or blast homology below 97 %, the strains can be considered as novel genus. Four TU were identified as *Micromonospora* (96.4 % MI), *Nonomuraea* (96.6 % MI), *Sphaerisporangium* (96.98 % MI) and "*Actinoallomurus*" (92.8 % MI), with one strain each except for *Sphaerisporangium* that has two strains. The strain with a very low blast homology value probably belongs to a novel genus.

Novel Species and Putative Novel Species

For Category 2 and 3, strains cannot be assigned to a validated species as the blast homologies are lower than 99 % (**Stackebrandt and Ebers, 2006**), but higher than 97 %. A main percentage of the strains (60 TU or 38 % of TU) are under this category. There were 23 TU and 37 TU under Category 2 and 3 respectively. For Category 2, majority of the TU (7 TU or 30.4 %) belongs to the genus *Sphaerisporangium* while in Category 3, most of the strains were from the genera *Streptomyces* (12 TU or 32.4 %) and *Micromonospora* (11 TU or 29.7 %). Novel species or putative novel species were found in 17 genera, comprising all genera that were isolated under the family *Micromonosporaceae* and *Streptomycetaceae*.

Known Species

There were 58 TU for Category 4 and 36 TU for Category 5 that could be assigned to a validated species. Under Category 4 (putative known species), there are possibly some novel species however, further test is required to define their taxonomic position. Category 5 are found in 13 genera, among which 5 genera contains only one TU each. TU assigned to a validated species under Category 5 represents only 22.6 % of the total.

A high level of novel species was observed: 64 TU among 158 corresponding to 40.5 %. In addition, among strains with over 99 % similarity but less than 99.8 %, a part can belong to novel species due mainly to another origin than the related, described species. Very recently diverged species may not be recognizable (**Fox *et al*., 1992**). Various strains with a coefficient of similarity higher than 99 % could be classified as novel species after delineation by other characters *e.g.* a species has to be defined by a series of phenotypic characters in a polyphasic approach including chemotaxonomy and DNA-DNA relatedness evaluation (**Tindall *et al*., 2010**).

Soil community structure

Reports states that in normal and healthy soil, the density of actinomycetes in population about 10^6 - 10^7 cfu g^{-1} soil (**Goodfellow and Williams, 1983; Labeda, 1990; Celentis Analytical, 2003; Handayanto and Hairiah, 2007**) as quantified by dilution plate counts. The reported total actinomycetes counts for paddy samples ranged from 1 to 3 x 10^6 cfu g^{-1} soil (**Xu *et al*., 1996; Saranraj *et al*., 2013**).

In the wetland paddy soil samples, the actinomycetes density was lower (6.84 x 10^5 cfu g^{-1} soil). Soil water content is closely associated with nutrient availability, as well as with soil aeration affecting the soil microbial community in combination (**Griffin, 1981; Uhlirova, 2005**). For hill paddy soil, the populations were slightly higher (1.61 x 10^6 cfu g^{-1} soil) as actinomycetes favour drier conditions due to their filamentous structure (**Waksman, 1959; Alexander, 1961; Potts, 1994**). Total colony count (TCC) among samples differ during the warm season, slightly lower for wet paddy while highest for hill paddy (see Figure 3).
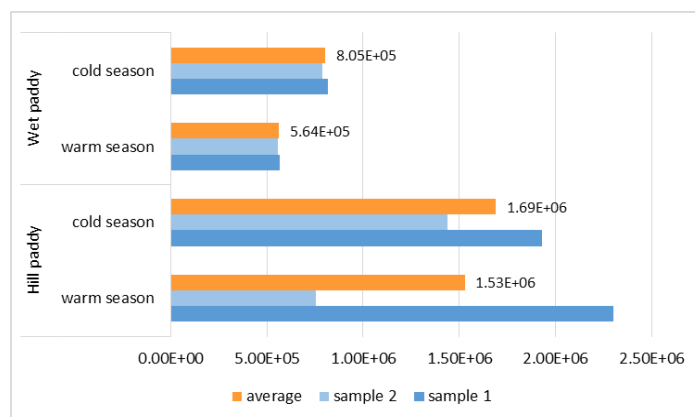

Figure 3 Actinomycetes Total Colony Count (TCC) based on sample type and season

Paddy Field Environment

Majority of the strains (44 %) isolated in this study belonged to the genus *Micromonospora* (40 TU). Ten TU and 5 TU were exclusive for wetland and hill paddy respectively. Various reports indicated that strains of *Micromonospora* are the dominant actinomycetes from aquatic environment (**Eccleston *et al*., 2008; Gebreyohannes *et al*., 2013**). The majority of the strains isolated (111 TU) were dependent on the location. Eleven genera were observed only in either one of the locations. Out of the 24 genera, 10 genera (30 %, 48 TU) were observed in both paddy environments, mainly from *Micromonospora* (16 %, 25 TU), followed by *Streptomyces* (6 %, 10 TU) and *Nocardia* (3 %, 4 TU). Under Category 1 (novel genus) three TU were identified from hill paddy and one TU from wet paddy respectively.

Effects of Seasonal Change

As with vegetation, microbial community structure is most influenced by soil temperature and moisture (**Sylvia *et al*., 2005**). Major differences are again observed in the total number of TU exclusively isolated during one season, 71 TU in cool season and 39 in warm season. High number of TU in the cool season indicates a higher diversity among the isolated strains (45 % non-redundant). TU exclusive by season were members from genera *Dactylosporangium*, *Streptacidiphilus*, *Mycobacterium* and *Kitasatospora*. Only a total of 49 TU (31 % from 11 genera) have members isolated from both cool and dry season. Representatives of the genus *Micromonospora* seems are well represented in both seasons. Three TU identified from cool season and one TU from warm season belong to under Category 1 (novel genus).

Strains from rhizosphere and roots

Representatives of 12 genera (17 TU) were isolated from rhizosphere and 9 genera (11 TU) from roots. Additional two genera could be detected, *Polymorphospora* and *Verrucosispora*, isolated from rhizospheric soil and root samples respectively. Eleven TU distributed among five genera were exclusive for root samples. Majority of the endophytes observed were *Micromonospora* (61 %) followed by *Streptomyces* (16 %). The high occurrence of endophytic *Micromonospora* isolated in this study also corresponds to a study made by Trujillo and her colleagues (2014) where they discovered that *Micromonospora* populations are potential plant growth promoters. The influence of *Micromonospora* on rice should be evaluated.

Among the isolated strains, one strain is categorised as a novel species (BLAST homology 96.98 %) in the genus *Sphaerisporangium* while two strains were

identified as novel species (Category 2 - 97.35% and 97.74%) *Sphaerisporangium* and *Streptomyces* respectively. Diversity and exclusiveness of strains were not significant for endophytes isolated in this study.

Distribution of Actinomycetes Isolated

114 TU (64 %) were exclusive to one type of sample, with a majority exclusive to soil samples (85 TU), followed by rhizospheric (17 TU) and root (11 TU) samples. The overlapping's were minimal. Highest TU overlapping was observed for the genus *Micromonospora* (21 TU, 13 %), followed by *Streptomyces* (6 TU, 4 %) and *Actinoallumurus* (3 TU, 2 %). This highlights the importance of diversifying the sample source for isolation.

Pre-treatment of the Samples

Most isolates were obtained from AD (89 TU), followed by HT (76 TU), PH (42 TU), and FM (37 TU). AD root samples (15 TU) and FD method (14 TU) gave satisfactory results. Nineteen genera were isolated using AD alone, whereby 5 genera were exclusively isolated by this method (*Kitasatospora, Blastococcus, Kocuria, Shimazuella*, and *Verrucosispora*). Other exclusive genera by pre-treatment are *Microtetraspora, Astrosporangium* and *Polymorphospora* for HT and, *Acrocarpospora* and *Actinomadura* for PH. AD pre-treatment gave the highest number of actinomycetes (3.65 x 10^6 cfu g^{-1} soil). Thirty-four TU was exclusively isolated from air dry treatment with the highest TU, 12 from *Streptomyces*.

Twenty-nine exclusive TU were isolated from HT followed by FM (12 TU), PH (7 TU), AD+S (4 TU) and FD (2 TU). The goal of the flooding (FM) method is to increase the percentage of motile actinomycetes. However, in this study only 4 motile genera (*Dactylosporangium, Microtetraspora, Planotetraspora* and *Actinoallumurus*) were isolated using other pre-treatment methods. Improvement on the current FM treatment will be needed to isolate more motile actinomycetes.

Isolation Media

Both HSV (humic acid as carbon and nitrogen source) and SEA (soil extract) media stimulated the differentiation of actinomycetes and allows direct microscopical observations. Most of the strains (178 TU) were isolated from such agar-plates. APA medium contains a mixture of nitrogen, carbon sources, vitamins and minerals; 86 strains (48 TU) have been isolated from this medium. Lastly, on REA medium, made from rice extract designed especially for this study, only 9 strains were isolated but with very low duplication (7 TU). Most genera were isolated from HSV media, 18 genera out of 21 genera, followed by SEA, 16 genera, APA 10 genera and REA three genera.

Soil Analysis

No significant variations were observed among paddy field types and season except for soil colour, moisture content, total organic content and exchangeable magnesium season (see Table 3). Available phosphorus was only detected in one sample for hill paddy field during the cold. **Mardi (2000)** has reported that the optimum soil chemical properties for paddy growth requirement are pH 5.5 - 6.0, organic carbon (2-3 %), available P (>40 mg kg^{-1}) and ExK (> 0.1 +cmol kg^{-1}) (**Aishah *et al*., 2010**). Soil moisture content for wetland paddy is almost double the value then for hill paddy. Hill paddy moisture content during the dry season is slightly higher than during the cool season. The acidity of soil samples ranged from pH 5.0-5.4. Acidic nature of Malaysian paddy soils have also been observed by other researchers (**Arnott, 1964; Kawaguch and Kyuma, 1974**). Organic matter can reach very high levels in soils that are usually waterlogged; such soils often smell sour and are oily (**Plaster, 2002**). TOC in both paddy environments ranged from two to seven percent. High CEC values for soil indicates higher capability of retaining nutrient therefore having better soil properties. As most soils used in plant production have a cation exchange capacity (CEC) between five and 30 (**Kawaguch and Kyuma, 1974**), the paddy soils collected with CEC values of 20 are of medium value, good quality for agriculture (**Hill Laboratories, nd**). Phosphorus is considered the prime limiting factor on plant growth in many areas, because it is the least mobile and available essential nutrient in soil (**Hinsinger, 2001**). Only soil sample from hill paddy during the cold season showed sufficient levels of available P (>10 mg kg^{-1}) (**Kamprath and Watson, 1980**).

Table 3 Soil analysis for wetland and hill paddy soil samples

Location	Alt (ft)	Season	pH	MC (%)	CEC (+cmol/kg)	TOC (%)	Exchangeable Ions (+cmol/ kg)				Avail. P
							Ca	Mg	K	Na	
Wetland paddy Kpg Jenan, Serian N01' 08.880" E110' 29.053"	90	Cold	5.4	52	19.08	7.28	3.4	0.51	0.13	0.1	0
		Warm	5.0	44	20.56	4.23	2.96	0.4	0.11	0.006	0
Hill paddy field Kpg Payang, Padawan N01' 13.248" E110' 18.680"	312	Cold	5.2	27	17.3	2.65	3.28	1.34	0.17	0.003	19
		Warm	5.3	30	16.96	3.17	3.85	1.41	0.21	0.003	0

Legend: Alt – Altitude, MC - Moisture Content, CEC - Cation Exchange Capacity, TOC - Total Organic Content

CONCLUSION

The results of this project demonstrate the potential of Sarawak for the discovery of novel actinobacteria species. The strategy followed in this study allowed the isolation and selection strains belonging to 24 genera with the percentage of novel and putative novel species is over 40 %, and thus high. This demonstrates that man-managed environments have a good diversity of microbial strains leaving a huge genetic pool yet to be discovered. The pre-treatments of the environmental samples and the media used should be extended in order to increase the diversity of the strains isolated. Horizontal gene transfer of up to 30-35 % especially in the accessory genome, results in organisms belonging to the same species with contrasting phenotypes and/or ecological potential (**Luo *et al*., 2011**). Strains within a species can be grouped by some independent specific characteristics; as biochemical, physiochemical, serological or pathological properties (biovars, biotypes, serovars, pathovars) can overlap each other (**Rosselló-Móra and Amann, 2001**). Additional unambiguous characters are necessary for accurate strain identification; the methods should be robust, inexpensive and not tedious. With advancement of technologies, a shift from evolutionary studies of rRNA genes to microbiome study is becoming more apparent. Massively parallel high throughput sequencing technologies, to pyrosequencing and now MISEQ and HISEQ has allowed us to interrogate the microbial composition of biological samples at a cheaper and unprecedented resolution (**Mizrahi-Man *et al*., 2013**). Approaches will be continually improved to diversify the Natural Product Library for bioprospecting by exploring various ecosystems. The strategy should be improved by evaluating the actinomycete diversity by culture-independent methods, including metagenomic (**Rondon *et al*., 2000**).

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